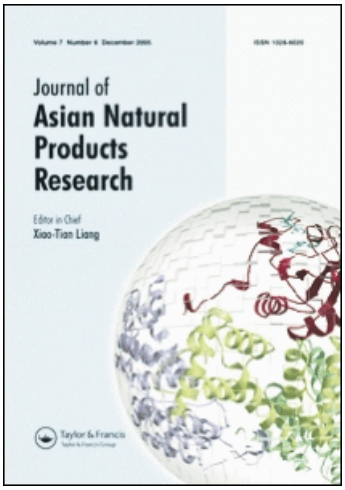

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Two new and four known polyphenolics obtained as new cell-cycle inhibitors from *Rubus aleaefolius* poir

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TWO NEW AND FOUR KNOWN POLYPHENOLICS OBTAINED AS NEW CELL-CYCLE INHIBITORS FROM *RUBUS ALEAEFOLIUS* POIR.

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Two new polyphenolics, rubuphenol (**1**) and sanguiin H-2 ethyl ester (**2**), were isolated together with ellagic acid (**3**), ethyl gallate (**4**), 1,2,3,4,6-penta-*O*-galloyl-β-D-glucopyranose (**5**) and 1,2,3,6-tetra-*O*-galloyl-β-D-glucopyranose (**6**) as new cell-cycle inhibitors from *Rubus aleaefolius* by bioassay-guided separation procedure and the structures of **1** and **2** were elucidated by spectroscopic method. Compounds **1**–**6** inhibited the cell cycle progression of tsFT210 cells at the G_0/G_1 phase with the MIC values of 14.6 μM (**1**), 22.1 μM (**2**), 10.3 μM (**3**), 7.8 μM (**4**), 7.9 μM (**5**) and 6.6 μM (**6**).

Keywords: Rosacae; *Rubus aleaefolius*; Rubuphenol; Sanguiin H-2 ethyl ester; Ellagitannin; Gallotannin; Cell cycle inhibitor

INTRODUCTION

Cell cycle, the sole pathway for cell proliferation, is a strictly-controlled bioprocess and cancers, in fact, are the undesired and unlimited proliferation of cancerous cells with deregulation of the cell cycle. New inhibitors of the cell cycle might, therefore, possess the potential to cure cancers. Thus, the screening of new cell-cycle inhibitors from natural resources has been undertaken by the bioassay using mammalian tsFT210 cells [1–4].

In the course of the screening, more than a thousand Chinese medicinal herbs were examined and *Rubus aleaefolius* Poir. (family Rosacae) was found to possess strong inhibitory activity on the cell-cycle progression of tsFT210 cells at the G_0/G_1 phase. *R. aleaefolius* is used as a traditional Chinese medicine (Cuye Xuangouzi in its Chinese name)

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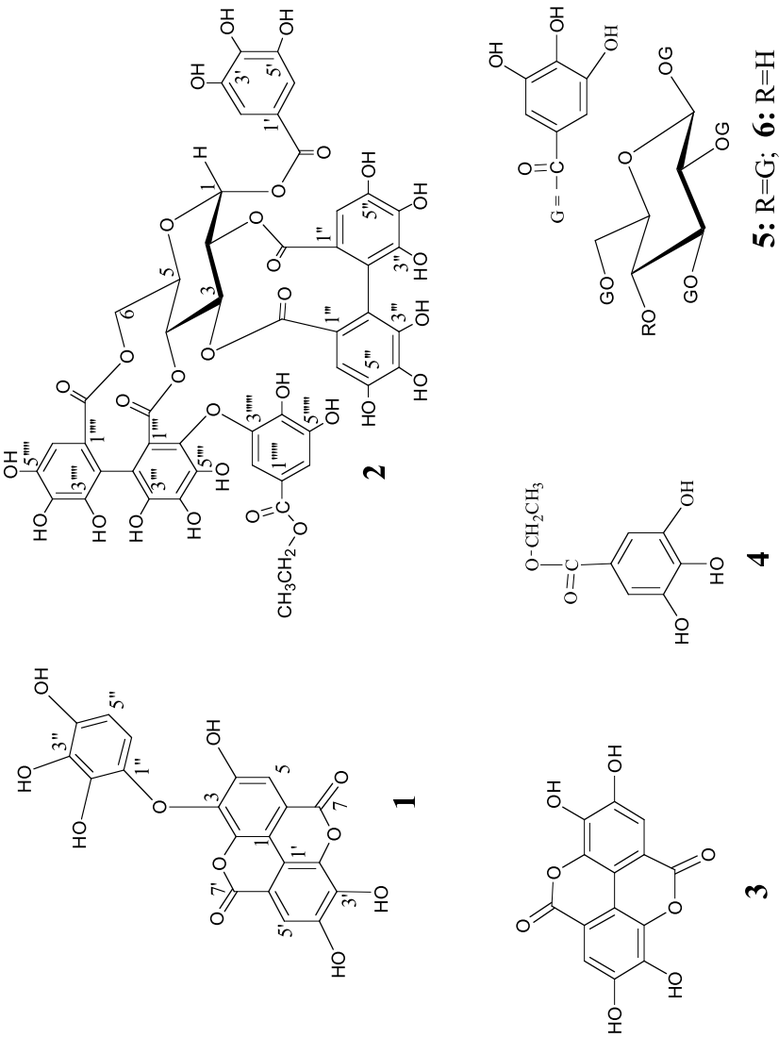

FIGURE 1 Chemical structures of compounds **1**–**6**.

to cure albuminuria, mastitis and chronic hepatitis, etc. [5] and in some areas of China, it is also used as a folk medicine to cure certain cancers. However, no research report had so far been seen on the active principles of the title plant. Thus, the studies on the bioactive constituents of *R. aleaefolius* were undertaken and we have now isolated two new polyphenolics, rubuphenol (**1**) and sanguiin H-2 ethyl ester (**2**), together with four known polyphenolics, ellagic acid (**3**) [6], ethyl gallate (**4**) [7], 1,2,3,4,6-penta-*O*-galloyl-β-D-glucopyranose (**5**) [8] and 1,2,3,6-tetra-*O*-galloyl-β-D-glucopyranose (**6**) [8] (Fig. 1), as new cell-cycle inhibitors from *R. aleaefolius* by a bioassay-guided separation procedure. In this paper, the isolation of **1**–**6**, structure elucidation of **1** and **2**, and biological properties of **1**–**6** are described.

RESULTS AND DISCUSSION

The air-dried roots (3 kg) of *R. aleaefolius* Poir. were extracted with 60% ethanol to give an aqueous ethanol extract (435 g). As the extract showed a strong G_0/G_1 inhibitory activity on the cell cycle of tsFT210 cells, the following separation procedure was monitored by the same activity. The whole extract was suspended in water and then extracted successively with chloroform, ethyl acetate and *n*-butanol to afford an active ethyl acetate extract (78 g). From the active extract, **1** (10 mg), **2** (83.7 mg), **3** (117 mg), **4** (33 mg), **5** (132 mg) and **6** (117 mg) were isolated through repeated solvent-extraction and column chromatography respectively on Sephadex LH-20 and ODS.

Rubuphenol (**1**) was obtained from MeOH solution as an amorphous powder and was decomposed above 275°C. It showed no optical activity and gave a blue color with the ferric chloride reagent, revealing that **1** is a phenolic compound. It gave quasi-molecular ion peaks at *m/z* 427 $[M + H]^+$ and at *m/z* 425 $[M - H]^-$, respectively, in the positive and negative ESI-MS measurements and its molecular formula, $C_{20}H_{10}O_{11}$, was determined by negative high resolution secondary ion MS (HR-SIMS) measurement (measured 425.0151, calcd for $C_{20}H_9O_{11}$ $[M - H]^-$ 425.0150). The UV spectrum of **1** in MeOH solution showed a characteristic absorption curve with maximum absorptions at 215 ($\log \varepsilon$ 4.99), 250sh, 258 (4.64), 272 (4.62) and 351 nm (4.13) and in the IR spectrum, **1** showed absorption bands at 3518, 3396 and 3262 cm^{-1} (free and hydrogen-bonded OH groups), 1737 and 1726 cm^{-1} (ester carbonyl groups), 1620, 1587 and 1494 cm^{-1} (aromatic rings), 1348, and 1243 cm^{-1} (=C–O– groups).

The ^1H and ^{13}C NMR data of **1** in methanol-d_4 solution (Fig. 2), analyzed by PFG ^1H–^1H COSY and PFG-HMQC spectroscopy, indicated the presence of two isolated sp^2 methine (δ_H 7.32 s, H-5, δ_C 111.78 d, C-5 and δ_H 7.50 s, H-5′, δ_C 111.85 d, C-5′) and two neighboring sp^2 methine (δ 6.39 d, $J = 8.8$ Hz, H-5″, δ_C 107.36 d, C-5″and δ 6.50 d, $J = 8.8$ Hz, H-6″, δ_C 112.80 d, C-6″) groups together with two conjugated ester carbonyl, 10 oxygenated sp^2 quaternary and four sp^2 quaternary carbons (Fig. 2) in **1**. Six proton signals have not been observed in the ^1H NMR spectrum in methanol-d_4 solution, revealing the presence of six free hydroxyl groups in **1**. These ^1H and ^{13}C NMR data suggested that **1** is an ellagic acid derivative with an *O*-phenyl moiety bearing adjacent trihydroxy groups [9].

Then, analysis of the PFG-HMBC spectrum enabled us readily to confirm the ellagic acid moiety and the *O*-trihydroxyphenyl group in **1**. In the PFG-HMBC spectrum, H-5 in **1** correlated with C-1, C-3, C-4, C-6, and C-7 and showed weak, but significant correlations with C-2 and C-1′, while H-5′ correlated with C-1′, C-3′, C-4′, C-6′ and C-7′ and gave weak, but significant correlation peaks with C-2′ and C-1. Moreover, H-5″ in **1** correlated with C-1″ C-3″ and C-4″ and gave a weak, but significant correlation peak with C-2″ in the PFG-HMBC spectrum, while H-6″ correlated with C-1″ C-2″ and C-4″ and further showed a weak, but

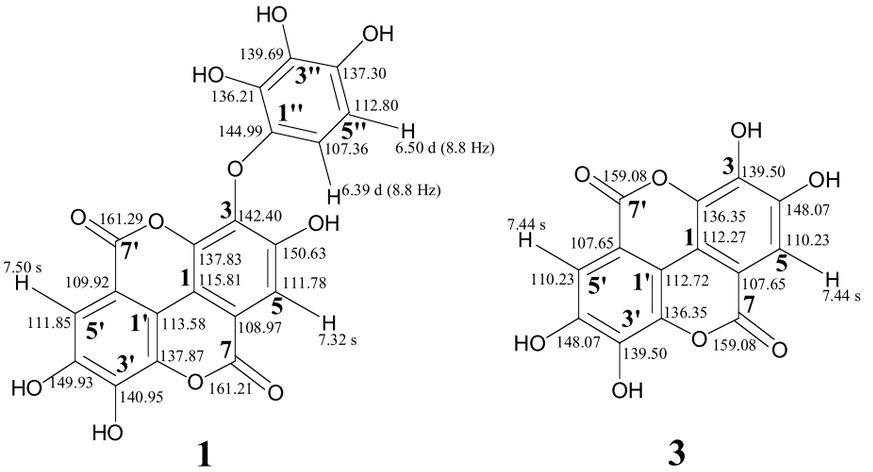

FIGURE 2 600 MHz ^{1}H and 150 MHz ^{13}C NMR data of **1** in CD_3OD and **3** in DMSO-d_6.

significant correlation peak with C-3″. These data established the unambiguous assignments of all ^{1}H and ^{13}C signals for the ellagic acid skeleton and the *O*-phenyl moiety bearing adjacent trihydroxy groups in **1** (Fig. 2). The data for the *O*-phenyl group in **1** (Fig. 2) are consistent with those of the phenyl group in the known 4-*O*-(2″,3″,4″-trihydroxyphenyl)-ellagic acid [9,10], indicating the same substitution pattern of the phenyl group in **1**.

The location of the *O*-phenyl group at the C-3 position in **1** could be demonstrated by the difference NOE experiments in methanol-d_4 solution, where no NOE was observed between H-5 and H-6″ in **1**[†]. This provided negative evidence for the ether linkage in **1** between C-4 and C-1″[†]. Thus the structure of **1** could be concluded to be 3-*O*-(2″,3″,4″-trihydroxyphenyl)-ellagic acid. To our best knowledge, rubuphenol (**1**) is a new phenolic compound and is the first example of ellagic acid derivative with a single ether linkage at the C-3 position to link an *O*-multihydroxyphenyl group [9,10].

Sanguiin H-2 ethyl ester (**2**), a tan amorphous powder, $[\alpha]_D^{25} + 16.12$ (*c* 0.98, acetone), showed a dark-blue color with the ferric chloride reagent and a red color with the Na_2SO_3–Na_2CO_3 reagent, revealing that **2** is an ellagitannin [11,12]. Its molecular formula, $C_{50}H_{36}O_{31}$, was determined by negative HR-FAB-MS (measured 1131.1191, *calcd* for $C_{50}H_{35}O_{31}$ $[M - H]^-$ 1131.1162). In the UV spectrum, **2** showed maximum absorptions at 222 (log ε 4.99) and 265 nm (4.67) and in the IR spectrum it showed absorptions due to sugar moiety (3378 and 1040 cm^{-1}, both strong), carbonyl groups (1745 and 1718 cm^{-1}) and *O*-substituted benzene rings (1617, 1508 and 1210 cm^{-1}).

The ^{1}H NMR spectrum of **2** in CD_3OD solution, analyzed by PFG ^{1}H–^{1}H COSY and PFG-NOESY, indicated the presence of an ethoxy group (δ 4.31 and 4.16 both dq, $J = 11, 7$ Hz; δ 1.35 t, $J = 7$ Hz) and a glucopyranose core (Table I) in **2**. Coupling constants of the glucose protons and their NOE's (Table I) suggested that the glucopyranose core should adopt the $^{4}C_1$ conformation [13] and its anomeric carbon should be the α-configuration on the basis of

[†]For the known 4-*O*-(2″,3″,4″-trihydroxyphenyl)-ellagic acid, the positive NOE was observed between H-5 and H-6″ in the methanol-d_4 solution, evidencing location of the ether linkage at the C-4 position (see Ref. [10]). While in the case of rubuphenol (**1**) in the present study, no NOE was observed between H-5 and H-6″ in the difference NOE experiments under the conditions, 11 mg of samples in 0.4 ml methanol-d_4 solution, 32 scans for each experiment, and variation of the mixing times between 0.05–0.5 s with 0.025 s steps, supporting that the ether linkage in **1** should be located at the C-3 position.

TABLE I 600 MHz ^{1}H NMR data and HMBC correlations for **2** in methanol-d_4

Positions	δ_H (*J* in Hz)	*NOE's**	*HMBC*†
1	6.58 d (3.9)	2′,6′	3, 5, 1′-*C*O
2	5.46 dd (9.3, 3.9)	4	3, 4, 1″*C*O
3	5.21 dd (Ca. 10, 9.3)	5, 2′, 6′,6″,6‴	4, 1‴-*C*O
4	5.13 t (10)	2	2, 6, 1⁗-*C*O
5	4.04 br dd (Ca. 10, 6.4)	3, 2′,6′	
6	5.54 dd (13.1, 6.4)	6⁗′	4, 1⁗′-*C*O
	3.94 br d (13.1)		4, 1⁗′-*C*O
2′	7.28 s	1, 3, 5, 6″	1′, 1′-*C*O, 3′, 4′, 6′
6′	7.28 s	1, 3, 5, 6″	1′, 1′-*C*O, 2′, 4′, 5′
6″	6.55 s	2′, 6′	1″, 1″-*C*O, 2″, 3″, 4″, 5″
6‴	6.55 s	3	1‴, 1‴-*C*O, 2‴, 3‴, 4‴, 5‴
6⁗′	6.87 s		1⁗′, 1⁗′-*C*O, 2⁗′ 3⁗′, 4⁗′, 5⁗′
2⁗′	7.15 d (1.5)	C*H*$_2$CH$_3$ (δ 4.31)	1⁗′, 1⁗′-*C*O, 3⁗′, 4⁗′, 5⁗′, 6⁗′
6⁗′	7.34 d (1.5)		1⁗′, 1⁗′-*C*O, 2⁗′, 3⁗′, 4⁗′, 5⁗′
C*H*$_2$CH$_3$	4.31 dq (11, 7)		1⁗′-*C*O
	4.16 dq (11, 7)		1⁗′-*C*O
CH$_2$C*H*$_3$	1.35 t (7)		*C*H$_2$CH$_3$

Signal assignments were based on the results of PFG ^{1}H–^{1}H COSY, PFG-HMQC, PFG-HMBC, and NOESY experiments.
* Numbers in the column indicate the protons at which NOE's were observed in the NOESYspectrum with the proton on the corresponding line.
† Numbers in the column indicate the carbons coupled with the proton on the corresponding line through two, three or four bonds, which were detected by the PFG-HMBC experiments (long-range J_{CH}: 7 or 5 Hz).

coupling constant of the anomeric proton ($J = 3.9$ Hz). Furthermore, the signals in the lower field region in the ^{1}H NMR spectrum revealed the presence of a galloyl (δ 7.28 s, 2H), a hexahydroxydiphenoyl (HHDP, δ 6.55 s, 2H) and a polyphenolic acid (δ 7.34 and 7.15 each d, $J = 1.5$ Hz; δ 6.87 s) moieties [14] in **2**. In parallel, the ^{13}C NMR spectrum of **2** confirmed the presence of the ethoxy group and the α-D-glucopyranose [14,15] core (Table II). In addition, the ^{13}C NMR spectrum showed the signals due to six carbonyl (δ 166.0, 166.8, 168.4, 169.2, 169.7, and 169.8) and seven sp^2 methine (δ 112.42 d, C-6⁗′; δ 110.68 d, C-2′ and C-6′; δ 109.96 d, C-2⁗′; δ 108.68 d, C-6⁗; δ 108.61, C-6‴; and δ 107.72 d, C-6″) carbons, along with 26 signals ascribable to 29 of quaternary sp^2 carbons including 19 of the oxygenated quaternary sp^2 carbons (Table II). These ^{13}C signals arisen from 42 of sp^2

TABLE II 150 MHz ^{13}C NMR data for **2** in methanol-d_4

Positions	δC	*Positions*	δC	*Positions*	δC	*Positions*	δC
		1″-CO	169.78 s	1⁗-CO	166.81 s	1⁗′-CO	168.47 s
1	91.14 d	1″	126.29 s	1⁗	121.21* s	1⁗′	121.10 s
2	74.54 d	2″	115.35 s	2⁗	116.47* s	2⁗′	109.96 d
3	76.01 d	3″	144.49 s	3⁗	142.86* s	3⁗′	148.78 s
4	69.91 d	4″	137.47 s	4⁗	138.97* s	4⁗′	140.44 s
5	71.44 d	5″	145.74 s	5⁗	135.25* s	5⁗′	145.74 s
6	63.48 t	6″	107.72 d	6⁗	139.65* s	6⁗′	112.42 d
1′-CO	166.01 s	1‴-CO	169.83 s	1⁗′-CO	169.21 s	*C*H$_2$CH$_3$	61.94 t
1′	119.95 s	1‴	126.20 s	1⁗′	125.88 s	CH$_2$CH$_3$	14.49 q
2′	110.68 d	2‴	115.59 s	2⁗′	116.21 s		
3′	146.65 s	3‴	145.51 s	3⁗′	144.76 s		
4′	140.86 d	4‴	137.47 s	4⁗′	137.58 s		
5′	146.65 s	5‴	145.78 s	5⁗′	146.19 s		
6′	110.68 d	6‴	108.61 d	6⁗′	108.68 d		

Signal assignments were based on the results of PFG ^{1}H–^{1}H COSY, PFG-HMQC, PFG-HMBC, and NOESY experiments. Multiplicities of the ^{13}C signals were determined by the DEPT method and are indicated as s (singlet), d (doublet), t (triplet) and q (quartet), respectively.
* Signal was assigned by the comparison with the data of neighboring benzene ring in the same O-substituted HHDP part.

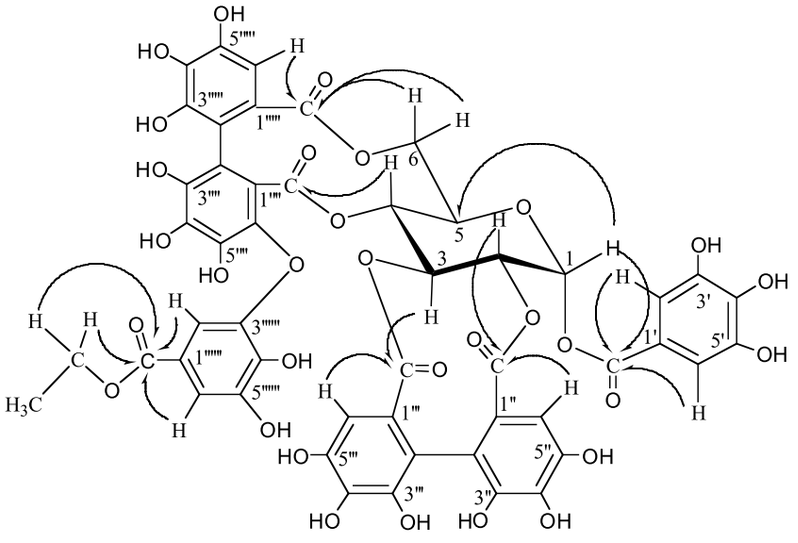

FIGURE 3 Key HMBC correlations for compound **2**.

carbons supported the presence of the galloyl, HHDP, and polyphenolic acid moieties in **2** and also further revealed that the polyphenolic acid moiety in **2** is a trimeric gallic acid moiety.

Then, detailed analysis of the PFG-HMBC spectrum led us readily to confirm the galloyl, HHDP and trimeric gallic acid moieties in **2** according to the long-range J_{CH} correlations summarized in Table I. Locations of the 1-*O*-galloyl and 2,3-*O*-HHDP groups could be determined by the long-range correlations (Table I and Fig. 3) observed in the PFG-HMBC spectrum between 1-H (δ 6.58) and 1′-*C*O (δ 166.01), 2-H (δ 5.46) and 1″-*C*O (δ 169.78), and between 3-H (δ 5.21) and 1‴-*C*O (δ 169.83), respectively. The trimeric gallic acid moiety with a substituted-HHDP skeleton located at the 4,6-positions in **2** could be deduced also by careful examination of HMBC correlations observed in the PFG-HMBC experiments (Table I and Fig. 3). It is noteworthy that in the PFG-HMBC spectrum measured with the long-range J_{CH} value of 5 Hz, the weak, but significant long-range correlations through four-bonds were detected for all aromatic protons with the related quaternary sp^2 carbons (Table I), which were very informative for distinguishing each galloyl carbon skeleton.

As to the absolute configuration of HHDP groups in ellagitannins structurally related to **2**, the sign of the Cotton effects around 235 and 265 nm has been demonstrated to be diagnostic, positive around 235 nm and negative around 265 nm for the *S*-configuration and negative around 235 nm and positive around 265 nm for the *R*-configuration [16]. In the CD spectrum in MeOH solution, **2** showed the Cotton effects, positive at 237 ($\theta + 46407$) and negative at 260 nm (-23519), demonstrating the *S*-configuration of HHDP groups in **2**.

Compounds **3**–**6** were identified respectively as ellagic acid (**3**) [6], ethyl gallate (**4**) [7], 1,2,3,4,6-penta-*O*-galloyl-β-D-glucopyranose (**5**) [8] and 1,2,3,6-tetra-*O*-galloyl-β-D-glucopyranose (**6**) [8] by comparison of their spectral data, respectively, with the corresponding values in the literatures [6–8] and by analyses of the two-dimensional NMR spectra.

Biological Activities of 1–6

The cell-cycle inhibitory activity was assayed for compounds **1**–**6** using tsFT210 cells by flow cytometry according to the previously reported method [2] with slight modification.

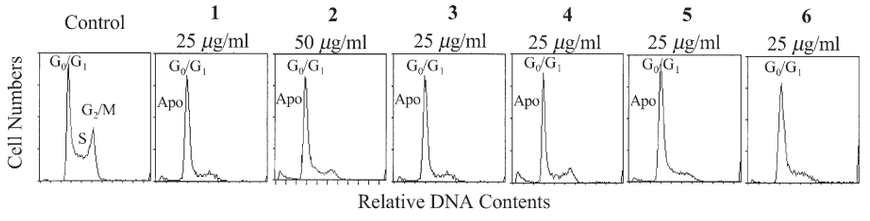

FIGURE 4 The tsFT210 cells in RPMI-1640 medium (20×10^5 cells ml^{-1}) were cultured for 17 h at 32°C (permissive temperature) in the humidified atmosphere of 5% CO_2 and 95% air under the absence (control) or presence of **1**–**6**, respectively. The cell nuclei were then stained with propidium iodide and their distribution within cell cycle was analyzed by flow cytometry.

Typical flow cytometric histograms for **1**–**6** are given in Fig. 4. Compounds **1**–**6** inhibited the cell-cycle progression of tsFT210 cells at the G_0/G_1 phase with MIC values of 14.6 μM (**1**), 22.1 μM (**2**), 10.3 μM (**3**), 7.8 μM (**4**) and 7.9 μM (**5**), and 6.6 μM (**6**). In addition, **2** and **4** also showed apoptosis-inducing activity at the concentrations over 25 μg/ml, as can be seen in the flow cytometric data shown in Fig. 4, and the apoptotic cell morphology was also demonstrated by light microscopy (data not given).

In the present paper, we reported two new compounds, rubuphenol (**1**) and sanguiin H-2 ethyl ester (**2**), isolated by a bioassay-guided separation procedure from *R. aleaefolius*, together with four known compounds, **3**–**6**, which inhibited the mammalian cell cycle at the G_0/G_1 phase. Compounds **3**–**6** are well-known plant polyphenolics and widespread biological activities have been reported for **3**–**6**. However, no research report had so far been seen on the cell-cycle inhibitory activity for compounds **3**–**6**. Thus the present result provides plant polyphenolics, **1**–**6**, as new G_0/G_1 phase inhibitors of mammalian cell cycle and the cell-cycle inhibitory activity of **5**–**6** may give an explanation for their *in vivo* anticancer effect reported [17].

On the other hand, compounds **1**–**6** were isolated for the first time from *R. aleaefolius* as cell-cycle inhibitory components in the present study, which also provided the first example of bioactive constituents of *R. aleaefolius* for its usage in Chinese people to cure cancers.

EXPERIMENTAL SECTION

General Experimental Procedures

Thin-layer chromatography (TLC) was done on silica gel 60 F254 plates (0.25 mm thick, 20×20 cm^2, Merck) or silica gel G plates (0.25 mm thick, 20×20 cm^2, Qingdao Haiyang Chemical group Co., China) and the spots were detected under UV light (254 and 365 nm) or by the use of 10% aqueous sulfuric acid reagent. Column chromatography was carried out on a glass open column and Sephadex LH-20 (Pharmacia) and SSC ODS-SS-1020T (Senshu Scientific Co., Ltd.) were used as adsorbents.

Melting points were measured using an XT-type micro melting point apparatus (Beijing Tech Instrument Co. Ltd., China) and are uncorrected. Optical rotations were determined on a JASCO P-1020 polarimeter in $CHCl_3$ solutions and UV spectra were recorded on a Shimadzu UV-2501PC UV–VIS recording spectrophotometer in MeOH solutions. IR spectra were taken on a Dynamin alignment FTS 175C Fourier transform infrared spectrophotometer in KBr discs. ESI-MS was measured on an Esquire LC mass spectrometer and HR-SIMS was measured on an APEX II mass spectrometer (Bruker). Fast atom bombardment (FAB) mass and HR-FAB mass spectra were measured on an Autospec Ultima-TOF mass spectrometer (Micromass Co. Ltd., UK) using a direct inlet system and

glycerol was used as a matrix in FAB mass measurement. ^{1}H and ^{13}C NMR spectra were taken on a JEOL Eclips-600 or Bruker AVANCE DRX-500 FT-NMR spectrometer using TMS as internal standard and chemical shifts are recorded in δ values (δ_H and δ_C for TMS 0.00). Two-dimensional NMR spectra were measured on a JEOL Eclips-600 FT-NMR spectrometer.

Plant Material

The roots of *R. aleaefolius* were collected at the Mengla area of Yunnan province, China, in September 1998. The original plant was identified by Professor Q.-S. Sun of Shenyang Pharmaceutical University, China, and a voucher specimen has been deposited in Shenyang Pharmaceutical University, China.

Extraction and Fractionation

Air-dried roots (3 kg) of *R. aleaefolius* were extracted at room temperature with 3 l of 60% alcohol (3 times) to give an extract (435 g). The whole extract was suspended in water (3 l) and partitioned successively with chloroform, ethyl acetate and butanol to afford an active ethyl acetate extract (78 g). This extract was extracted with methanol to give methanol-soluble (74 g) and methanol-insoluble (4 g) parts. The former methanol-soluble part (74 g) was further divided into water-soluble (7.5 g) and water-insoluble (66.4 g) parts by dissolving in water followed by sonication and then centrifugation.

The water-insoluble part (66.4 g) was subjected to column chromatography over Sephadex LH-20 (50 g, bed $4.2 \times 50\,cm^2$) and eluted with MeOH to give two fractions, **RA-4** (the early fraction, 55 g) and **RA-5** (the later fraction, 11.4 g). **RA-5** (11.4 g) was subjected again to the column chromatography over Sephadex LH-20 (50 g, bed $4.2 \times 50\,cm^2$) and eluted with EtOH–H_2O–acetone in a stepwise manner (100:0:0 to 30:20:50, v/v, each 500 ml) to obtain 22 fractions, **Fr.1**–**Fr.22**, among which five are bioactive, namely **Fr.3** (340 mg, 80:20:0 eluent), **Fr.8** (316 mg, 75:25:0 eluent), **Fr.9** (240 mg, 54:36:10 eluent), **Fr.14**–**15** (240 mg, 42:28:30 eluent), and **Fr.18**–**20** (820 mg, 36:24:40 eluent).

Isolation of Compounds 1–6

Fr.3 (340 mg) was further separated by ODS column ($3 \times 25\,cm^2$) chromatography using MeOH–H_2O as eluting solvent to give **2** (33 mg) from MeOH–H_2O (35:65) eluent.

Fr.8 (316 mg) was separated by ODS column ($3 \times 25\,cm^2$) chromatography using MeOH–H_2O as eluting solvent to give **1** (10 mg) from MeOH–H_2O (36:64) eluent.

Fr.9 (410 mg) was separated by ODS column ($3 \times 25\,cm^2$) chromatography using MeOH–H_2O as eluting solvent to give **4** (64 mg) from MeOH–H_2O (40:60) eluent.

Fr.14–**15** (240 mg) was further separated by ODS column ($3 \times 25\,cm^2$) chromatography using MeOH–H_2O as eluting solvent to give **6** (84 mg) from MeOH–H_2O (26:74) eluent.

Fr.18–**20** (820 mg) was subjected to ODS column ($3 \times 25\,cm^2$) chromatography and eluted with MeOH–H_2O as eluting solvent to give **3** (132 mg) from MeOH–H_2O (22:78) eluent and **5** from MeOH–H_2O (24:76) eluent.

Rubuphenol (**1**). Tan amorphous powder from MeOH, 10 mg, mp 275°C (dec.), $C_{20}H_{10}O_{11}$. Positive ESI-MS *m/z*: 427 $[M + H]^+$. Negative ESI-MS *m/z*: 425 $[M - 1]^-$. HR-SIMS *m/z*: measured 425.0151, *calcd* for $C_{20}H_9O_{11}$ 425.0150. UV λ_{max} nm ($\log \varepsilon$) in MeOH: 351 (4.13), 271 (4.61), 257 (4.64), 203 (4.68). IR (KBr) ν_{max} cm^{-1}: 3395, 3262 (OH), 1736, 1725 (C=O), 1619, 1586, 1494 (aromatic rings), 1440, 1347, 1242, 1187, 1114, 1067, 1033, 969, 921, 826, 755. ^{1}H and ^{13}C NMR data in CD_3OD: see Fig. 2.

Sanguiin H-2 ethyl ester (**2**). Tan amorphous powder from MeOH, 33 mg, $C_{50}H_{36}O_{31}$, mp 280°C (dec.), $[\alpha]_D^{25} + 16.12$ (*c* 0.98, acetone). FAB-MS *m/z*: 1155 $[M + Na]^+$. Negative FAB-MS *m/z*: 1131 $[M - H]^-$ and 1154 $[M - H + Na]^-$. Negative HR-FAB-MS *m/z*: measured 1131.1191, *calcd* for $C_{50}H_{35}O_{31}$ $[M - H]^-$ 1131.1162; measured 1154.1075, *calcd* for $C_{50}H_{35}O_{31}Na$ $[M - H + Na]^-$ 1154.1060. UV λ_{max} nm ($\log \varepsilon$) in MeOH: 222 (4.99), 265 (4.67). IR (KBr) ν_{max} cm^{-1}: 3378 (OH), 1745 and 1718 (C=O), 1617 and 1508 (benzene ring), 1210, 1040. ^1H and ^{13}C NMR data: see Table I. CD θ (nm) in MeOH: +46407 (237), −23519 (260), +82386 (282), −11776 (308).

Ellagic acid (**3**). Light yellow needles, 123 mg, mp 357–358°C. ESI-MS *m/z*: 303 $[M + H]^+$. UV λ_{max} nm ($\log \varepsilon$) in MeOH: 365 (4.29), 255 (3.78). IR (KBr) ν_{max} cm^{-1}: 3090 (OH), 1716 (C=O), 1620, 1582, 1507 (benzene ring), 1443, 1337, 1187, 1103, 1054, 1015, 984, 917, 758. ^1H and ^{13}C NMR δ in DMSO-d_6: see Fig. 2. The carbon NMR data in Fig. 2 were assigned by PFG-HMBC spectroscopy.

Ethyl gallate (**4**). Colorless needles from chloroform, 64 mg, mp 160–161°C. ESI-MS *m/z*: 221 $[M + Na]^+$. UV λ_{max} nm ($\log \varepsilon$) in MeOH: 275 (3.99), 224 (4.26). IR (KBr) ν_{max} cm^{-1}: 3449, 3309 (OH), 2980 (CH_2 and CH_3), 1707 (C=O), 1620 and 1535 (benzene ring), 1384, 1318, 1256, 1200, 1040, 967, 758 cm^{-1}. ^1H NMR δ in CD_3OD: 1.53 (3H, t, $J = 7.0$ Hz, CH_3), 4.46 (2H, q, $J = 7.0$ Hz, CH_2), 7.24 (2H, s, aromatic protons).

1,2,3,4,6-penta-*O*-gally-β-D-glucopyranose (**5**). Light brown powder from MeOH, 117 mg, mp 203–204°C, $[\alpha]_D^{25} + 19.8$ (*c* 2.00, MeOH). ESI-MS *m/z*: 963 $[M + Na]^+$. UV λ_{max} nm ($\log \varepsilon$) in MeOH: 220 (5.13), 281 (4.80). IR (KBr) ν_{max} cm^{-1}: 3394 (OH), 1708 (C=O), 1615, 1536, 1450 (aromatic rings), 1349, 1322, 1207, 1094, 1032, 869, 804, 762. ^1H-NMR δ in CD_3OD: 7.31, 7.26, 7.18, 7.15, 7.10 (all 2H and s, aromatic protons), 6.43 (d, $J = 8.3$ Hz, H-1), 6.10 (t, $J = 9.7$ Hz, H-3), 5.82 (t, $J = 9.7$ Hz, H-4), 5.78 (dd, $J = 9.7$, 8.3 Hz, H-2), 4.72 (br d, $J = 14.7$ Hz, H-6), 4.61 (m, H-5), 4.58 (dd, $J = 14.7$, 4.3 Hz, H-6). ^{13}C-NMR δ in CD_3OD: 167.98, 167.35, 167.06, 166.98, 166.28 (5 × *C*O), 146.57, 146.48, 146.46, 146.40, 146.30 (5 × C-3′,5′), 140.79, 140.38, 140.33, 140.16, 140.03 (5 × C-4′), 121.18, 120.49, 120.37, 120.35, 119.86 (5 × C-1′), 110.77, 110.61, 110.52, 110.52, 110.48 (5 × C-2′,6′), 93.9 (C-1), 74.5 (C-5), 74.2 (C-3), 72.3 (C-2), 69.9 (C-4), 63.2 (C-6).

1,2,3,6-tetra-*O*-gally-β-D-glucopyranose (**6**). Light brown powder from MeOH, 84 mg, mp 226°C (dec.), $[\alpha]_D^{25} + 32.4$ (*c* 0.85, MeOH). ESI-MS *m/z*: 811 $[M + Na]^+$. UV λ_{max} nm ($\log \varepsilon$) in MeOH: 221 (4.87), 279 (4.52). IR (KBr) ν_{max} cm^{-1}: 3382 (OH), 2965, 1706 (C=O), 1614, 1535 (aromatic rings), 1450, 1348, 1213, 1090, 1033, 804, 763. ^1H NMR δ in CD_3OD: 7.31, 7.23, 7.22, 7.13 (all 2H and s, aromatic protons), 6.29 (d, $J = 8.0$ Hz, H-1), 5.67 (dd, $J = 10.0$, 9.0 Hz, H-3), 5.63 (dd, $J = 10.0$, 8.0 Hz, H-2), 4.61 (dd, $J = 12.0$, 2.0 Hz, H-6), 4.71 (dd, $J = 12.0$, 4.0 Hz, H-6), 4.20 (m, H-5), 4.15 (t, $J = 9.0$ Hz, H-4). ^{13}C NMR δ in CD_3OD: 168.21, 167.76, 167.21, 166.35 (4 × *C*O), 146.53, 146.53, 146.53, 146.42, 146.38, 146.36, 146.28, 146.28 (8 × C-3′,5′), 140.69, 140.21, 140.00, 139.97 (4 × C-4′), 121.36, 121.12, 120.54, 119.99 (4 × C-1′), 110.70, 110.52, 110.49, 110.49, 110.49, 110.42, 110.34, 110.34 (8 × C-2′,6′), 93.98 (C-1), 76.72 (C-5), 76.58 (C-3), 72.43 (C-2), 69.75 (C-4), 64.05 (C-6).

Cell Culture and Bioassay

A mouse temperature-sensitive $p34^{cdc2}$ mutant cell line, tsFT210 [1], was used for bioassay. The tsFT210 cells were routinely maintained at 32°C in RPMI-1640 medium supplemented with 10% FBS under a humidified atmosphere of 5% CO_2 and 95% air.

5 μl of sample solutions in MeOH was added, respectively, into each well of the 24-well plate containing the exponentially growing tsFT210cells at the density of 2×10^5 cell ml^{-1} in 0.5 ml of fresh medium and the cells were cultured at 32°C for 17 h. Then the cells were

transferred into the 1.5 ml Eppendorf centrifuge tubes, harvested by centrifugation at 3000 rpm for 3 min at 4°C, washed once with cold phosphate-buffered saline (PBS), and harvested again by centrifugation under the same condition. Then, 150 μl of propidium iodide in water solution (propidium iodide 50 μg/ml, sodium citrate 0.1% and Nonidet p-40 0.2%) was added into each of the tubes and the cells were stained at 4°C for 30 min. The cells were then subjected to a flow cytometric analysis after dilution with the same volume of PBS and the distribution within the cell cycle was analyzed by WinCycle software (Coulter).

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