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Research Paper

# Isolation, modification and cytotoxic evaluation of flavonoids from *Rhododendron hainanense*

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**Keywords**
cytotoxicity; flavonoids; *Rhododendron hainanense*; structure–activity relationship

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## Abstract

**Objectives** The aim of this study was to search for antitumour activity of flavonoid compounds. The cytotoxic activity of these compounds *in vitro* was evaluated against the human leukaemia (HL-60) and human hepatoma (SMMC-7721) cell lines.

**Methods** Eight natural flavonoids (**1**–**8**) were isolated from the aerial parts of *Rhododendron hainanense* and a series of modified flavonoid derivatives (**9**–**18**) were obtained from the natural product matteucinol (**1**), using simple synthetic methods. Antitumour inhibitory activity of these flavonoids was assessed using the sulforhodamine B method.

**Key findings** Most of the compounds exhibited good pharmacological activity and the preliminary structure–activity relationships were described. Within the series of flavonoid derivatives in this study, compounds **3** (2,3-dihydro-5-hydroxy-7-methoxy-2-(4-methoxyphenyl)-6,8-dimethyl-4H-1-benzopyran-4-one) and **16** (5-hydroxy-7, 4′-dimethoxy-6, 8-dimethylflavan) exhibited strong inhibitory activity against the HL-60 cell line with IC50 values (the drug concentration that resulted in a 50% reduction in cell viability or inhibition of the biological activity) of 15.2 and 13.2 μM, respectively.

**Conclusions** Renewed attention to flavonoid derivatives revealed the possibility that compounds **3** and **16** could be considered as lead compounds for the development of new antitumour agents. Our results have not only enriched the family of active flavonoids from natural sources, but have encouraged the synthesis of flavonoid analogues for improving cytotoxic activity.

## Introdution

Flavonoids are a large class of plant secondary metabolites belonging to the wider family of natural polyphenols. They occur naturally in fruit, vegetables, nuts, seeds, flowers and bark, and are an integral part of the human diet. Recently, interest in natural polyphenolic compounds has increased steadily owing to their antioxidant capacity, and to their ability to modulate a number of molecular pathways primarily involved in the regulation of cell proliferation, cell function and inflammatory reactions. Considerable attention is thus now concentrated on their possible beneficial implications on human health, such as in the prevention and treatment of cancer, cardiovascular diseases, neurodegenerative processes and other chronic diseases affecting the liver, gut, kidney and lung.[1]

*Rhododendron hainanense*, belonging to the family Ericaceae, is distributed throughout southern regions of China. Some species of the genus *Rhododendron* have been used as medicinal plants. The dried leaves of *R. dauricum* have been used medicinally as an expectorant and for treatment of acute-chronic bronchitis.[2,3] Both flowers and fruits of *R. molle* have been recorded in ancient and modern monographs as analgesics in traditional uses. The chemical compound rhodojaponin, isolated from *R. molle*, has been shown to have significant blood pressure lowering and heart rate slowing effects.[4] The ethanolic extract of *R. arboretum* possessed a hypotensive effect and respiratory stimulant in cats and dogs. It was reported also to produce contraction of the guinea-pig ileum and have an effect on the central nervous

 

system (CNS).[5] The methanolic extract of the leaves and twigs of *R. dauricum* was found to display significant anti-HIV activity.[6] The characteristic compounds of the genus *Rhododendron* are flavonoids, coumarins, and tannins. During our program of screening for antitumour activity of flavonoid compounds from natural sources, the alcoholic (EtOH) extract of the leaves and twigs of *R. hainanense* led to the isolation of eight flavonoids (**1**–**8**). We found that the content of matteucinol (**1**) in *R. hainanense* was high and accounted for 0.166%, and had a weak cytotoxic activity *in vitro* against the human leukaemia (HL-60) and human hepatoma (SMMC-7721) cell lines. We therefore modified the chemical structure of matteucinol in an attempt to improve its activity. In this study, we report on the isolation, modification and in-vitro cytotoxicity of a series of natural and synthetic flavonoid derivatives, as well as the primary structure-activity relationships.

## Materials and Methods

### Materials

Acetic acid, anhydrous magnesium sulfate, sodium cyanoborohydride ($NaBH_3CN$), boron tribromide ($BBr_3$), anhydrous sodium carbonate, HCl (36~38%) and $H_2SO_4$ (95%~98%) were purchased from Lanzhou Aihua Chemical Reagent Co., Ltd. (China). EtOH, MeOH and $CH_2Cl_2$ were purchased from Tianjing Chemical Reagent Co., Ltd. (China). All the reagents and chemicals were of analytical grade. The HL-60 and SMMC-7721 cell lines used for the bioassays were offered to us by Chun-ming Wang, at the School of Life Sciences, Lanzhou University, Lanzhou, China. RPMI-1640 medium was purchased from Gibco Co., Ltd. (Grand Island, NY, USA). Fetal calf serum (FCS) was purchased from Hangzhou Sijiqing Biological Engineering Materials Co., Ltd. (China). Trichloroacetic acid (TCA), dimethyl sulfoxide (DMSO), mitomycin and tris(hydroxymethyl) aminomethane were purchased from Sigma Aldrich (St. Louis, MO, USA).

### General procedures

Column chromatography: silica gel (200–300 mesh; Qingdao Marine Chemical Factory, China). TLC: silica gel $GF_{254}$ plates (10–40 lm; Qingdao Marine Chemical Factory, China). $^1H$ and $^{13}C$ NMR spectra: Varian Mercury-300BB (300 MHz) spectrometer, in $CDCl_3$ and DMSO-$d_6$; d in ppm rel. to TMS, *J* in Hz. EI-MS: VG ZAB-HS instrument, at 70 eV, in m/z. HRESIMS: Bruker APEX-II mass spectrometer; in m/z.

### Collection and extraction of plant material

Aerial parts of *R. hainanense* were collected from Jianfeng town of Hainan Province, China, in the summer of 2008. They were identified by Guo-Liang Zhang, at the School of Life Science, Lanzhou University China. A voucher specimen (No. 08-08) has been deposited with the Natural Organic Academy of Lanzhou University.

Aerial parts of *R. hainanense* (3 kg) were kept until air dry. The dry materials were ground into a powder and extracted three times with 80% EtOH at room temperature, each for three days. After concentration of the combined extracts under reduced pressure, the residue (289.9 g) was suspended in $H_2O$, then extracted with EtOAc and n-BuOH, respectively. The EtOAc-soluble fraction (93 g) was subjected to a silica gel column chromatography (eluted with PE–EtOAc, from 40 : 1 to 1 : 1, then with MeOH) to give nine fractions (fractions 1–9). Further purification of fraction 3 by column chromatography ($SiO_2$; PE–acetone, 15 : 1) yielded compound **3** (10 mg). Fraction 3-1 was subjected to Sephadex LH-20 column chromatography to afford compound **1** (5 g). Fraction 4 was purified in a similar procedure ($SiO_2$; PE–acetone, 3 : 1) to give pure compounds **2** (30 mg), **4** (56 mg) and **6** (10 mg). Fraction 5-2 afforded compound **5** (50 mg) by Sephadex LH-20 column chromatography. Compound **7** (2 mg) was separated from fraction 5-3. Fraction 6 was purified by column chromatography on silica gel eluted with PE–acetone (1 : 1) to give compound **8** (5 mg).

### Synthesis

To determine the changes of bioactivity resulting from different substituents of A, B ring and the type of C ring, matteucinol (**1**) was modified to the target compounds according to the method described in Figure 1. Matteucinol (**1**) was oxidized to flavone **9** upon exposure to iodine in DMSO at 100°C in a 95% yield.[7] The demethylation reaction of flavone **9** was carried out using HBr in AcOH to obtain the corresponding 5,7,4′-trihydroxyflavone **10**.[8] Matteucinol was derivatized according to Fujise's mild acetylation to give diacetate **12** as colourless needles.[9] Removal of the carbonyl group was carried out by reduction of **12** with 2 equiv $NaBH_4$ in tetrahydrofuran (THF) and $H_2O$ at 0°C for 30 min to give a 90% yield of the monoacetate **17**.[10]

Matteucinol with 1.5 equiv $(CH_3)_2SO_4/K_2CO_3$ in acetone afforded 7-O-methylmatteucinol (**3**), acetylation of compound **3** using Fujise's conditions gave **13**, removal of the carbonyl group was carried out by reduction of **13** with 2 equiv $NaBH_4$ in THF and $H_2O$ at 30 min to give an 85% yield of **16**, flavan **18** was prepared by deprotection of the methyl ether using $BBr_3/CH_2Cl_2$ protocol.[11] Matteucinol with 10 equiv $(CH_3)_2SO_4$ was refluxed overnight to generate 5,7-O-dimethylmatteucinol (**11**). According to the literature, compound **11** with sodium borohydride in EtOH furnished the desired compound **14**, demethylation of **14** with boron tribromide furnished compound **15**.[12]

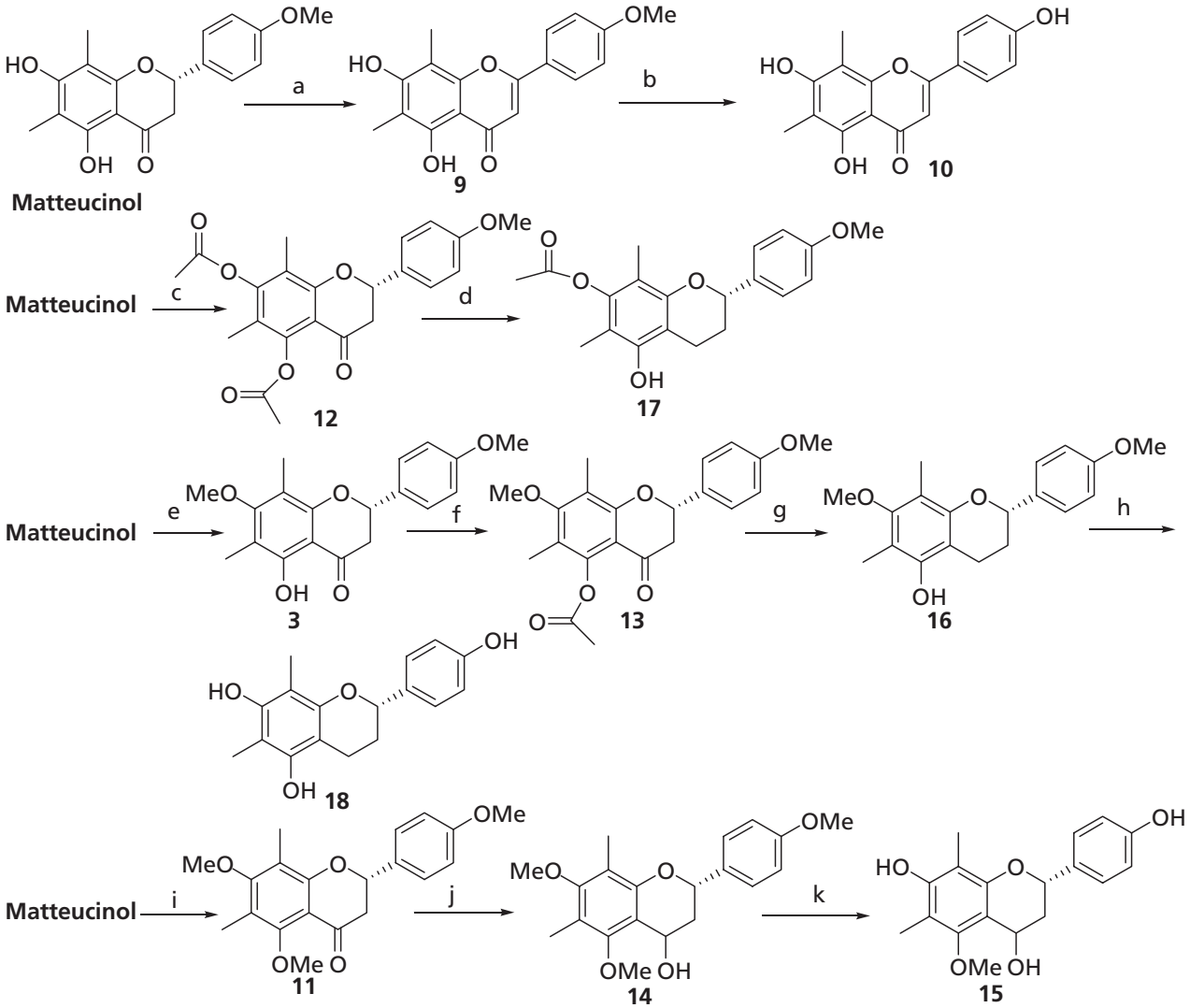


**Figure 1** Matteucinol (compound **1**) extracted from *Rhododendron hainanense* was modified to the target compounds according to various methods. Reagent and conditions: (a) $I_2$, DMSO, $H_2SO_4$, 100°C, 95%; (b) HOAC, HBr, reflux, 50%; (c) $Ac_2O$, $H_2SO_4$, 0°C, 95%; (d) $NaBH_4$, THF/$H_2O$, 0°C, 90%; (e) $(CH_3)_2SO_4$, acetone, $K_2CO_3$, reflux, 98%; (f) $Ac_2O$, $H_2SO_4$, 0°C, 82%; (g) $NaBH_4$, THF/$H_2O$, 0°C, 85%; (h) $BBr_3$, $CH_2Cl_2$, –78°C to room temperature (rt), 60%; (i) $(CH_3)_2SO_4$, acetone, $K_2CO_3$, reflux, 80%; (j) $NaBH_4$, EtOH, rt, 80%; (k) $BBr_3$, $CH_2Cl_2$, –78°C to rt, 50%.

## $^1$H and $^{13}$C NMR spectral data of compounds 1–18

Matteucinol (**1**) mp 168–170°C. $^1$H NMR (DMSO-$d_6$, 300 MHz): $\delta$ (ppm): 12.37 (s, 1H, 5-OH), 9.67 (s, 1H, OH), 7.44 (d, $J$ = 8.6 Hz, 2H, Ar-2′-H, Ar-6′H), 6.98 (d, $J$ = 8.8 Hz, 2H, Ar-3′-H, Ar-5′-H), 5.48 (dd, $J$ = 12.0, 3.0 Hz, 1H, 2-CH), 3.77 (s, 3H, 4′-$CH_3$), 3.20 (dd, $J$ = 16.7, 12.5 Hz, 1H, 3-CH), 2.78 (dd, $J$ = 16.7, 3.0 Hz, 1H, 3-CH), 1.95 (s, 6H, $CH_3$); ESI-MS (m/z) 313.1 ([M-H]$^-$).

Farrerol (**2**) mp 228–231°C. $^1$H NMR (DMSO-$d_6$, 300 MHz): $\delta$ (ppm): 12.39 (s, 1H, 5-OH), 9.67 (s, 1H, OH), 9.58 (s, 1H, OH), 7.33 (d, $J$ = 7.3 Hz, 2H, Ar-2′-H, Ar-6′-H), 6.80 (d, $J$ = 8.3 Hz, 2H, Ar-3′-H, Ar-5′-H), 5.41 (dd, $J$ = 12.4, 2.4 Hz, 1H, 2-CH), 3.20 (dd, $J$ = 17.0, 12.5 Hz, 1H, 3-CH), 2.75 (dd, $J$ = 17.0, 2.4 Hz, 1H, 3-CH), 1.95 (s, 6H, 6-$CH_3$, 8-$CH_3$); ESI-MS (m/z) 299.1 ([M-H]$^-$).

2,3-Dihydro-5-hydroxy-7-methoxy-2-(4-methoxyphenyl)-6,8-dimethyl-4H-1-benzopyran-4-one (**3**) $^1$H NMR (DMSO-$d_6$, 300 MHz): $\delta$ (ppm):12.37 (s, 1H, 5-OH), 9.67 (s, 1H, OH), 7.44 (d, $J$ = 8.6 Hz, 2H, Ar-2′-H, Ar-6′-H), 6.98 (d, $J$ = 8.8 Hz, 2H, Ar-3′-H, Ar-5′-H), 5.48 (dd, $J$ = 12.0, 3.0 Hz, 1H, 2-CH), 3.77 (s, 3H, 4′-$CH_3$), 3.62 (s, 3H, 7-$CH_3$), 3.20 (dd, $J$ = 16.7, 12.0 Hz, 1H, 3-CH), 2.78 (dd,

$J$ = 16.7, 3.0 Hz, 1H, 3-CH), 1.95 (s, 6H, $CH_3$). ESI-MS (m/z) 327.1 ([M-H]$^-$).

4′-Methylnaringenin (**4**) $^1$H NMR (DMSO-$d_6$, 300 MHz): $\delta$ (ppm): 12.39 (s, 1H, 5-OH), 9.67 (s, 1H, OH), 9.58 (s, 1H, OH), 7.33 (d, $J$ = 7.3 Hz, 2H, Ar-2′-H, Ar-6′-H), 6.95 (d, $J$ = 8.3 Hz, 2H,Ar-3′-H, Ar-5′-H), 5.98 (d, $J$ = 2.5 Hz, 2H, 6-CH, 8-CH), 5.41 (dd, $J$ = 12.4, 2.4 Hz, 1H, 2-CH), 3.77 (s, 3H, 4′-$OCH_3$), 3.20 (dd, $J$ = 17.0, 12.4 Hz, 1H, 3-CH), 2.75 (dd, $J$ = 17.0, 2.4 Hz, 1H, 3-CH). ESI-MS (m/z) 285.1 ([M-H]$^-$).

Naringenin (**5**) $^1$H NMR (DMSO-$d_6$, 300 MHz): $\delta$ (ppm): 12.39 (s, 1H, 5-OH), 9.67 (s, 1H, OH), 9.58 (s, 1H, OH), 7.33 (d, $J$ = 7.3 Hz, 2H, Ar-2′-H, Ar-6′-H), 6.95 (d, $J$ = 8.3 Hz, 2H, Ar-3′-H, Ar-5′-H), 5.98 (d, $J$ = 2.5 Hz, 2H, 6-CH, 8-CH), 5.41 (dd, $J$ = 12.4, 2.4 Hz, 1H, 2-CH), 3.20 (dd, $J$ = 17.0, 12.4 Hz, 1H, 3-CH), 2.75 (dd, $J$ = 17.0, 2.4 Hz, 1H, 3-CH). ESI-MS (m/z) 271.0 ([M-H]$^-$).

(2*S*)-2,3-Dihydro-5,7-dihydroxy-2-(4-methoxyphenyl)-8-methyl-4H-1-benzopyran-4-one (**6**) $^1$H NMR (DMSO-$d_6$, 300 MHz): $\delta$ (ppm): 12.37 (s, 1H, 5-OH),9.67 (s, 1H, OH), 7.37 (d, $J$ = 8.6 Hz, 2H, Ar-2′-H, Ar-6′-H), 6.94 (d, $J$ = 8.8 Hz, 2H, Ar-3′-H, Ar-5′-H), 5.97 (s, 8-CH), 5.48 (dd, $J$ = 12.0, 3.0 Hz, 1H, 2-CH), 3.83 (s, 3H, 4′-$OCH_3$), 3.20 (dd, $J$ = 16.7, 12.0 Hz, 1H, 3-CH), 2.78 (dd, $J$ = 16.7, 3.0 Hz, 1H, 3-CH), 2.05 (s, 8-$CH_3$). ESI-MS (m/z) 299.1 ([M-H]$^-$). $[\alpha]_D^{17}$: −21.0° ($c$ 0.41, acetone).

(2*S*)-2,3-Dihydro-5,7-dihydroxy-2-(4-hydroxyphenyl)-8-methyl-4H-1-benzopyran-4-one (**7**) $^1$H NMR (DMSO-$d_6$, 300 MHz): $\delta$ (ppm): 12.37 (s, 1H, 5-OH), 9.67 (s, 1H, OH), 7.37 (d, $J$ = 8.6 Hz, 2H, Ar-2′-H, Ar-6′-H), 6.94 (d, $J$ = 8.8 Hz, 2H, Ar-3′-H, Ar-5′-H), 5.97 (s, 8-CH), 5.48 (dd, $J$ = 12.0, 3.0 Hz, 1H, 2-CH), 3.20 (dd, $J$ = 16.7, 12.0 Hz, 1H, 3-CH), 2.78 (dd, $J$ = 16.7, 3.0 Hz, 1H, 3-CH), 2.05 (s, 8-$CH_3$). ESI-MS (m/z) 285.0 ([M-H]$^-$). $[\alpha]_D^{17}$: −22.3° ($c$ 0.41, acetone).

(2*R*,3*S*)-2-(3,4-Dihydroxyphenyl)-3,4-dihydro-2*H*-chromene-3,5,7-triol (**8**) $^1$H NMR ($CDCl_3$, 400 MHz): $\delta$ (ppm): 7.05 (d, 1H, $J$ = 1.8 Hz, Ar-6′-H), 6.83 (dd, 1H, $J$ = 7.8, 1.8 Hz, Ar-2′-H), 6.78 (d, 1H, $J$ = 7.8 Hz, Ar-5′-H), 6.02 (d, 1H, $J$ = 1.8 Hz, H-6), 5.92 (d, 1H, $J$ = 1.8 Hz, H-8), 4.78 (s,1H, H-2), 4.20 (s, 1H, H-3), 2.89 (dd, 1H, $J$ = 4.8, 17.1 Hz, H-4a), 2.72 (dd, 1H, $J$ = 3.0, 17.1 Hz, H-4b). ESI-MS (m/z) 289.0 ([M-H]$^-$). $[\alpha]_D^{22}$: +14° ($c$ 0.41, acetone).

5,7-Dihydroxy-2-(4-methoxyphenyl)-6,8-dimethyl-4H-1-benzopyran-4-one (**9**) $^1$H NMR (DMSO- $d_6$, 300 MHz): $\delta$ (ppm): 12.37 (s, 1H, 5-OH), 9.67 (s, 1H, OH), 7.85 (d, $J$ = 8.6 Hz, 2H, Ar-2′-H, Ar-6′-H), 7.02 (d, $J$ = 8.8 Hz, 2H, Ar-3′-H, Ar-5′-H), 6.58 (s, 3-CH), 3.89 (s, 3H, 4′-$CH_3$), 1.95 (s, 6H, $CH_3$); $^{13}$C NMR (DMSO- $d_6$, 75 MHz): $\delta$ (ppm): 182.0 (C-4), 163.2 (C-7), 160.9 (C-4′), 159.7 (C-2), 155.9 (C-5), 152.3 (C-9), 128.2 (C-2′/C-6′), 121.5 (C-1′), 115.9 (C-3′/C-5′), 106.9 (C-10), 103.4 (C-6), 102.4 (C-3), 101.8 (C-8), 55.1($CH_3$), 8.2 ($CH_3$-6), 7.9 ($CH_3$-8). ESI-MS (m/z) 311.1 ([M-H]$^-$).

Syzalterin (**10**) yellow needles (MeOH); $C_{17}H_{14}O_5$; $^{13}$C NMR (DMSO- $d_6$, 75 MHz): $\delta$ (ppm): 182.0 (C-4), 163.2 (C-7), 160.9 (C-4′), 159.7 (C-2), 155.9 (C-5), 152.3 (C-9), 128.2 (C-2′/C-6′), 121.5 (C-1′),115.9 (C-3′/C-5′), 106.9 (C-10), 103.4 (C-6), 102.4 (C-3), 101.8 (C-8), 8.2 ($CH_3$-6), 7.9 ($CH_3$-8). ESI-MS (m/z) 297.0 ([M-H]$^-$).

2,3-Dihydro-5,7-dimethoxy-2-(4-methoxyphenyl)-6,8-dimethyl-4H-1-benzopytan-4-one (**11**) $^1$H NMR (DMSO-$d_6$, 300 MHz): $\delta$ (ppm): 12.37 (s, 1H, 5-OH), 9.67 (s, 1H, OH), 7.44 (d, $J$ = 8.6 Hz, 2H, Ar-2′-H, Ar-6′-H), 6.98 (d, $J$ = 8.8 Hz, 2H, Ar-3′-H, Ar-5′-H), 5.48 (dd, $J$ = 12.0, 3.0 Hz, 1H, 2-CH), 3.82 (s, 3H, 5-$CH_3$), 3.80 (s, 3H, 7-$CH_3$), 3.77 (s, 3H, 4′-$CH_3$), 3.20 (dd, $J$ = 16.7, 12.0 Hz, 1H, 3-CH), 2.78 (dd, $J$ = 16.7, 3.0 Hz, 1H, 3-CH), 1.95 (s, 6H, $CH_3$); $^{13}$C NMR (DMSO-$d_6$, 125 MHz): $\delta$(ppm): 196.6 (C-4), 160.8 (C-7), 159.8 (C-4′), 159.3 (C-5), 157.8 (C-9), 131.0 (C-1′), 127.5 (C-2′/C-6′), 114.1 (C-3′/C-5′), 102.9 (C-10), 102.0 (C-6 and C-8), 78.5 (C-2), 55.4 (OMe-5, OMe-7 and OMe-4′), 43.3 (C-3), 8.9 ($CH_3$-6), 7.6 ($CH_3$-8). ESI-MS (m/z) 341.1 ([M-H]$^-$).

Matteucinol diacetate (**12**) mp 177–178°C. $^1$H NMR ($CDCl_3$, 300 MHz): $\delta$(ppm): 7.35–7.41 (m, 2H, Ar-2′-H, Ar-6′-H), 6.93–6.99 (m, 2H, Ar-3′-H, Ar-5′-H), 5.42 (d, $J$ = 13.6 Hz, 1H), 3.83 (s, 3 H, OMe), 3.03 (dd, $J$ = 16.6, 13.7 Hz, 1 H), 2.75 (dd, $J$ = 16.6, 2.3 Hz, 1H), 2.42 (s, 3H, Me), 2.03 (s, 3H, Me), 1.95 (s, 3 H, Me);$^{13}$C NMR ($CDCl_3$, 75 MHz): $\delta$(ppm):190.4 (C-4), 169.4 (C-OAc), 167.9 (C-OAc), 159.9 (C-7), 159.1 (C-4′), 153.6 (C-5), 146.5 (C-9), 130.6 (C-1′), 127.5 (C-2′/C-6′), 118.0 (C-3′/C-5′), 117.8 (C-10), 114.2 (C-6), 111.6 (C-8), 79.0 (C-2), 55.4 (OMe-4′), 45.2 (C-3), 21.0 ($CH_3$-OAc), 20.4 ($CH_3$-OAc), 9.6 ($CH_3$-6), 9.2 ($CH_3$-8). ESI-MS (m/z) 397.1 ([M-H]$^-$).

5-Acetoxy-2,3-dihydro-7-dimethoxy-2-(4-methoxyphenyl)-6,8-dimethyl-4H-1-benzopytan-4-one (**13**) $^1$H NMR ($CDCl_3$, 300 MHz): $\delta$ (ppm): 7.35–7.41 (m, 2 H, Ar-2′-H, Ar-6′-H), 6.93–6.99 (m, 2 H, Ar-3′-H, Ar-5′-H), 5.42 (d, $J$ = 13.6 Hz, 1 H), 3.83 (s, 3 H, OMe), 3.80 (s, 3H, 7-$CH_3$), 3.03 (dd, $J$ = 16.6, 13.7 Hz, 1 H), 2.75 (dd, $J$ = 16.6, 2.3 Hz, 1 H), 2.42 (s, 3 H, Me), 2.03 (s, 3 H, Me), 1.95 (s, 3 H, Me); $^{13}$C NMR ($CDCl_3$, 75 MHz): $\delta$(ppm): 190.4 (C-4), 167.9 (C-OAc), 159.9 (C-7), 159.1 (C-4′), 153.6 (C-5), 146.5 (C-9), 130.6 (C-1′), 127.5 (C-2′/C-6′), 118.0 (C-3′/C-5′), 117.8 (C-10), 114.2 (C-6), 111.6 (C-8), 79.0 (C-2), 55.4 (OMe-4′), 45.2 (C-3), 20.4 ($CH_3$-OAc), 9.6 ($CH_3$-6), 9.2 ($CH_3$-8). ESI-MS (m/z) 369.1 ([M-H]$^-$).

5, 7, 4′-Trimethoxy-6, 8-dimethylflavan-4-ol (**14**) $^1$H NMR ($CDCl_3$, 300 MHz): $\delta$ (ppm): 7.38 (d, 2H, $J$ = 8.7 Hz, Ar-2′-H, Ar-6′-H), 6.93 (d, 2H, $J$ = 8.7 Hz, Ar-3′-H, Ar-5′-H), 5.25 (t, 1H, $J$ = 8.5 Hz, H-4), 4.97 (dd, 1H, $J$ = 12.0, 1.7 Hz, H-2), 3.87 (s, 3H, OMe-5), 3.82 (s, 3H, OMe-7), 3.74 (s, 3H, OMe-4′), 2.48 (ddd, 1H, $J$ = 13.5, 8.5, 1.7 Hz, H-3a), 2.25 (ddd, 1H, $J$ = 13.5, 12.0, 8.5 Hz, H-3b), 1.95 (s, 6H, 6-$CH_3$, 8-$CH_3$); $^{13}$C NMR ($CDCl_3$, 75 MHz): $\delta$(ppm): 160.6 (C-7), 159.6 (C-4′), 159.3 (C-5), 156.7 (C-9), 132.4 (C-1′), 127.7 (C-2′ and C-6′),

114.0 (C-3′and C-5′), 107.2 (C-10), 116.0 (C-8), 115.4 (C-6), 76.9 (C-2), 63.5 (C-4), 55.6, 55.3 (OMe-5 and OMe-7), 55.4 (C-4′), 37.6 (C-3). ESI-MS (m/z) 343.1 ([M-H]$^-$).

7, 4′-Dihydroxy-5-methoxy-6, 8-dimethylflavan-4-ol (**15**) $^1$H NMR ($CDCl_3$, 300 MHz): $\delta$ (ppm): 7.38 (d, 2H, $J$ = 8.7 Hz, Ar-2′-H, Ar-6′-H), 6.93 (d, 2H, $J$ = 8.7 Hz, Ar-3′-H, Ar-5′-H), 5.25 (t, 1H, $J$ = 8.5 Hz, H-4), 4.97 (dd, 1H, $J$ = 12.0, 1.7 Hz, H-2), 3.91 (s, 1H, OH), 3.87 (s, 3H, OMe-5), 2.48 (ddd, 1H, $J$ = 13.5, 8.5, 1.7 Hz, H-3a), 2.25 (ddd, 1H, $J$ = 13.5, 12.0, 8.5 Hz, H-3b), 1.95(s, 6H, 6-$CH_3$, 8-$CH_3$); $^{13}$C NMR ($CDCl_3$, 75 MHz): $\delta$(ppm): 160.6 (C-7), 159.6 (C-4′), 159.3 (C-5), 156.7 (C-9), 132.4 (C-1′), 127.7 (C-2′and C-6′), 116.0 (C-8), 115.4 (C-6), 114.0 (C-3′and C-5′), 107.2 (C-10), 76.9 (C-2), 63.5 (C-4), 55.6 (OMe-5), 37.6 (C-3). ESI-MS (m/z) 315.1 ([M-H]$^-$).

5-Hydroxy-7, 4′-dimethoxy-6, 8-dimethylflavan (**16**) $^1$H NMR ($CDCl_3$, 300 MHz): $\delta$ (ppm): 7.41 (d, 2H, $J$ = 8.6 Hz, Ar-2′-H, Ar-6′-H), 7.09 (d, 2H, $J$ = 8.6 Hz, Ar-3′-H, Ar-5′-H), 4.98 (dd, 1H, $J$ = 10.3, 2.4 Hz, H-2), 3.71 (s, 3H, $OCH_3$), 2.6-2.7 (m, 2H), 2.2-2.3 (m,1H), 1.9-2.1 (m, 1H). 1.95 (s, 6H, 6-$CH_3$, 8-$CH_3$); $^{13}$C NMR ($CDCl_3$, 75 MHz): $\delta$(ppm): 159.1 (C-9), 156.4 (C-7), 154.4 (C-4′), 150.1 (C-5), 139.0 (C-1′), 127.1 (C-2′and C-6′), 121.5 (C-3′and C-5′), 107.5 (C-6), 106.9 (C-8), 101.8 (C-10), 77.1 (C-2), 55.3 (OMe-5, 4′), 29.5 (C-4), 21.2 (C-3), 8.9 ($CH_3$-6), 8.8 ($CH_3$-6). ESI-MS (m/z) 313.1 ([M-H]$^-$).

7-Acetoxy-5-hydroxy-4′-methoxy-6, 8-dimethylflavan (**17**) $^1$H NMR ($CDCl_3$, 300 MHz): $\delta$ (ppm): 7.31 (d, 2H, $J$ = 8.6 Hz, Ar-2′-H, Ar-6′-H), 6.93 (d, 2H, $J$ = 8.6 Hz, Ar-3′-H, Ar-5′-H), 4.98 (dd, 1H, $J$ = 10.3, 2.4 Hz, H-2), 3.71 (s, 3H, $OCH_3$), 2.6-2.7 (m 2H), 2.29 (s, 3H, OAc), 2.2-2.3 (m, 1H), 1.9-2.1 (m, 1H), 1.95 (s, 6H, 6-$CH_3$, 8-$CH_3$). $^{13}$C NMR ($CDCl_3$,75 MHz): $\delta$(ppm): 169.4 (C-OAc), 159.1 (C-9), 156.4 (C-7), 154.4 (C-4′), 150.1 (C-5), 134.0 (C-1′), 127.1 (C-2′and C-6′), 113.8 (C-3′and C-5′), 110.4 (C-10), 107.5 (C-6), 106.9 (C-8), 77.1 (C-2), 55.3 (OMe-5, 4′), 29.5 (C-4), 21.2 (C-3), 8.9 ($CH_3$-6), 8.8 ($CH_3$-6). ESI-MS (m/z) 341.1 ([M-H]$^-$).

5, 7, 4′-Trihydroxy-6, 8-dimethylflavan-4-ol (**18**) $^1$H NMR ($CDCl_3$, 300 MHz): $\delta$ (ppm): 7.32 (d, 2H, $J$ = 8.6 Hz, Ar-2′-H, Ar-6′-H), 6.93 (d, 2H, $J$ = 8.6 Hz, Ar-3′-H, Ar-5′-H), 4.98 (dd, 1H, $J$ = 10.3, 2.4 Hz, H-2), 2.6-2.7 (m, 2H), 1.9-2.1 (m, 1H), 2.2-2.3 (m,1H), 1.95 (s, 6H, 6-$CH_3$, 8-$CH_3$); $^{13}$C NMR ($CDCl_3$, 75 MHz): $\delta$ (ppm): 159.1 (C-9), 156.4 (C-7), 154.4 (C-4′), 150.1 (C-5), 134.0 (C-1′), 127.1 (C-2′and C-6′), 113.8 (C-3′and C-5′), 110.4 (C-10), 107.5 (C-6), 106.9 (C-8), 77.1 (C-2), 29.5 (C-4), 21.2 (C-3), 8.9 ($CH_3$-6), 8.8 ($CH_3$-6). ESI-MS (m/z) 285.1 ([M-H]$^-$).

## Cell culture and treatment

Acute promyelocytic leukaemia (HL-60) cell lines and human hepatoma (SMMC-7721) were grown and maintained in RPMI-1640 medium, pH 7.4. The media was supplemented with FCS (10%), penicillin (100 U/ml) and streptomycin (100 mg/ml), being referred to as complete medium. The cells were grown in a $CO_2$ incubator (Hera Cell, Heraeus, Germany) at 37°C with 90% humidity and 5% $CO_2$. All experiments were performed with cells in the logarithmic growth phase.

### *Cytotoxicity assay*

The cytotoxicity of compounds **1–18**, toward HL-60 and SMMC-7721 cell lines was determined in 96-well microtitre plates by the sulforhodamine B method. Briefly, exponentially growing HL-60 and SMMC-7721 cells were harvested and seeded in 96-well plates with the final volume 100 µl containing $4 \times 10^3$ cells/well. After 24-h incubation, cells were treated with various concentrations of **1–18** for 48 h. The cultures were fixed at 4°C for 1 h by addition of ice-cold 50% TCA to give a final concentration of 10%. Fixed cells were rinsed five times with de-ionized $H_2O$ and stained for 10 min with 0.4% sulforhodamine B dissolved in 0.1% HOAc. The wells were washed five times with 0.1% HOAc and left to dry overnight. The absorbed sulforhodamine B was dissolved in 150 µl unbuffered 1% Tris base (tris(hydroxymethyl) aminomethane) solution in $H_2O$ (pH 10.5). The absorbance of extracted sulforhodamine B at 515 nm was measured on a microplate reader (Bio-Rad). The experiments were carried out in triplicate. Each run entailed five or six concentrations of the compounds being tested. The percentage survival rates of cells exposed to the compounds were calculated by assuming the survival rate of untreated cells to be 100%.

## Statistical analysis

All experiments were carried out three times unless mentioned in the procedure. Continuous variables were presented as mean ± SD. The IC50 was determined as the drug concentration that resulted in a 50% reduction in cell viability or inhibition of the biological activity. IC50 values were calculated using a four parameter logistic curve (SigmaPlot 11.0). All data were statistically evaluated using Student's *t*-test or the Kruskal–Wallis test (GraphPad Prism 5.01; GraphPad Software, Inc., San Diego, CA, USA), followed by Dunn's post-hoc multiple comparison test when the significance value was < 0.05, using the same significance level. The criterion for statistical significance was generally taken as $P < 0.05$.

# Results

The alcoholic extract of the leaves and twigs of *R. hainanense* led to the isolation of eight flavonoids (**1**–**8**), matteucinol (**1**), farrerol (**2**), 2,3-dihydro-5-hydroxy-7-methoxy-2-(4-methoxyphenyl)-6,8-dimethyl-4H-1-benzopyran-4-one (**3**), 4′-methylnaringenin (**4**), naringenin (**5**), (2*S*)-2,3-dihydro-

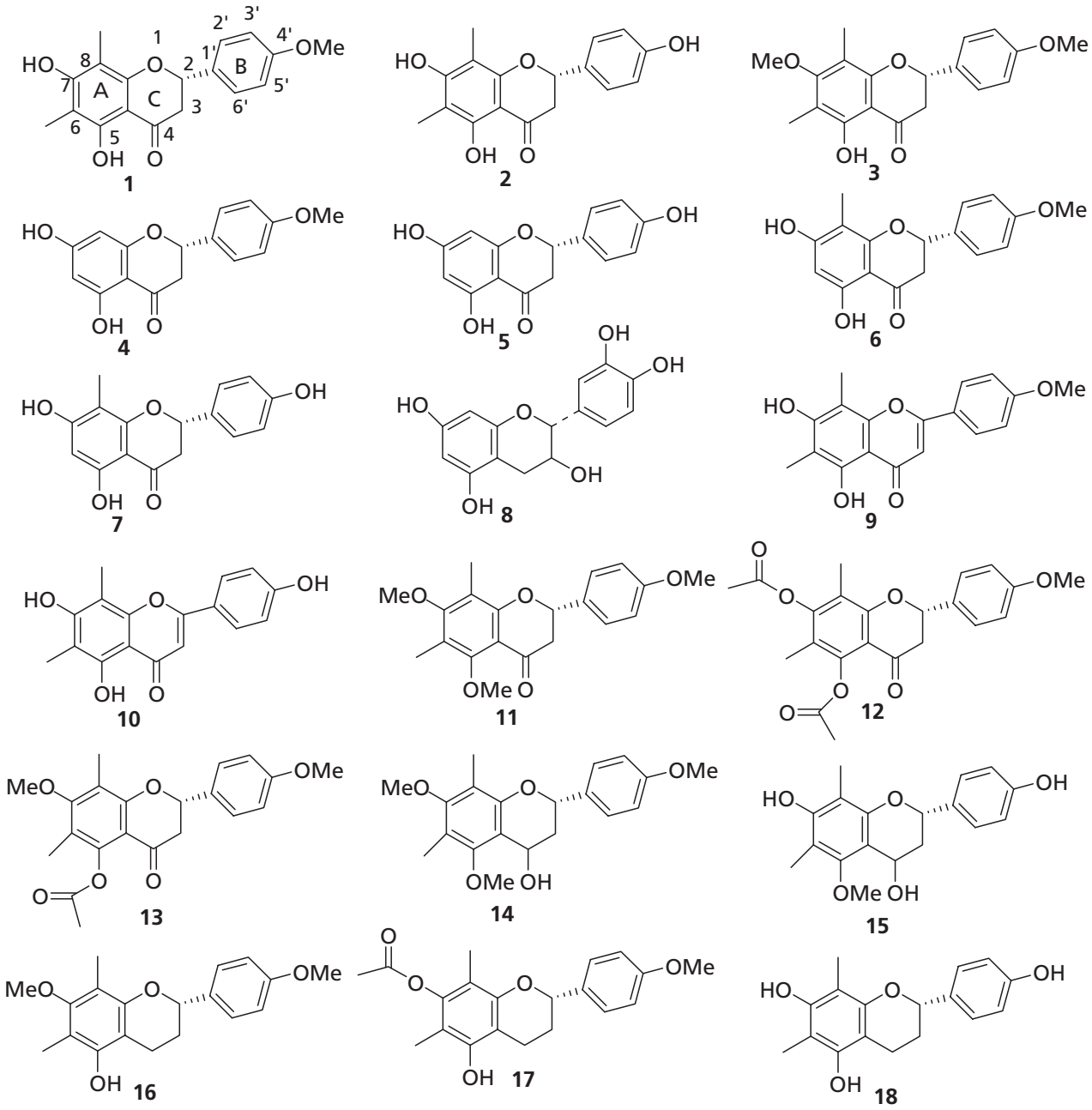


**Figure 2** The structures of flavonoids **1**–**18** extracted from the leaves and twigs of *Rhododendron hainanense*.

5,7-dihydroxy-2-(4-methoxyphenyl)-8-methyl-4H-1-benzopyran-4-one (**6**), (2*S*)-2,3-dihydro-5,7-dihydroxy-2-(4-hydroxyphenyl)-8-methyl-4H-1-benzopyran-4-one (**7**) and (2*R*,3*S*)-2-(3,4-dihydroxyphenyl)-3,4-dihydro-2H-chromene-3,5,7-triol (**8**).[13–19] To determine the changes of bioactivity resulting from different substituents of A, B ring and the type of C ring, matteucinol (**1**) was modified to the target compounds according to the methods described in Figure 1. The structures of all compounds have been described in Figure 2. The compounds were assayed for their inhibitory activity against HL-60 and SMMC-7721 cell lines, and the results are presented in Table 1. Mitomycin was used as the positive control in this assay. Compounds **3** and **16** exhibited potent inhibitory activity against HL-60 cells with lower IC50 values of 15.2 and 13.2 μM, respectively. Compound **8** showed moderate inhibitory activity against HL-60 and SMMC-7721 cells with IC50 values of 61.7 and 78.9 μM, respectively. Compounds **9**, **11**, **14** and **15** showed weak

**Table 1** Cytotoxicity of compounds **1–18** extracted from the leaves and twigs of *Rhododendron hainanense*

| Flavonoids | IC50 (μM) | |
|---|---|---|
| | HL-60 | SMMC-7721 |
| Mitomycin[a] | 1.67 ± 0.58 | 5.53 ± 1.58 |
| **1** | 160.1 ± 3.8 | 131.6 ± 1.2 |
| **2** | 169.3 ± 3.3 | 227.4 ± 27.0 |
| **3** | 15.2 ± 2.4 | 83.5 ± 22.5 |
| **4** | 186.7 ± 13.3 | 304.1 ± 0.3 |
| **5** | 217.6 ± 4.0 | 661.0 ± 16.1 |
| **6** | 205.0 ± 9.6 | 214.0 ± 10.0 |
| **7** | NT[b] | NT[b] |
| **8** | 61.7 ± 1.7 | 78.9 ± 2.3 |
| **9** | 93.8 ± 5.1 | 132.9 ± 25.3 |
| **10** | ND[c] | |
| **11** | 89.4 ± 26.0 | 90.0 ± 11.9 |
| **12** | ND[c] | |
| **13** | 135.0 ± 3.7 | 247.0 ± 18.3 |
| **14** | 89.2 ± 16.8 | 149.3 ± 17.1 |
| **15** | 84.4 ± 3.4 | 198.8 ± 4.5 |
| **16** | 13.2 ± 0.9 | 103.7 ± 3.1 |
| **17** | ND[c] | |
| **18** | 264.6 ± 4.2 | 445.5 ± 25.3 |

[a]Positive control. [b]Not Tested. [c]ND: The IC50 value was not detected because of poor sample solubility. IC50 value: the drug concentration that resulted in a 50% reduction in cell viability or inhibition of the biological activity. The data shown are means ± SD obtained from three independent experiments.

cytotoxic activity against HL-60 and SMMC-7721 cells, with IC50 values in the range of 100–200 μM. The IC50 values of compounds **10**, **12** and **17** were not detected because of the poor sample solubility.

## Discussion

The compounds were assayed for their inhibitory activity against HL-60 and SMMC-7721 cell lines (see Table 1). Compounds **3** and **16** exhibited the strongest inhibitory activity against HL-60 with lower IC50 values of 15.2 and 13.2 μM, respectively. Comparison of the activity of compounds **1**/**2**, **4**/**5**, **6**/**7** and **16**/**17** suggested that the presence of a methoxy group instead of the hydroxyl group at $C_4'$ of the B ring increased the antitumour activity. The loss of this methoxy substituent reduced the antitumour potency with respect to the parent compound **3**. The fact that the cytotoxicity of flavonoids was enhanced by methoxyl substitution on the B ring and reduced by hydroxyl substitution led us to believe that the influence of the substituents was not due to their electronic properties. In addition, compounds **3** and **11**, with $C_7$-methoxy of the A ring, showed more activity compared with matteucinol (**1**). From the compounds of the same skeleton, compound **3** was more active than compounds **11** and **13**, indicating that the phenolic hydroxyl group at C-5 was required for the activity. The most striking evidence was the favourable influence of lipophilicity on cytotoxicity (the methoxy group at 7 and to a lesser extent at 4′ made compounds more lipophilic; the same applied for an OH at 5 because this OH formed a hydrogen bond with the carbonyl group, making the structure more lipophilic). When examining the influence of C-methyl group, compounds **1** and **2**, with the C-methyl group at the 6, 8-position, were more potent than compounds **6** and **7** with a C-methyl group at the 8-position. Therefore, the existence of the C-methyl substitutents at C-6 and C-8 was also important for antitumour activity. The carbonyl group on the C ring may not affect the activity because compound **11** showed slightly higher activity than compound **14** with a hydroxyl group. These results confirmed that methylation of 7, 4′-hydroxyl groups together with the presence of the C-methyl group at the 6, 8-position seemed to be the most favourable structural condition for antitumour activity.

## Conclusions

Flavonoids are an important class of natural compounds with significant potential to cure, treat and prevent tumour, senescence and cardiovascular diseases. Flavanones have been a potential source in the search for lead compounds and biologically active components, and have been the focus of much research and development in the last 30 years.[20] We have isolated and modified the flavonoids from *R. hainanense* and evaluated their cytotoxicity. Within the series of flavonoid derivatives in this study, we found that compounds **3** and **16** were the most active. Compound **3** is a natural product isolated from *R. hainanense* and to our knowledge, little antitumour activity data has been reported for it. Compound **3** could also be synthesized from matteucinol using a simple synthetic method. Compound **16** was a new modified compound from the natural product matteucinol. This renewed attention to flavonoid derivatives revealed the possibility that compounds **3** and **16** could be considered as lead compounds for the development of new antitumour agents. Our results not only enrich the family of active flavonoids from natural sources, but encourage the synthesis of flavonoid analogues for improving cytotoxic activity.

## Declarations

### Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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