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Cytotoxic and anti-HIV-1 constituents from leaves and twigs of *Gardenia tubifera*

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Abstract—Two new natural cycloartanes, tubiferolide methyl ester (**1**) and tubiferaoctanolide (**2**), together with the known coronalolide (**3**) and coronalolide methyl ester (**4**) have been isolated from leaves and twigs of *Gardenia tubifera*. In addition, a new flavone 5,3′,5′-trihydroxy-7,4′-dimethoxyflavone (**5**), five known flavones **6**–**10** and hexacosyl 4′-hydroxy-*trans*-cinnamate (**11**) were also obtained from the same source. The structures were assigned on the basis of spectroscopic methods. Compounds **3**, **7**, **9**, and **10** showed significant cytotoxic activities only in P-388 cell line. Compound **1** was cytotoxic against P-388, KB, Col-2 and Lu-1, while **4** was active in P-388 and BCA-1. Compounds **3** and **4** displayed significant anti-HIV activities in the HIV-1RT assay; compound **7** showed moderate activity in this assay. Compounds **5**–**10** were also found to be active in the $^{\Delta Tat/Rev}$MC 99 syncytium assay.

1. Introduction

Several species of plants in *Gardenia* genus (Rubiaceae) have been used ethnomedically in various countries, primarily for abortifacient[1] and contraceptive[1–3] purposes. Some species are used as a febrifuge[4] and a larvicide,[5] as well as for the treatment of headaches,[6] asthma[7] and malaria.[8] Extracts of various species exhibiting anti-implantation and abortifacient effects,[9] anti-ulcer,[10] anti-bacterial,[11] diuretic,[12] analgesic,[12] hypertensive,[12] and larvicidal activity[13] have been previously reported. Our investigations of *G. coronaria* and *G. sootepensis*[14] led to the isolation of four ring-A *seco*-cycloartanes, and the work on *G. obtusifolia*[15] yielded one ring-A *seco*-cycloartane, two cycloartanes and five flavones. Herein we describe the investigation of hexane and chloroform extracts of *G. tubifera* which led to the isolation of tubiferolide methyl ester (**1**), tubiferaoctanolide (**2**), coronalolide (**3**), coronalolide methyl ester (**4**), 5,3′,5′-trihydroxy-7,4′-dimethoxyflavone (**5**), 5,3′,5′-trihydroxy-3,6,7,4′-tetramethoxyflavone (**6**), 5,7,4′-trihydroxy-6-methoxyflavone (**7**), 5,7,3′-trihydroxy-6,4′,5′-trimethoxyflavone (**8**), 5,3′-dihydroxy-7,4′,5′-trimethoxyflavone (**9**), 5,3′-dihydroxy-6,7,4′,5′-tetramethoxyflavone (**10**), and hexacosyl 4′-hydroxy-*trans*-cinnamate (**11**) (see Fig. 1). Compounds **1**, **2** and **5** have not been isolated previously. The structures of all isolated compounds were elucidated on the basis of spectroscopic methods. The results from cytotoxic and anti-HIV-1 activity evaluations are also included.

2. Results and discussion

Tubiferolide methyl ester (**1**) was shown to possess a molecular formula $C_{31}H_{46}O_4$ by HR-FABMS ($[M+H]^+$= 483.3458). Its IR ($CHCl_3$) spectrum displayed two carbonyl absorptions at 1755 and 1734 cm^{-1}, corresponding to the C=O stretching of α,β-unsaturated lactone and C=O stretching of ester, respectively. The ^1H NMR spectral data of **1** (Table 1) were similar to those of coronalolide methyl ester (**4**) obtained from the same species and previously isolated from *G. coronaria* and *G. sootepensis*.[14] Compound **1** exhibited a characteristic pair of doublets at δ 0.17 and 0.43 (J=5.3 Hz each), assignable to C-19 methylene protons of the cyclopropane ring of a cycloartane triterpene.[16–23] Two doublets at δ 6.34 (J=2.5 Hz) and 5.74 (J=2.1 Hz) were ascribed to H-28a and H-28b in exocyclic methylene γ-lactone ring, while the signals of the β and γ-methine protons of the lactone ring appeared at δ 3.24 (br d,

Keywords: Ring-A *seco*-cycloartane; Octanolide; Flavones; Cytotoxic activity; Anti-HIV activity.
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Table 1. ^{1}H and ^{13}C NMR data of compounds **1** and **2**

Position	δ_H[a]		δ_C[b]	
	1[c]	**2**[d]	**1**[e]	**2**[f]
1	(a) 2.23 (m) (b) 1.59 (obsc.)	(a) 2.10 (obsc.) (b) 1.30 (obsc.)	30.95	29.07
2	(a) 2.53 (m) (b) 2.44 (m)	(a) 2.61 (m) (b) 2.15 (obsc.)	31.25	32.00
3	—	—	173.42	173.66
4	—	—	139.27	147.33
5	3.24 (br d, 8.3)	2.35 (m)	39.14	38.61
6	4.75 (td, 8.3, 6.5)	(a) 1.75 (obsc.) (b) 0. 67 (obsc.)	74.46	29.47
7	(a) 1.78 (obsc.) (b) 1.53 (obsc.)	(a) 1.25 (obsc.) (b) 0.94 (obsc.)	27.26	25.30
8	2.14 (br t, 5.7)	1.52 (obsc.)	38.37	47.89
9	—	—	25.18	21.00
10	—	—	28.26	27.42
11	(a) 1.75 (obsc.) (b) 1.64 (obsc.)	(a) 2.13 (obsc.) (b) 1.21 (obsc.)	26.61	26.74
12	(a) 1.70 (obsc.) (b) 1.60 (obsc.)	(a) 1.65 (obsc.) (b) 0.88 (obsc.)	33.01	32.94
13	—	—	45.70	45.11
14	—	—	48.66	48.87
15	1.30 (obsc.)	(a) 1.23 (obsc.) (b) 0.89 (obsc.)	34.84	35.70
16	(a) 1.93 (obsc.) (b) 1.32 (obsc.)	(a) 1.84 (obsc.) (b) 1.24 (obsc.)	27.72	28.05
17	1.62 (obsc.)	1.55 (obsc.)	51.48	52.23
18	0.93 (s)	0.91 (s)	15.91	18.13
19	(a) 0.43 (d, 5.3) (b) 0.17 (d, 5.3)	(a) 0.76 (d, 4.0) (b) 0.46 (d, 4.0)	23.18	30.23
20	1.47 (obsc.)	1.35 (obsc.)	35.87	35.86
21	0.89 (d, 6.4)	0.83 (d, 6.3)	18.40	18.19
22	(a) 1.42 (obsc.) (b) 1.06 (obsc.)	1.37 (obsc.)	36.33	36.29
23	(a) 2.08 (obsc.) (b) 1.86 (obsc.)	(a) 1.98 (obsc.) (b) 1.77 (obsc.)	24.88	24.92
24	5.10 (br t, 7.0)	5.04 (br t, 6.9)	125.05	125.20
25	—	—	131.02	130.90
26	1.69 (br s)	1.62 (br s)	25.66	25.71
27	1.61 (br s)	1.54 (br s)	17.60	17.62
28	(a) 6.34 (d, 2.5) (b) 5.74 (d, 2.1)	(a) 5.22 (br s) (b) 5.11 (br s)	122.95	116.13
29	—	(a) 4.56 (d, 11.3) (b) 4.30 (d, 11.3)	170.64	68.23
30	0.91 (s)	0.86 (s)	20.07	19.35
OMe	3.70 (s)	—	51.75	—

[a] Chemical shift given in ppm using TMS as internal reference; multiplicities and coupling constants (Hz) are given in parentheses; obsc.=obscured signal.
[b] Chemical shift given in ppm; $CDCl_3$ signal at δ_C 77.00 as reference.
[c] Recorded at 500 MHz.
[d] Recorded at 300 MHz.
[e] Recorded at 125 MHz.
[f] Recorded at 75 MHz.

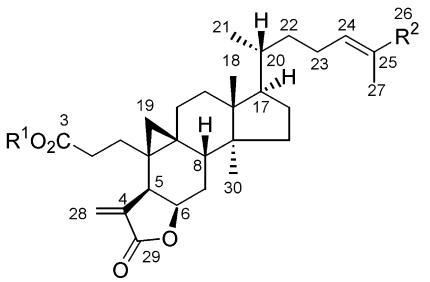
Table 2. Observed HMBC correlations in compounds **1** and **2**

HMBC		
C	**1** correlated H	**2** correlated H
1	5, 2a, 2b, 8 (w), 11b, 19a, 19b	19a, 19b
2	1a, 1b	1b
3	1a, 1b, 2a, 2b, 31	2a, 2b, 29a, 29b
4	5, 6, 28a	5, 28a, 28b, 29a, 29b
5	1a, 1b, 6 (w), 7a, 28a, 28b	1b, 19a, 19b, 28a, 28b
6	5, 7a, 7b, 8	5, 7a, 8, 28a, 28b
7	5, 8	8, 11b
8	1a, 6, 7a, 7b, 19a, 19b, 30	7a, 19a, 19b
9	1a, 8, 7a, 7b, 11a, 11b, 12b, 19a, 19b	1a, 7a, 8, 11a, 12a, 19a, 19b
10	1a, 1b, 2a, 2b, 5, 6, 7a, 8b, 11b, 19a, 19b, 28a	5, 1b, 19a, 19b
11	7a, 7b, 19a, 19b	12a, 19a, 19b
12	11a, 11b, 17, 18	18, 11a
13	8, 11a, 12a, 12b, 15b, 16a, 16b, 30	12a, 18, 20, 30
14	8, 7a, 7b, 12b, 15, 16b, 18	8, 12a, 17, 18, 30
15	8, 16a, 16b, 30	30
16	15, 17, 20	15a, 20
17	16a, 16b, 18, 20, 21	18, 30
18	12b, 17	12a, 17, 20
19	5, 7a, 8, 11a, 11b, 12b	1b, 11a, 8
20	17, 21, 22a, 22b	21
21	20, 22a	17, 20, 22
22	21, 23a, 23b, 24	21
23	22a, 22b	17, 22
24	20, 22a, 22b, 23a, 23b, 26, 27	26, 27
25	23a, 23b, 26, 27	26, 27
26	24, 27	24, 27
27	22a, 24, 26	26
28	5	5, 29a, 29b
29	5, 6, 28a, 28b	5, 28a, 28b
30	8, 15	8, 15a
31	31	—

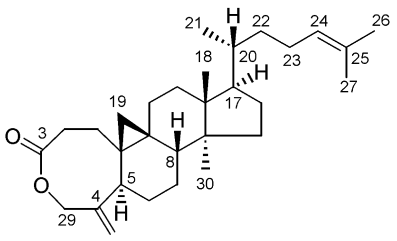
w=weak correlation.

J=8.3 Hz, H-5) and 4.75 (td, J=8.3, 6.5 Hz, H-6), respectively. When compared to the data of the known compound **4**, differences were found only for the side-chain signals. A broad triplet of an olefinic proton (H-24) at δ 5.10 (J=7 Hz), together with two broad singlets of two methyl groups at δ 1.69 (26-Me) and 1.61 (27-Me) indicated the presence of a terminal dimethylvinyl group in the side-chain of **1**. The structural feature of the side-chain as shown in **1** was confirmed by the EI-MS fragment ions at *m/z* 371 $(M-C_8H_{15})^+$ and 111 $(C_8H_{15})^+$ which were consistent with a cleavage of the bond between C-17 and C-20. Apart from the singlets of 18-Me and 30-Me at δ 0.93 and 0.91, a doublet of a secondary methyl group (21-Me) was observed at δ 0.89 (J=6.4 Hz). The ^{13}C NMR spectrum of **1** (Table 1) was analyzed and found to consist of two carbonyl carbons, five methyl carbons, eleven methylene carbons, six methine carbons and six quarternary carbons. Assignments of the ^{1}H and ^{13}C signals as shown in Table 1 were carried out on the basis of 2D-NMR spectral data (see Section 5 and Table 2) and by direct comparison of the chemical shifts with those of similar compounds reported in the literature.[14] The HMBC correlations of 18-Me signal to C-12, C-14 and C-17 signals confirmed the assignment of 18-Me signal, while the correlations of 30-Me signals to C-8, C-13 and C-15 supported the identification of 30-Me. The relative stereochemistry of **1** was determined on the basis of NOE difference data (see Fig. 2).

Tubiferaoctanolide **2** ($C_{30}H_{46}O_2$) showed an $[M+H]^+$ peak at 439.3546 in the HRMS. The IR absorptions at 1724 and 1647 cm^{-1} indicated the presence of C=O and C=C units in the structure. The ^{1}H NMR spectral data (Table 1) of **2** displayed a characteristic pair of doublets (δ 0.46 and 0.76, J=4.0 Hz each) of the methylene protons in cyclopropane ring of a cycloartane triterpene. Compound **2** was found to possess the same side-chain as **1**, as a broad triplet of



1 $R^1 = R^2 = Me$
3 $R^1 = H$, $R^2 = CHO$
4 $R^1 = Me$, $R^2 = CHO$



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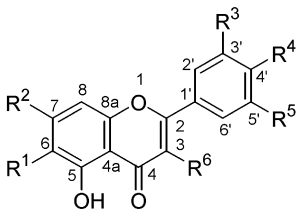
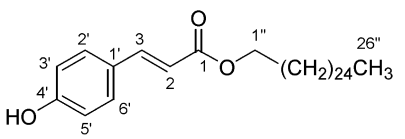


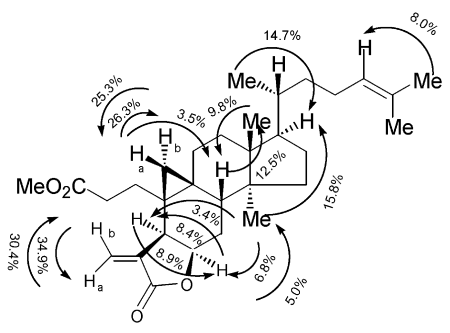
5 $R^1 = R^6 = H$, $R^2 = R^4 = OMe$, $R^3 = R^5 = OH$
6 $R^1 = R^2 = R^4 = R^6 = OMe$, $R^3 = R^5 = OH$
7 $R^1 = OMe$, $R^2 = R^4 = OH$, $R^3 = R^5 = R^6 = H$
8 $R^1 = R^4 = R^5 = OMe$, $R^2 = R^3 = OH$, $R^6 = H$
9 $R^1 = R^6 = H$, $R^2 = R^4 = R^5 = OMe$, $R^3 = OH$
10 $R^1 = R^2 = R^4 = R^5 = OMe$, $R^3 = OH$, $R^6 = H$



11

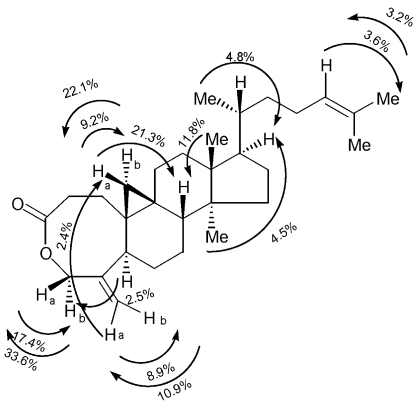
Figure 1.

olefinic H-24 (δ 5.04, J=6.9 Hz), together with two broad singlets of two methyls (δ 1.62 for 26-Me and 1.54 for 27-Me) in dimethylvinyl moiety were observed. The signal of 21-Me in the side-chain appeared as a doublet at δ 0.83 (J=6.3 Hz). The fragment ions due to the loss of side-chain at m/z 327 for $(M-C_8H_{15})^+$ and 111 $(C_8H_{15})^+$ in the EI-MS confirmed the skeleton of the side-chain. In addition, the two singlets at δ 0.91 (3H) and 0.86 (3H) were assigned to the signals of 18-Me and 30-Me, respectively. The HMBC correlations of 18-Me to C-12, C-13 and C-14; C-18 to H-12a, H-17 and H-20; 30-Me to C-13, C-14, C-15 and C-17; C-30 to H-8 and H-15a confirmed the assignments of these two angular methyls. Information from the ^{13}C NMR and DEPT spectra indicated the presence of carbonyl carbon of an ester or a lactone at δ 173.66 which was confirmed to be at C-3 by the observed HMBC correlations of H-2a and H-2b to C-3. Instead of having the signals corresponding to gem-dimethyl groups at C-4 as in typical cycloartanes, two broad singlets of sp^2 methylene signals of H-28a and H-28b (δ 5.22 and 5.11) and a pair of low-field doublets (δ 4.56 and 4.30, J=11.3 Hz each) of *gem*-methylene (H-29a and H-29b) on a carbon bearing an oxygen atom were observed. The observed HMBC correlations of H-28a and H-28b signals to C-4 signal, as well as H-29a and H-29b signals to C-4 signal confirmed the connection of C-28 and C-29 to C-4. The oxygen atom on C-29 was found to be attached to C-3, as HMBC correlations of H-29a and H-29b to C-3 were observed. Other connectivities which established the structure of **2** were obtained from HMBC correlation data (Table 2). The NOE difference data which support the assignments of the relative stereochemistry are shown in Figure 3.



1

Figure 2. NOE enhancements observed in compound **1**.



2

Figure 3. NOE enhancements observed in compound **2**.

Table 3. Cytotoxic and antimitotic activities (ASK assay) of the isolated compounds **1**–**11**

Compound	Cytotoxicity (ED_{50}, μg/mL)					ASK assay
	Cell line					
	P-388	KB	Col-2	BCA-1	Lu-1	
1	0.89	1.48	2.28	8.39	1.89	−
2	>20	>20	>20	>20	>20	−
3	1.73	9.11	12.06	>20	10.83	−
4	1.50	15.10	10.70	3.59	18.22	−
5	9.07	13.32	>20	>20	>20	−
6	6.82	10.97	>20	>20	18.83	−
7	2.38	6.53	18.33	>20	19.95	−
8	7.03	16.11	17.27	18.65	>20	−
9	3.00	7.17	>20	>20	>20	−
10	2.82	6.31	14.78	10.08	8.18	−
11	>20	>20	>20	>20	>20	−
Ellipticine	0.58	0.56	0.58	0.77	0.47	

Cytotoxic assay: $ED_{50} \leq 5$ μg/mL is considered active; P-388: murine lymphocytic leukemia, KB: human nasopharyngeal carcinoma, Col-2: human colon cancer, BCA-1: human breast cancer, Lu-1: human lung cancer, Ellipticine, an anticancer drug, was used as a positive control in the cytotoxicity test; ASK: rat glioma, ASK assay: +=active; −=inactive.

The new flavone **5** exhibited an $[M]^+$ peak at *m*/*z* 330 in the EI-MS, corresponding to a molecular formula $C_{17}H_{14}O_7$. The UV maxima at 269 and 330 nm were consistent with those observed in flavone derivatives. The presence of phenolic and the conjugated carbonyl groups in flavone **5** were indicated in its IR spectrum at 3466 (phenolic O–H), 1667 (conj. C=O), 1614 (C=C) and 1199 (C–O of phenol) cm^{-1}. The presence of a chelated 5-OH signal at δ 12.83 (1H) and a free OH signal at δ 9.59 (2H) in the ^1H NMR spectrum (see Section 5) confirmed that **5** was triphenolic. In addition, **5** was found to possess two methoxyl groups; the two methoxyl signals resonated at δ 3.83 (3H) and 3.75 (3H). A characteristic singlet signal of H-3 in flavone was observed at δ 6.66. The *J* value of 2.1 Hz observed at the aromatic signals [δ 6.67 (H-6) and 6.33 (H-8)] indicated the *meta*-oxygenation pattern in ring A. The remaining aromatic signal at δ 6.97 (s, 2H) was assigned to H-2′and H-6′of ring B. A series of NOE enhancement experiments was performed to prove the oxy-genation pattern in **5**. The signals of H-6 and H-8 were enhanced by 15.2 and 14%, respectively, when the signal of 7-OMe was irradiated. The locations of the two free hydroxyl groups were proved to be at C-3′and C-5′, as en- hancements of 2.0 and 7.1% were observed at the free OH and H-3 signals, respectively, when H-2′ and H-6′ signals were irradiated. Thus, the remaining methoxyl group was assigned to C-4′. Interpretation of 15 signals for 17 carbons was facilitated by analyses of the HETCOR and COLOCspectra (see Section 5). The assignments of ^1H and ^{13}C signals accomplished by the 2D-NMR spectal analyses established the structure of **5** as 5,3′,5′-trihydroxy-7,4′-dimethoxyflavone.

Compounds **3** and **4** are ring-A *seco*-cycloartane triterpenes which have been reported previously from leaves and/or stems of *G. coronaria* and *G. sootepensis*.[14] Compound **6** is the only 3-methoxyflavone found in this plant, while compounds **7**–**10** are flavone derivatives. The structures were identified by direct comparison of their melting points and spectral data to the values reported in the literature.[24–28] Compound **11** is a phenolic ester which has been isolated from *Dikamali* gum, the exudation of the leaf-bud of *Gardenia lucida*.[29]

3. Biological evaluations

Pure isolated compounds **1**–**11** were tested for cytotoxic effects against a panel of cultured mammalian cell lines,[30] antimitotic[31] and anti-HIV-1 activities. The results are given in Tables 3 and 4. It was found that compounds **1**, **3**, **4**, **7**, **9**, and **10** exhibited cytotoxic acitivity against P-388 cell line and **1** also showed cytotoxic activity against KB, Col-2 and Lu-1; while compound **4** was also active in BCA-1 cell line. Compounds **2**, **5**, **6**, **8**, and **11** were found inactive in all tested cell lines. In the ASK assay, the tested compounds did not exhibit antimitotic effects. All isolated compounds were also tested, employing HIV-1 reverse transcriptase (RT),[32] and a syncytium assay[33] using $^{\Delta Tat/Rev}$MC99 virus and 1A2 cell line system[33,34] (see Table 4). The results indicated that compounds **5**–**10** were active in the $^{\Delta Tat/Rev}$MC99 syncytium assay, while compounds **1** and **3** were toxic. However, compounds **3** and **4** were very active (99.9% and 71.1% inhibition at 200 μg/mL; with IC_{50} values of 17.0

Table 4. Anti-HIV-1 activities of the isolated compounds **1**–**11** by syncytium and HIV-1 RT assays

Compound	Syncytium ($^{\Delta Tat/Rev}$MC99+1A2) assay				HIV-1 RT assay	
	IC_{50} (μg/mL)	EC_{50} (μg/mL)	TI (IC_{50}/EC_{50})	Activity	Inhibition (%)	Activity
1	<3.9	—	—	T	9.9	I
2	27	—	—	I	2.6	I
3	4.4	—	—	T	99.9	VA
4	12.5	—	—	I	71.1	VA
5	>125	8.8	>14.1	A	12.1	I
6	21.1	9.8	2.1	A	16.2	I
7	8.8	<3.9	>2.3	A	57.9	M
8	16.7	9.5	1.8	A	25.5	I
9	72.5	6.8	10.7	A	5.9	I
10	>125	48.7	>2.6	A	18.6	I
11	>125	—	—	I	23.4	I

Syncytium assay: IC_{50}=dose of compound that inhibited 50% metabolic activity of uninfected cells. EC_{50}=dose of compound that reduced 50% syncytium formation by $^{\Delta Tat/Rev}$MC99 virus. A=active; I=inactive, <50% reduction at the IC_{50} indicated, T=Toxic; EC_{50} AZT, averaged from 2 experiments, 4×10^{-9} M. RT assay: inhibition (%) at 200 μg/mL; VA=very active (>70% inhibition), M=moderately active (>50–70% inhibition), W=weakly active (30–50% inhibition), I=inactive (<30% inhibition); positive control, fagaronine chloride, IC_{50} 9.8 μg/mL and non-nucleoside reverse transcriptase inhibitor, nevirapine, IC_{50} 1.8 μg/mL.

and 49.7 μg/mL, respectively) and compound **7** was moderately active in the HIV-1 RT assay.

4. Conclusion

Although cycloartane triterpenes and flavones are commonly found in plants, 3,4-*seco*-cycloartanes with α-methylene-γ-butyrolactone fused at C-5 and C-6 as in compound **1** are particularly rare. The extraordinary cycloartane **2** with oxygen insertion between C-29 and C-3 to form an eight membered ring-A lactone represents a novel skeleton which has not been previously reported. The highly significant anti-HIV-1 activity of compounds **3** and **4** is reported for the first time.

5. Experimental

5.1. General procedure

Mps: uncorr.; UV: EtOH or MeOH; IR: $CHCl_3$ or KBr. NMR spectra were recorded on either Bruker DPX 300 or Bruker Avance 500 spectrometer, using TMS as an internal standard, unless otherwise stated; CC and prep. TLC were carried out on silica gel 60 (63–200 μm) and silica gel 60 PF_{254} (5–40 μm), respectively.

5.2. Plant material

The leaves and twigs of *Gardenia tubifera* were collected from Kalasin province of Thailand in January 1998 and identified by one of the authors (T. S.). The voucher specimen (BKF 25199) has been deposited at the Forest Herbarium, Royal Forest Department, Bangkok, Thailand.

5.3. Extraction and isolation

The air-dried and finely powdered leaves and twigs of *G. tubifera* (1.85 kg) were successively percolated with hexane (4×3 L), $CHCl_3$ (8×3.5 L) and MeOH (3×4 L), respectively. Removal of solvents gave a crude hexane fraction (110 g), a $CHCl_3$ fraction (190 g) and a MeOH fraction (190 g), respectively.

The hexane fraction (69 g) was separated by CC (silica gel, 1.5 kg), eluting with 0–100% acetone–hexane, followed by 0–100% MeOH–acetone to afford fractions A1–A10. Fr. A6 (eluted with 10% acetone–hexane, 2.60 g) gave **11** (179.6 mg) upon recrystallization from CH_2Cl_2–hexane. Fr. A7 (eluted with 10% acetone–hexane, 8.98 g) was further separated by CC over silica gel (acetone–hexane and MeOH–acetone gradients, respectively) to give fractions B1–B5. Fr. B2 (eluted with 5–8% acetone–hexane, 1.26 g) yielded **1** (115.5 mg) and **11** (229.1 mg) after prep. TLC (silica gel, 90% CH_2Cl_2–hexane, 2 elutions). Fr. A8 (eluted with 15% acetone–hexane, 8.99 g) was separated by CC over silica gel ($CHCl_3$–hexane and MeOH–$CHCl_3$ gradients, respectively) to give fractions C1–C4. Fr. C1 (eluted with 1% MeOH–$CHCl_3$, 1.14 g) provided **4** (45.2 mg) after prep. TLC (silica gel, 90% CH_2Cl_2–hexane, 2 elutions).

The $CHCl_3$ fraction (188 g) was subjected to a coarse separation by CC (silica gel, 1.3 kg), eluting with 0–100% CH_2Cl_2–hexane, followed by 0–100% MeOH–CH_2Cl_2, to yield fractions C1–C7. Further separation of Fr. C3 (eluted with 1.5–2% MeOH–CH_2Cl_2, 68.9 g) by CC over silica gel (acetone–hexane and MeOH–acetone gradients) gave fractions D1–D8. Fr. D4 (eluted with 14–20% acetone–hexane, 14.3 g) was separated by CC over silica gel ($CHCl_3$–hexane and MeOH–$CHCl_3$ gradients) to give fractions E1–E5. Fr. E2 (eluted with 0.5% MeOH–$CHCl_3$, 2.71 g) afforded **4** (107.7 mg) after prep. TLC (silica gel, 30% acetone–hexane) and recrystallization from CH_2Cl_2–hexane. Fr. D5 (eluted with 22–25% acetone–hexane, 12.1 g) was further separated by CC (CH_2Cl_2–hexane and MeOH–CH_2Cl_2 gradients, respectively) to provide **9** (eluted with 60–70% CH_2Cl_2–hexane, 2.01 g). Fr. D6 (eluted with 40–80% acetone–hexane, 13.7 g) yielded fractions F1–F4 after separation by CC over silica gel (CH_2Cl_2–hexane and MeOH–CH_2Cl_2 gradients, respectively). Fr. F2 (eluted with 1% MeOH–CH_2Cl_2, 2.62 g) afforded **10** (1.48 g) after prep. TLC (silica gel, 40% acetone–hexane) and recrystallization from MeOH. Fr. D7 (eluted with 5–20% MeOH–acetone, 16.9 g) gave fractions G1–G4 after CC (silica gel, $CHCl_3$–hexane and MeOH–$CHCl_3$ gradients). Fr. G3 (eluted with 2–5% MeOH–$CHCl_3$, 2.62 g) yielded **3** (247.8 mg) after prep. TLC (silica gel, 30% acetone–hexane). Fr. C4 (eluted with 2–3% MeOH–CH_2Cl_2, 6.49 g) was separated by CC (silica gel, EtOAc–hexane and MeOH–EtOAc gradients, respectively) to give fractions H1–H7. Fr. H3 (eluted with 25% EtOAc–hexane, 659.1 mg) gave **5** (56.1 mg) upon recrystallization in MeOH. Fr. H5 (eluted with 25–30% EtOAc–hexane, 2.28 g) afforded **6** (1.06 g) after recrystallization from EtOH. Fr. C5 (eluted with 3–3.5% MeOH–CH_2Cl_2, 35.05 g) was purified by CC over silica gel (CH_2Cl_2–hexane and MeOH–CH_2Cl_2 gradients, respectively) to give fractions I1–I7. Fr. I1 (eluted with 3–5% acetone–hexane, 612.2 mg) yielded **2** (36.0 mg) upon recrystallization from CH_2Cl_2–hexane. Fr. I5 (eluted with 15% acetone–hexane, 8.39 g) was separated by CC (silica gel, acetone–hexane and MeOH–acetone gradients, respectively) to provide fractions J1–J7. Fr. J5 (eluted with 20% acetone–hexane, 1.03 g) afforded **7** (327.5 mg) upon recrystallization from MeOH. Fr. J6 (eluted with 25–35% acetone–hexane, 9.41 g) was further separated by CC (silica gel, acetone–hexane and MeOH–acetone gradients, respectively) to give **8** (eluted with 30–40% acetone–hexane, 792.9 mg).

5.3.1. Tubiferolide methyl ester (1). White solid from CH_2Cl_2–hexane, mp 125.7–126.3 °C. $[\alpha]_D^{30}$=+142.0 (*c* 0.26, $CHCl_3$). UV (EtOH) λ_{max} nm (log ε): 206 (3.96). IR ($CHCl_3$) ν_{max}: 3027, 2952, 2875, 1755 (C=O stretching of α,β-unsaturated lactone), 1734 (C=O stretching of ester), 1654 (C=C stretching), 1457, 1438, 1377, 1297, 1270, 1171, 1149, 1017, 982, 945, 823 cm^{-1}. ^1H and ^{13}C NMR: see Table 1. COSY correlations: H/H; 1a/1b, 2a, 2b; 1b/1a, 2a, 2b; 2a/1a, 1b, 2b; 2b/1a, 1b, 2a; 5/6, 28a, 28b; 6/5, 7a, 7b; 7a/6, 8, 7b; 7b/6, 7a, 8; 8/7a, 7b, 30; 11a/11b, 12a, 12b; 11b/11a, 12a, 12b; 12a/11a, 12b, 18; 12b/11b, 12a; 15/16a, 16b; 16a/16b, 17; 16b/16a, 17; 17/16a, 16b, 18, 20, 22a; 18/12a, 17; 19a/19b; 19b/5, 19a; 20/17, 21, 22a, 22b; 21/20; 22a/21, 22b, 23a; 22b/20, 22a, 23a; 23a/22a, 22b, 23b, 24; 23b/22a, 22b, 23a; 24/23a, 26, 27; 26/24; 27/24; 28a/5;

28b/5; 30/8. HMBC correlations: see Table 2. EIMS (70 eV) *m/z* (%): 482 ([M]$^+$, 30), 398 (25), 371 (32), 286 (2), 111 (8), 69 (100). HRFABMS calcd for $C_{31}H_{47}O_4$ [M+H]$^+$483.3474, found 483.3458.

5.3.2. Tubiferaoctanolide (2). White solid from CH_2Cl_2–hexane, mp 139.5–140.3 °C. $[\alpha]_D^{28}$=+32.0 (*c* 0.25, $CHCl_3$). IR ($CHCl_3$) ν_{max}: 3029, 2936, 2875, 1724 (C=O stretching of lactone), 1647 (C=C stretching), 1457, 1379,1361, 1235, 1161, 997, 921, 839 cm^{-1}. ^{1}H and ^{13}C NMR: see Table 1. COSY correlations: H/H; 1a/1b, 2a, 2b; 1b/1a, 2a, 2b; 2a/1a, 1b, 2b; 2b/1a, 2a; 5/6a, 6b; 6a/5, 6b, 7a; 6b/5, 6a, 7b; 7b/6a, 7a, 8; 8/7a, 7b; 11a/11b, 12a; 11b/11a, 12a, 12b; 12a/11a, 11b; 15a/15b, 16a; 15b/15a, 16a; 16a/16b, 17; 17/20; 19a/19b; 19b/19a; 20/21; 21/20; 22/23a; 23a/22; 23b/22, 23a; 24/23a, 23b, 26, 27; 26/24; 27/24; 28a/28b; 28b/29a, 29b; 29a/28b, 29b; 29b/28a, 29a. HMBC correlations see Table 2. EIMS (70 eV) *m/z* (%): 438 ([M$^+$], 4), 410 (4), 340 (4), 327 (15), 111 (14), 69 (77), 55 (100). HRFABMS calcd for $C_{30}H_{47}O_2$ [M+H]$^+$439.3576, found 439.3546.

5.3.3. Coronalolide (3). White powder from CH_2Cl_2–hexane, mp 82.2–83.1 °C (lit.[14] 82.5–83.0 °C). $[\alpha]_D^{30}$=+126.9 (*c* 0.26, $CHCl_3$) [lit.[14] $[\alpha]_D^{25}$=+119.1 (*c* 0.69, $CHCl_3$)]. UV (EtOH) λ_{max} nm (log ε): 225 (4.05). IR ($CHCl_3$) ν_{max}: 3688, 3508 (OH-stretching of carboxylic acid), 3026, 2950, 2876 (C–H stretching of aldehyde), 1755 (C=O stretching of α,β-unsaturated lactone), 1711 (C=O stretching of carboxylic acid), 1681 (C=O stretching of α,β-unsaturated aldehyde), 1644 (C=C stretching), 1459, 1379, 1349, 1270 cm^{-1}. EIMS (70 eV) *m/z* (%): 482 ([M]$^+$, 7), 454 (11), 384 (24), 357 (70), 125 (7), 69 (31).

5.3.4. Coronaloride methyl ester (4). White solid from CH_2Cl_2–hexane, mp 91.5–93.2 °C (lit.[14] mp 91.0–92.5 °C). $[\alpha]_D^{28}$=+113.9 (*c* 0.26, $CHCl_3$). [lit.[14] $[\alpha]_D^{25}$=+121.6 (*c* 0.86, $CHCl_3$). UV (EtOH) λ_{max} nm (log ε): 215 (4.92). IR ($CHCl_3$) ν_{max}: 3026, 2987, 2952, 2875 (C–H stretching of aldehyde), 1755 (C=O stretching of α,β-unsaturated lactone), 1735 (C=O stretching of ester), 1681 (C=O stretching of α,β-unsaturated aldehyde), 1644 (C=C stretching), 1459, 1377, 1358, 1270 cm^{-1}. EIMS (70 eV) *m/z* (%): 496 ([M]$^+$, 1), 478 (2), 468 (2), 398 (4), 371 (22), 125 (15), 69 (27), 59 (11).

5.3.5. 5,3′,5′-Trihydroxy-7,4′-dimethoxyflavone (5). Yellow needle from MeOH, mp 281.5–282.2 °C dec. UV (MeOH) λ_{max} nm (log ε): 330 (4.22), 269 (4.19), 210 (4.58). IR (KBr) ν_{max}: 3466 (O–H stretching), 1667 (C=O stretching of conjugated ketone), 1614, 1595, 1560, 1467, 1369, 1216, 1199, 1072 cm^{-1}. 300 MHz ^{1}H NMR (DMSO-d_6): δ 12.83 (1H, s, 5-OH), 9.59 (1H each, s, 3′-OH, 5′-OH), 6.97 (2H, s, H-2′, H-6′), 6.67 (1H, d, *J*=2.1 Hz, H-6), 6.66 (1H, s, H-3), 6.33 (1H, d, *J*=2.1 Hz, H-8), 3.83 (3H, s, 7-OMe), 3.75 (3H, s, 4′-OMe). 75 MHz ^{13}C NMR (DMSO-d_6): δ 181.86 (C-4), 165.26 (C-7), 163.83 (C-2), 161.25 (C-8a), 157.26 (C-5), 151.25 (C-3′, C-5′), 139.08 (C-4′), 125.64 (C-1′), 105.91 (C-2′, C-6′), 104.77 (C-4a), 104.45 (C-3), 98.06 (C-8), 92.53 (C-6), 59.93 (4′-OMe), 56.04 (7-OMe). COLOC correlations: C/H; 2/3, 2′,6′; 4/3; 5/6; 6/3,8; 7/6, 8, 7-OMe; 4a/6, 8, 5-OH; 8a/8, 5-OH; 1′/3; 3′/2′; 4′/2′, 6′, 4′-OMe; 5′/6′. EIMS (70 eV) *m/z* (%): 330 ([M$^+$], 100), 287 (29), 259 (25), 167 (7), 138 (2), 95 (4). HRFABMS calcd for $C_{17}H_{15}O_7$ [M+H]$^+$331.0817, found 331.0819.

5.3.6. 5,3′,5′-Trihydroxy-3,6,7,4′-tetramethoxyflavone (6). Yellow needle from MeOH, mp 175.5–176.3 °C (lit.[24] mp 175–176 °C). UV (MeOH) λ_{max} nm (log ε): 336 (4.47), 272 (4.43), 212 (5.83). IR (KBr) ν_{max}: 3392 (O–H stretching), 1655 (C=O stretching of conjugated ketone), 1592, 1556, 1460, 1431, 1358, 1268, 1220, 1180, 1118, 1096, 1018, 859 and 818 cm^{-1}. EIMS (70 eV) *m/z* (%): 390 ([M]$^+$, 100), 375 (46), 373 (9), 347 (14), 153 (28), 125 (8). HRFABMS calcd for $C_{19}H_{19}O_9$ [M+H]$^+$391.1029, found 391.1023.

5.3.7. 5,7,4′-Trihydroxy-6-methoxyflavone (hispidulin) (7). Yellow needle from MeOH, mp 293–294 °C (lit.[25] mp 291–292 °C). UV (MeOH) λ_{max} nm (log ε): 336 (2.03), 275 (1.96), 216 (2.19). IR (KBr) ν_{max}: 3332 (O–H stretching), 1652 (C=O stretching of conjugated ketone), 1611, 1558, 1492,1439, 1368, 1250, 1155, 1110, 827, 882 cm^{-1}. EIMS (70 eV) *m/z* (%): 300 ([M]$^+$, 100), 285 (47), 257 (59), 121(11), 119 (26), 118 (20), 93 (6). HRFABMS calcd for $C_{16}H_{13}O_6$ [M+H]$^+$301.0712, found 301.0709.

5.3.8. 5,7,3′-Trihydroxy-6,4′,5′-trimethoxyflavone (8). Yellow needles from acetone, mp 243–244 °C (lit.[26] mp 243–245 °C). UV (MeOH) λ_{max} nm (log ε): 332 (4.26), 277 (4.20), 215 (4.59). IR (KBr) ν_{max}: 3470 (OH-stretching), 1656 (C=O stretching of conjugated ketone), 1619, 1588, 1496, 1204 cm^{-1}. EIMS (70 eV) *m/z* (%): 360 (M$^+$, 100), 345 (62), 342 (30), 331 (7), 317 (46), 167 (5), 139 (9), 111 (2), 178 (2), 181 (2). HRFABMS calcd for $C_{18}H_{17}O_8$ [M+H]$^+$361.0923, found 361.0907.

5.3.9. 5,3′-Dihydroxy-7,4′,5′-trimethoxyflavone (9). Yellow needle from EtOH, mp 194–195 °C (lit.[27] mp 194–195 °C). UV (EtOH) λ_{max} nm (log ε): 332 (3.99), 270 (3.94), 210 (4.39). IR (KBr) ν_{max}: 3432 (O–H stretching), 1651 (C=O stretching of conjugated ketone), 1623, 1587, 1557, 1448, 1430, 1355, 1195, 1110, 1049, 836, 746. EIMS (70 eV) *m/z* (%): 334 ([M]$^+$, 100), 329 (7), 315 (7), 301 (17), 283 (3), 273 (12), 258 (8), 227 (14), 178 (1), 167 (10), 158 (10), 135 (6), 95 (7). Anal. Calcd for $C_{18}H_{16}O_7$: C, 62.79; H, 4.68. Found: C, 62.45; H, 5.01.

5.3.10. 5,3′-Dihydroxy-6,7,4′,5′-tetramethoxyflavone (10). Pale yellow powder from MeOH, mp 217.4–218.3 °C dec. (lit.[28] m.p 216 °C dec.). UV (MeOH) λ_{max} nm (log ε): 330 (3.16), 277 (3.05), 214 (3.45). IR (KBr) ν_{max}: 3384 (OH-stretching), 1665 (C=O stretching of conjugated ketone), 1591, 1523, 1496, 1450, 1366, 1210, 1129. EIMS (70 eV) *m/z* (%): 374 ([M]$^+$, 100), 359 (88), 331 (24), 181 (23), 153 (47). HRFABMS calcd for $C_{19}H_{19}O_8$ [M+H]$^+$375.1079, found 375.1082.

5.3.11. Hexacosyl 4′-hydroxy-*trans*-cinnamate (11). White powder from CH_2Cl_2–hexane, mp 95–96 °C (lit.[29] mp 95–96 °C). UV (MeOH) λ_{max} nm (log ε): 312 (4.15), 227 (3.87), 211 (3.84). IR ($CHCl_3$) ν_{max}: 3589, 3310 (O–H stretching), 1698 (C=O stretching of α,β-unsaturated ester), 1635 (C=C stretching), 1607, 1588, 1514, 1436, 1322, 1169, 1102, 983, 832, 760, 720 cm^{-1}. EIMS (70 eV)

m/z (%): 528 ([M^+], 28), 164 (100), 147 (67). HRFABMS calcd for $C_{35}H_{61}O_3$ [M+H]$^+$529.4620, found 529.4613.

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