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Full Length Research Paper

Two new phenolic constituents from *Clematis connata* DC.

Lin Yuan[1], Wenzhong Huang[1], Yinhai Ma* and Zhizhi Du[2]

[1]Chemical Science and Technology Department, Kunming University, Kunming 650214, P. R. China.
[2]State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650204, P. R. China.

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Two new phenolic compounds, 3,4-dihydroxyphenethyl 5-hydroxy-4-oxopentanoate (1) and 3-(4-hydroxy-2,5-dimethoxyphenyl)-3-oxopropyl acetate (2), together with fifteen known compounds, 3 to 17, were isolated from the dried whole plants of *Clematis connata* DC. Their structures were elucidated by extensive spectral analysis, especially 2D NMR techniques. Compounds 1 to 5 were evaluated for their *in vitro* cytotoxicities against acute promyelocytic leukemia HL-60, hepatocellular carcinoma Hep-G2, oropharyngeal epidermoid carcinoma KB and breast cancer cells MDA-MB-231. Compounds 1 and 3 exhibited the most potential with IC_{50} values of 0.63 and 1.27 μM against MDA-MB-231 and HepG2, respectively. The antibacterial activities experiment of all the isolated compounds showed that only Compounds 3 to 5 exhibited weak activities against *Staphylococcus aureus*, methcillin-resistant *S. aureus* and β-lactamase positive *S. aureus*, and Compound 9 and 17 showed weak activities against *S. aureus*.

Key words: *Clematis connata* DC., phenolic constituents, cytotoxicities, antibacterial activities.

INTRODUCTION

The plants of genus *Clematis*, which belongs to the family of Ranunculaceae, comprise more than 300 species all around the world. There are over 130 species mainly distributed in southwest and the central region of China (Hou, 1984; Wang and Li, 2005). The chemical compounds separated from the genus *Clematis* involved lignans, flavonoids, cumarins, saponins, alkaloids and organic acids. The pharmacological effects of the genus *Clematis* included antibiotic, antitumor, anti inflammatory and analgesic, and antiviral activity etc (Huang, 2002). In previous studies, we reported new saponins with antifungal effects from *Clematis tangutica* (Du et al., 2003). Here we investigate another species of the genus *Clematis*, *Clematis connata* DC, which is commonly used as a traditional folk medicine to treat indigestion, chronic gastropathy, carbuncles and ulcers (Hou, 1984). There has been no report on the chemical constituents of this species. Hence we carried out comprehensive phytochemical study of ethyl acetate (EtOAc) extracts of *C. connata* to investigate the potential bioactive chemical constituents in it.

The isolation and structure elucidation of the two new compounds, 3,4-dihydroxyphenethyl 5-hydroxy-4 -oxopentanoate (**1**) and 3-(4-hydroxy-2,5-dimethoxyphenyl)-3-oxopropyl acetate (**2**) were described along with fifteen known compounds, hydroxytyrosol (**3**) , 2-(4'-O-β-D-glucopyranosyl-3'-hydroxyphenyl)-ethanol (**4**) (Bianco et al., 1998), 2-(3'-O-β-D-glucopyranosyl-4'-hydroxyphenyl)-ethanol (**5**) (Kim et al., 2008), caffeic acid (**6**) (Tao et al., 2006), isoferulic acid (**7**) (Tao et al., 2006), ferulic acid (**8**) (He et al., 2005), 3-hydroxy-4-methoxybenzoic acid (**9**) (Luo et al., 2009), 4-

*Corresponding author. E-mail: myh5929251@hotmail.com. Tel: +86-871-5098482. Fax: +86-871-5098474.

Abbreviations: NMR, Nuclear magnetic resonace; **UV**, ultraviolet; **IR**, infrared; **TLC,** thin layer chromatography; **^{1}H-^{1}H COSY**: ^{1}H-^{1}H correlation spectroscopy; **HSQC,** heteronuclear single quantum correlation; **HMBC,** heteronulear multiple bond correlation.

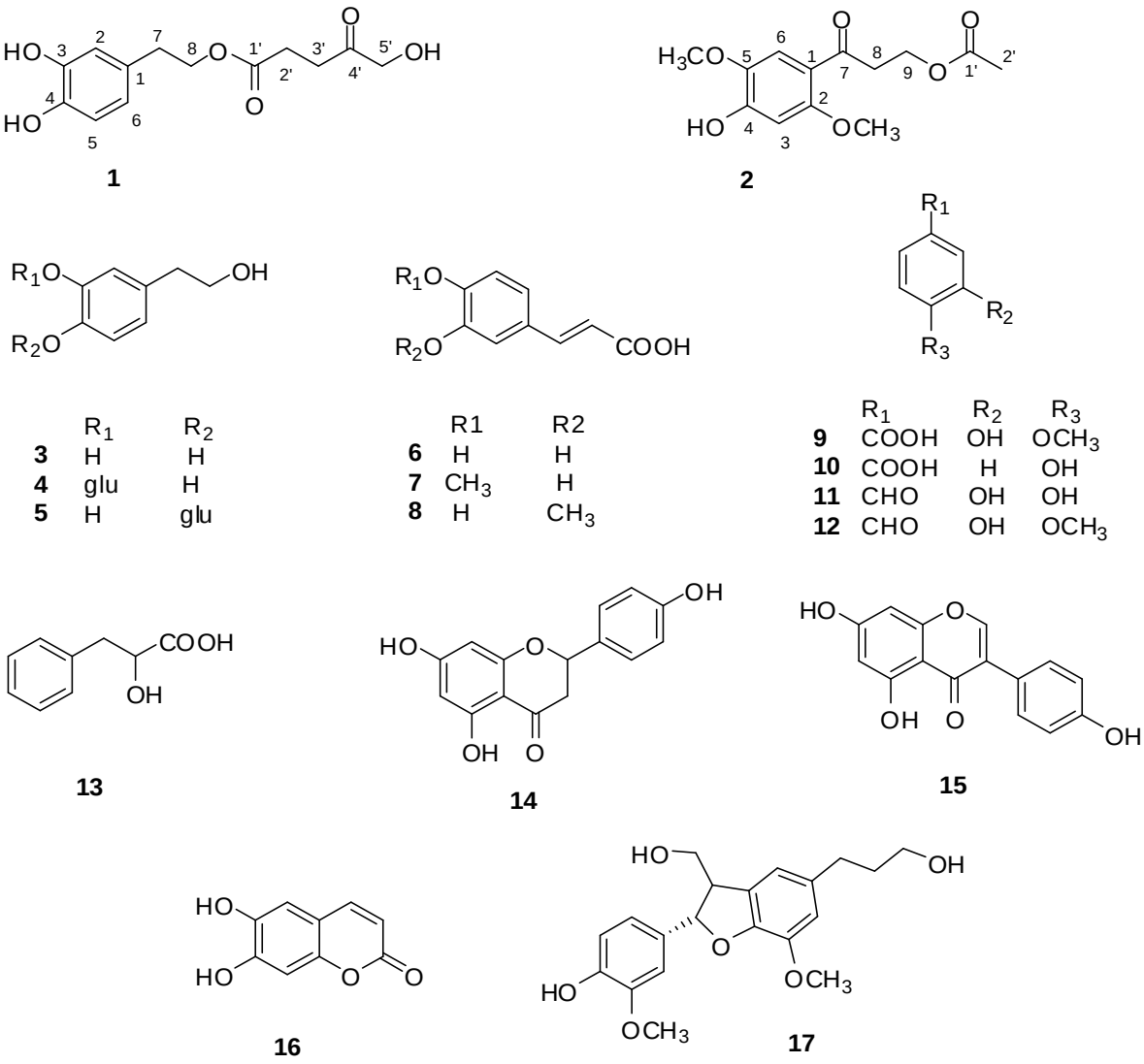

Figure 1. The structures of the isolated Compounds **1** to **17** from *C. connata* DC.

hydroxybenzoic acid (**10**) (Luo et al., 2009), 3,4-dihydroxybenzaldehyde (**11**) (Luo et al., 2009), 3-hydroxy-4-methoxybenzaldehyde (**12**) (Li et al., 2010), 2-hydroxy-3-phenylpropionic acid (**13**) (Baderschneider and Winterhalter, 2001), naringenin (**14**) (Lee et al., 2001), genistein (**15**) (Li and Song, 2009), 6,7-dihydroxycoumarin (**16**) (Wang et al., 2011) and (7*S*, 8*R*)-dihydrodehydrodiconiferyl alcohol (**17**) (Kuang et al., 2009) (Figure 1) in *C. connata*. The structures of the new compounds were determined by means of spectral analysis and the known ones were identified by comparison of their NMR data with those reported in the literature. In addition, Compounds **1** to **5** were evaluated for their cytotoxicities against acute promyelocytic leukemia (HL-60), hepatocellular carcinoma (Hep-G2), oropharyngeal epidermoid carcinoma (KB) and breast cancer cells (MDA-MB-231). The antibacterial activity of all the compounds was tested against *Staphylococcus aureus*, methcillin-resistant *S. aureus* and β-lactamase positive *S. aureus*.

MATERIALS AND METHODS

General experimental procedures

UV Spectra were obtained on Shimadzu 2401PC spectrophotometer; λ_{max} log (ε) in nm. IR Spectra were recorded on Bio-Rad FTS-135 spectrophotometer with KBr discs; in cm^{-1}. 1D- and 2D-NMR Spectra were recorded on Bruker AM-400 and DRX-500 instruments; chemical shifts δ in ppm relative to residual solvent signals, coupling constants *J* in Hz. ESI-MS and HR-ESI-MS were measured on VG Auto-Spec-3000 mass spectrometers, in *m/z*.

Table 1. ^{1}H and ^{13}C-NMR data for Compounds **1** in CD_3OD and **2** in C_5D_5N (500 and 125 MHz, *J* in Hz and δ in ppm, HMBC (H to C)).

No.	1			2		
	δ_H	δ_C	HMBC	δ_H	δ_C	HMBC
1	-	130.7	-	-	111.4	-
2	6.64 (1H, d, *J* = 1.85)	117.0	C-4, 6, 7	-	155.0	-
3	-	146.2	-	6.69 (1H, s)	102.1	C-1, 2, 4, 5
4	-	144.9	-	-	153.6	-
5	6.68 (1H, d, *J* = 7.91)	116.4	C-1, 4, 6	-	142.6	-
6	6.53 (1H, dd, *J* = 7.91, 1.80)	121.2	C-4, 5, 7	7.42 (1H, s)	112.2	C-1, 2, 4, 5, 7
7	2.75 (2H, t, *J* = 7.02)	35.3	C-1, 2, 6, 8	-	198.8	-
8	4.18 (2H, t, *J* = 7.06)	66.7	C-1, 1′, 7	3.39 (2H, t, *J* = 6.10)	40.1	C-1, 7, 9
9	-	-	-	4.62 (2H, t, *J* = 6.10)	60.6	C-1′, 7, 8
1′	-	174.3	-	-	169.5	-
2′	2.70 (2H, t, *J* = 6.35)	33.7	C-1′, 3′, 4′	1.99 (3H, s)	20.3	C-1′
3′	2.59 (2H, t, *J* = 6.40)	28.5	C-1′, 2′, 4′	-	-	-
4′	-	210.8	-	-	-	-
5′	4.20 (2H, s)	68.6	C-4′	-	-	-
2-OMe	-	-	-	3.73 (3H, s)	55.6	C-2
5-OMe	-	-	-	3.78 (3H, s)	55.7	C-5
4-OH	-	-	-	10.31 (3H, br s)	-	C-3, 4, 5

Column chromatography was performed on silica gel (200 to 300 mesh or 10 to 40 μm; Qingdao Marine Chemicals, Co. Ltd, P.R. China), RP-18 (reversed-phase C18 SiO_2) (40 to 63 μm; Merck, Germany) and Sephadex LH-20 (Amersham Pharmacia, Sweden). Fractions were monitored by TLC and spots were visualized by heating plates spraying with 5% H_2SO_4 in ethanol.

Plant material

The whole plant of *C. connata* was collected in Luquan county of Yunnan province in September, 2009 and identified by associate professor Chunxia Pu at Yunnan University of Traditional Chinese Medicine. A voucher specimen (CP20090903) was deposited at Chemical Science and Technology Department, Kunming University, Yunnan, China.

Extraction and isolation

The dry and powdered plants of *C. connata* (7.5 kg) were extracted with 70% aqueous acetone three times (10 L × 3). The extract was filtered, concentrated in *vacuo* to suitable volume and then partitioned with ethyl acetate (EtOAc) and *n*-butanol in sequence. The EtOAc extract (72.8 g) was subjected to column chromatography of MIC-gel CHP-20P eluting with 95% ethanol. The eluate was concentrated, and the residue (68.5 g) was fractioned on column chromatography of silica gel (200 to 300 mesh) eluting with petroleum ether and acetone in gradient (9:1, 4:1, 2:1, 1:1, 1:2 and 0:1) to afford fractions **A** to **J**. Compound **12** (19 mg) was isolated from fraction **C** by repeated chromatographic column of silica gel (petroleum ether/EtOAc). Fraction **F** was subjected to column chromatography of silica gel (petroleum ether/EtOAc) and Sephadex LH-20 (methanol (MeOH)/chloroform ($CHCl_3$)) to afford **2** (5 mg) and **10** (10 mg). Fraction **G** was further purified by column chromatography of RP-18 (MeOH/H_2O), Sephadex LH-20 (MeOH/$CHCl_3$) and silica gel (petroleum ether/acetone) to afford **7** (10 mg), **9** (9 mg), **11** (10 mg), **14** (6 mg), **15** (4 mg) and **17** (11 mg). Fraction **H** was also subjected to column chromatography of RP-18 (MeOH/H_2O), Sephadex LH-20 (MeOH) and silica gel (petroleum ether/acetone) to afford **6** (9 mg), **8** (10 mg), **13** (13 mg), **16** (11 mg). Fraction **I** was chromatographed on Sephadex LH-20 (MeOH) and silica gel ($CHCl_3$/MeOH) columns to yield **1** (15 mg), and **3** (6 mg). Fraction **J** was subjected to column chromatography of RP-18 (MeOH/H_2O), Sephadex LH-20 (MeOH) and silica gel ($CHCl_3$/MeOH) to afford **4** (6 mg) and **5** (5 mg).

Compound **1** (3,4-dihydroxyphenethyl 5-hydroxy-4-oxopentanoate): Colorless oil (MeOH); UV (MeOH) λ_{max} (log ε): 367 (2.55), 282 (3.49), 202 (4.38); IR bands (KBr): 3495, 3419, 2925, 1731, 1716, 1612, 1522, 1477, 1413, 1344, 1281, 1187, 1121, 1088, 977, 818 cm^{-1}; ^{1}H and ^{13}C NMR data (CD_3OD, 500 and 125 MHz) (Table 1); HR-ESI-MS *m/z* 291.0839 $[M+Na]^+$ (calc. 291.0844 for $C_{13}H_{16}O_6Na$).

Compound **2** (3-(4-hydroxy-2,5-dimethoxyphenyl)-3-oxopropyl acetate): Pale yellow gum (pyridine, MeOH); UV (MeOH) λ_{max} (log ε): 324 (2.84), 289 (4.05), 250 (3.18), 210 (4.72); IR bands (KBr): 3380, 2921, 2854, 1713, 1705, 1636, 1506, 1450, 1435, 1362, 1284, 1173, 1139, 1086, 1047, 974, 822 cm^{-1}. ^{1}H and ^{13}C NMR data (C_5D_5N, 500 and 125 MHz) (Table 1); HR-ESI-MS *m/z* 291.0840 $[M+Na]^+$ (calc. 291.0844 for $C_{13}H_{16}O_6Na$).

Biological studies

Cytotoxic activity

Four human cancer cell lines, HL-60, Hep-G2, KB and MDA-MB-231, were used in the cytotoxic assay, which was performed according to the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method with doxorubicin as positive control (Mosmann, 1983). Each tumor cells were plated in the 96-well plate at the density of 5000 cells per well and incubated at 37℃ for 24 h. After treatment and continuously expo sed to various concentrations of compounds for 72 h, cell proliferation was

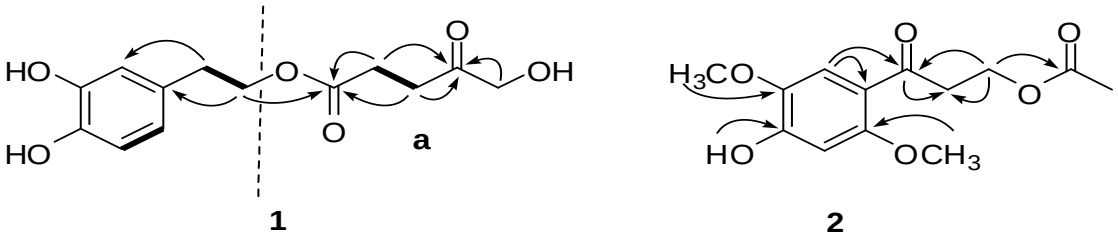

Figure 2. Key COSY (▬) and HMBC (→) correlations of **1** and **2**.

analyzed by the Cell Proliferation Kit I (MTT) according to the manufacturers instructions. The optical densities were measured at 570 nm with a microplate reader. Growth inhibition rate for cell proliferation was calculated as $(OD_{control} - OD_{treated})/OD_{control} \times 100\%$. Results are expressed as IC_{50} in μM, which is defined as the concentration of compounds that resulted in 50% inhibition of growth rate. All assays were done in triplicate.

Antibacterial activity

All the compounds were dissolved in dimethyl sulfoxide (DMSO) to the concentration of 50 mg/ml. The antibacterial activity was assessed against *S. aureus*, methcillin-resistant *S. aureus* and *β*-lactamase positive *S. aureus* at the concentration of 50 μg/disk, by means of the disk diffusion assay on agar plates, as described previously (Du et al., 2003). The diameter of the disk was 6 mm. The results of antibacterial activity were observed after incubation at 37℃ for 24 h. The experiments were performed in tri plicate, and the results are presented as mean values of the three measurements.

RESULTS

The investigation of chemical constituents of *C. connata* led to the isolation of seventeen compounds, including two new phenolic compounds, which have never been reported and have been identified by SciFinder search system.

Compound **1** was obtained as colorless oil, and its molecular formula was determined as $C_{13}H_{16}O_6$ by positive-ion HR-ESI-MS ($[M+Na]^+$ at *m/z* 291.0839; calc. 291.0844). The IR spectrum of **1** suggested the presence of hydroxyl groups (3495, 3419 cm^{-1}), benzene ring (1612, 1522 and 1477 cm^{-1}), and carbonyl groups (1731, 1716 cm^{-1}). The ^{1}H-NMR spectrum (Table 1) showed typical proton signals from trisubstituted phenyl (δ_H 6.68 (1H, d, *J* = 7.91); 6.64 (1H, d, *J* = 1.85) and 6.53 (1H, d, *J* = 7.91, 1.80). ^{13}C-NMR and DEPT spectra (Table 1) exhibited signals for one phenyl, five methylene (two oxygenated) and two carbonyl groups. Comparison of the ^{1}H- and ^{13}C-NMR spectroscopic data of **1** with those of the known compound hydroxytyrosol (**3**) (Bianco et al., 1998), which was also isolated in this study, indicated that **1** contained the structure of **3**, and the structure of **1** included three more methylene (δ_C 68.6, 33.7 and 28.5) and two more carbonyl groups (δ_C 210.8 and 174.3) than **3**. ^{1}H,^{1}H-COSY correlation between δ_H 2.70 (H-2′) and δ_H 2.59 (H-3′), HMBC correlations from δ_H 2.70 (H-2′) and δ_H 2.59 (H-3′) to both δ_C 174.3 (C-1′) and δ_C 210.8 (C-4′), and HMBC correlation from the oxygenated methylene (δ_H 4.20 (H-5′)) to δ_C 210.8 (C-4′) formed the fraction **a** (Figure 2). The HMBC correlation from δ_H 4.18 (H-8) to δ_C 174.3 (C-1′) connected hydroxytyrosol to fraction **a**. Thus the structure of **1** was established as 3,4-dihydroxyphenethyl 5-hydroxy-4-oxopentanoate.

Compound **2** was isolated as pale yellow gum with the molecular formula $C_{13}H_{16}O_6$, as derived from positive HR-ESI-MS ($[M+Na]^+$ at *m/z* 291.0840; calc. 291.0844). The IR spectrum displayed the absorption bands at 3380 cm^{-1} for a hydroxyl group, 1713 and 1705 cm^{-1} for two carbonyls, 1636, 1506 and 1450 cm^{-1} for a benzene group.

In the ^{1}H-NMR spectrum (Table 1), the same coupling constant of δ_H 3.39 (H-8) and δ_H 4.62 (H-9) indicated that the only two methylene groups were connected together. The HMBC correlations from δ_H 3.39 (H-8) to the carbonyl (δ_C 198.8 (C-7) and δ_C 60.6 (C-9), and from the oxygenated methylene (δ_H 4.62 (H-9)) to δ_C 198.8 (C-7), δ_C 40.1 (C-8) and δ_C 169.5 (C-1′) , which was from one acetyl group including δ_C 169.5 (C-1′) and δ_C 20.3 (C-2′), indicated 3-oxopropyl acetate group in **2**. The rest proton signals displayed two methoxyl group (δ_H 3.73 and 3.78), two aromatic proton (δ_H 6.69 and 7.42) and one phenolic hydroxyl (δ_H 10.31). In addition to the rest carbon signals (δ_C 55.6, 55.7, 102.1, 111.4, 112.2, 142.6, 153.6, 155.0) (Table 1), a tetrasubstituted benzene group was deduced. Two methoxyl groups were established to be located at C-2 and C-5 of benzene group by the HMBC correlations of OMe (δ_H 3.73, 3H, s) to C-2 (δ_C 55.6) and OMe (δ_H 3.78, 3H, s) to C-5 (δ_C 55.7). The HMBC correlation of phenolic hydroxyl (δ_H 10.31, 1H, br s) to C-3, 4 and 5 (δ_C 102.1, 153.6 and 142.6) showed that hydroxyl group was located at C-4. Finally, the HMBC correlations from aromatic δ_H 7.42 (H-6) to δ_C 198.8 (C-7) and from δ_H 3.39 (H-8) to δ_C 111.4 (C-1) showed the 3-oxopropyl acetate group was connected with benzene group at C-1(Figure 2). Hence, on the basis of the aforementioned evidence, Compound **2** was identified as 3-(4-hydroxy-2,5-dimethoxyphenyl)-3-oxopropyl acetate.

Table 2. IC_{50} values (μM) of Compounds **1 to 5** in four cell lines.

Compound	Cell lines			
	HL-60	HepG2	KB	MDA-MB-231
1	1.87	5.26	2.21	0.63
2	4.68	5.24	16.03	8.40
3	2.16	1.27	4.42	3.21
4	1.22	3.27	8.35	3.27
5	2.74	6.87	2.96	2.20
Doxorubicin	0.1	0.2	0.1	0.1

Most of the isolated known compounds were found to have been measured for their cytotoxic activities against different cell lines after a large amount of literature research. As a result, two new compounds (**1** and **2**) and three known compounds (**3** to **5**), which were structure related with compound **1** were tested for cytotoxic activities against HL-60, Hep-G2, KB and MDA-MB-231. The cytotoxicity tests for the isolated compounds were performed using a previously reported procedure (Mosmann, 1983). The cytotoxic abilities by MTT-assay were shown in Table 2, with doxorubicin as the positive control.

All the tested compounds exhibited different extent activities to four cell lines. Among them, the best cytotoxic activities against HL-60, HepG2, KB and MDA-MB-231 were Compounds **4**, **3** and **1**. Generally, Compounds **1** and **3** to **5**, which were structurally related with hydroxytyrosol (**3**), showed stronger activities than **2**. Compound **1** and **3** showed the most potent with IC_{50} values of 0.63 and 1.27 μM, which were nearly sextuple of positive control, against MDA-MB-231 and HepG2 respectively. All the others activities were more than tenfold compared with positive control.

All the isolated compounds were tested for their antibacterial activities against *S. aureus*, methcillin-resistant *S. aureus* and β-lactamase positive *S. aureus* by the disk diffusion method (Du et al., 2003). At the concentration of 50 μg/disk, Compounds **3** to **5** show weak antibacterial activities with the inhibitory zone of a diameter of 8 mm against *S. aureus*, methcillin-resistant *S. aureus* and β-lactamase positive *S. aureus*, and Compounds **9** and **17** only showed weak activities against *S. aureus* with the inhibitory zone of 8 mm. The other compounds were inactive against all organisms.

DISCUSSION

The phytochemical investigation of *C. connata* afforded two new phenolic compounds, 3,4-dihydroxyphenethyl 5-hydroxy-4-oxopentanoate (**1**) and 3-(4-hydroxy-2,5-dimethoxyphenyl)-3-oxopropyl acetate (**2**), along with fifteen known compounds. Most of these compounds belongs to phenolic constituents, flavonoids, coumarins and lignans. The cytotoxic evaluation of Compounds **1** to **5** showed that Compounds **1** and **3** exhibited important potential cytotoxic activity against MDA-MB-231 and HepG2, respectively. In the antibacterial activity experiment of all the isolated compounds, Compounds **3** to **5** displayed weak activities against *S. aureus*, methcillin-resistant *S. aureus* and β-lactamase positive *S. aureus*, and Compounds **9** and **17** showed weak activities against *S. aureus*. The results of this study could provide chemical foundation for better medical usage of *C. connata*. What is mentionable is hydroxytyrosol (**3**), which was the key anti-HIV component of olive leaf extract and then was identified as a unique class of HIV-1 inhibitor effective against viral fusion and integration (Sylvia et al., 2007). Whether its derivatives, new Compound **1** and Compounds **4** to **5**, share the similar effect is worth further investigation.

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