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Terpene and lignan glycosides from the twigs and leaves of an endangered conifer, *Cathaya argyrophylla*

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ABSTRACT

Labdane diterpene glycosides cathargyroside A and cathargyroside B, monoterpene glycosides vervenone-10-*O*-*β*-D-glucopyranoside and vervenone-10-*O*-*β*-D-apiofuranosyl-(1″→6′)-*β*-D-glucopyranoside, as well as lignan glycosides cedrusinin-4-*O*-*α*-L-rhamnopyranoside and (+)-cyclo-olivil-9′-*O*-*β*-D-xylopyranoside, along with 39 known compounds, were obtained from the methanol extract of the twigs and leaves of *Cathaya argyrophylla*. These compounds were identified mainly by analyzing their NMR and MS data. Almost all of these compounds were hitherto unknown in this genus. The isolated compounds were screened against *Candida albicans* and *Staphylococcus aureus* for antimicrobial assay, and against K562, HT-29, BEL-7402, SGC-7901, B16, BGC-823, U251 and A549 cancer cell lines for cytotoxic activities. One compound showed antimicrobial activity against *C. albicans*, and four of them displayed cytotoxicity. Similarity analysis on the chemical constituents of the genera *Cathaya*, *Picea* and *Pinus* supported their close phylogenetic relationships.

1. Introduction

In our program, aimed to characterize new active compounds from gymnosperms, many natural compounds have been isolated and identified, and some have exhibited inhibitory activities against cancer cell lines (Fu et al., 2008; He et al., 2011a,b; Xu et al., 2006a,b; Xu and Tan, 2007; Zhang and Tan, 2007; Zhang et al., 2004, 2005a,b, 2007, 2009). In this regard, *Cathaya argyrophylla* Chun et Kuang Gord. (Pinaceae) is a gymnosperm indigenous to China and classified as a palaeoendemic plant. It has also been listed as one of the eight most endangered plant species in China (Wang and Xie, 2004) with the total number of individual plants being less than 4,000 and trivially named as "giant panda" in the plant kingdom. The history of its fossil record dates back to the Cretaceous period (Chun and Kuang, 1962; Wang and Ge, 2006). It is considered as being important in its conservation and molecular phylogeny (Ge et al., 1998; Kan et al., 2007; Wang and Ge, 2006; Wang et al., 1998, 1997). Nuclear ribosomal DNA (nrDNA) has also been used to study phylogenetic relationships at many taxonomic levels. Phylogenetic relationship studies of GC content in the ITS1 region indicated that the genera *Cathaya*, *Picea* and *Pinus* are closely related (Kan et al., 2007; Wang et al., 1998). Chemotaxonomic study of the above genera using serratane-type triterpenes as a chemotaxonomic marker confirmed further their close taxonomic phylogenetic relationships (He et al., 1981). There are only five serratane-type triterpenes (Ma et al., 1981) and two flavonoids (Ma et al., 1982; Qiu et al., 1981) previously isolated from *C. argyrophylla*. In the current study, 45 compounds from the twigs and leaves of *C. argyrophylla* were isolated and characterized. They were used to screen their cytotoxic and antimicrobial activities, and to consider the phylogenetic relationships of the genera *Cathaya*, *Picea* and *Pinus* based on the chemical constituent analysis.

2. Results and discussion

The methanol extract of the twigs and leaves of *C. argyrophylla* was subjected to silica gel, MCI, RP-8, Sephadex LH-20 column chromatographic (CC) and semipreparative HPLC purification steps to give compounds **1**–**45**. The structures of the new compounds **1**–**6** (Fig. 1) were elucidated by examining both their spectroscopic data and other chemical evidence. In addition to the six new compounds, two known megastigmane glycosides icariside B5 (**7**) (Matsunami et al., 2010) and dihydrovomifoliol-*O*-*β*-D-glucopyranose (**8**) (Miyase et al., 1988), six known lignan glycosides

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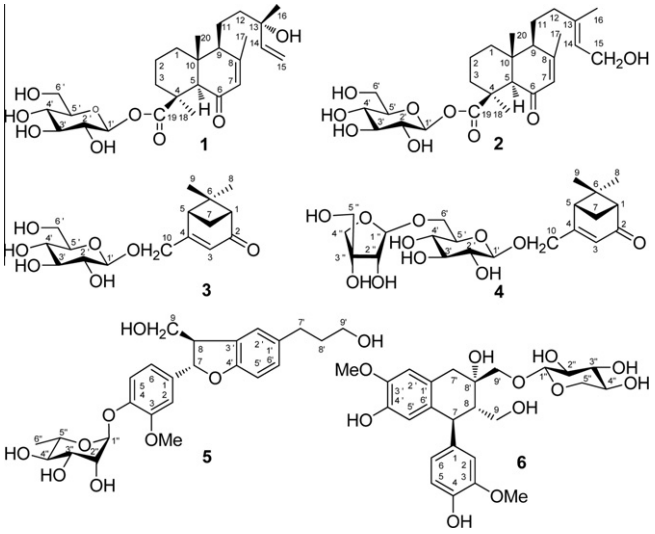

Fig. 1. Structures of compounds **1**–**6** isolated from twigs and leaves of *C. argyrophylla*.

massonianoside B (**9**) (Bi et al., 2001), (2*S*,3*R*)-2,3-dihydro-7-hydroxy-2-(4′-hydroxy-3′-methoxyphenyl)-3-hydroxymethyl-5-benzofuranpropanol 4′-*O*-*β*-D-glucopyranoside (**10**) (Machida et al., 2002), (2*S*,3*R*)-2,3-dihydro-7-methoxy-2-(4′-hydroxy-3′-methoxyphenyl)-3-hydroxymethyl-5-benzofuranpropanol 4′-*O*-*β*-D-glucopyranoside (**11**) (Machida et al., 2002), (−)-(7*R*,8*R*)-*threo*-7,9,3′,9′-tetrahydroxy-3-methoxy-8-4′-oxyneolignan-4-*O*-*β*-D-xylopyranoside (**12**) (Lundgren et al., 1981; Pan and Lundgren, 1995, 1996; Thomas and Olof, 1977) and a mixture of (−)-(7*S**,8-*R**)-*erythro*-7,9,3′,9′-tetrahydroxy-3-methoxy-8-4′-oxyneolignan-4-*O*-*β*-D-xylopyranoside (**13**) and (−)-(7*R**,8*S**)-*erythro*-7,9,3′,9′-tetrahydroxy-3-methoxy-8-4′-oxyneolignan-4-*O*-*β*-D-xylopyranoside (**14**) (Lundgren et al., 1981; Pan and Lundgren, 1995, 1996; Thomas and Olof, 1977), three known phenylpropanoid glycosides dihydroconiferyl alcohol 4-*O*-*β*-D-glucopyranoside (**15**) (Higuchi et al., 1977), 2-*O*-[4′-(*α*-hydroxypropyl)-2′-methoxyphenyl]-1-*O*-*β*-D-glucopyranosyl glycerol (**16**) (Pan and Lundgren, 1996) and *β*-hydroxypropiovanillone 3-*O*-*β*-D-glucopyranoside (**17**) (Xiang et al., 2004), one known flavonoid glycoside quercitrin (**18**) (Yin et al., 2002), three known aromatic glycosides 1′-*O*-benzyl-*α*-L-rhamnopyranosyl-(1″→6′)-*β*-D-glucopyranoside (**19**) (Hamerski et al., 2005), 3,4-dimethoxyphenyl-*β*-D-glucopyranoside (**20**) (Pan and Lundgren, 1995) and tachioside (**21**) (Pan and Lundgren, 1995), eight known lignans (+)-berchemol-9-acetate (**22**) (Ando et al., 2007), (−)-berchemol (**23**) (Ando et al., 2007), (−)-secoisolariciresinol (**24**) (Achenbach et al., 1983), (−)-carinol (**25**) (Achenbach et al., 1983), (+)-isolariciresinol (**26**) (Fonseca et al., 1978), (+)-cyclo-olivil (**27**) (Kanchanapoom et al., 2006), (+)-dihydrodehydrodiconiferyl alcohol (**28**) (Fu et al., 2008) and (+)-1-hydroxypinoresinol (**29**) (Tsukamoto et al., 1984), three known diterpenes 12,13E-ozic acid (**30**) (Deng et al., 1997), 13-epicupressic acid methyl ester (**31**) (Su et al., 1994) and 13-hydroxy-8,11,13-podocarpatrien-18-oic acid (**32**) (Cheung et al., 1993), eight known triterpenes 3*α*-methoxyserrat-14-en-21*β*-ol (**33**) (Tanaka et al., 1994a), 3*β*,21*β*-dihydroxyserrat-14-ene (**34**) (Tanaka et al., 1994b), 21*β*-methoxyserrat-14-en-3*β*-ol (**35**) (Conner et al., 1980; Rowe et al., 1972), 3*β*-hydroxyserrat-14-en-21-one (**36**) (Chernenko et al., 1993), serratenediol-3*β*-acetate (**37**) (Pei et al., 2004), 21*β*-hydroxyserrat-14-en-3-one (**38**) (Tanaka et al., 1994b), 21*β*-methoxyserrat-14-en-3-one (**39**) (Tanaka et al., 1994a) and serrat-14-en-3,21-dione (**40**) (Tanaka et al., 1997), two known flavonols kaempferol (**41**) (Fu et al., 2008) and quercetin (**42**) (Xu and Tan, 2007), two known aromatic compounds vanillin (**43**) (Fu et al., 2008) and benzoic acid (**44**), and *β*-sitosterol (**45**) (Fu et al., 2008) were also isolated from *C. argyrophylla*. Their structures were confirmed by comparison of their ^{1}H NMR, ^{13}C NMR, $[\alpha]_D$ and MS data with the corresponding authentic sample or literature data. Using HPLC, compound **22** was not detected in the crude extract and should thus be an artifact during isolation. All compounds were tested against *Candida albicans* and *Staphylococcus aureus* for their antimicrobial activities, and against K562, HT-29, BEL-7402, SGC-7901, B16, BGC-823, U251 and A549 cancer cell lines for cytotoxic activities. Compound **17** showed antimicrobial activity against *C. albicans* with the IC_{50} value of 10.74 μM. Compounds **33**, **34**, **39** and **40** displayed cytotoxicity against the HT-29 cell line with the IC_{50} values of 1.26, 1.12, 1.19, and 2.01 μM, respectively (Table 1).

Table 1
Antimicrobial and cytotoxic activities of selected compounds from *C. argyrophylla*.[a]

Compounds	IC_{50} (μM) *C. albicans*	IC_{50} (μM) HT-29
17	10.74 ± 1.25	–
33	–	1.26 ± 0.18
34	–	1.12 ± 0.15
39	–	1.19 ± 0.06
40	–	2.01 ± 0.18
Miconazole nitrate salt	2.50 ± 0.28	
Taxol		0.006 ± 0.0002

[a] HT-29: human colon cancer cell line; "–" means not active; IC_{50} > 10 μg/mL was considered to be inactive.

Table 2
^{13}C NMR spectroscopic data for compounds **1**–**4** in CD_3OD.

No.	**1** δ_C, mult.	**2** δ_C, mult.	**3** δ_C, mult.	**4**[a,b] δ_C, mult.
1	39.2 *t*	39.1 *t*	59.5 *d*	58.5 *d*
2	19.9 *t*	19.9 *t*	206.6 *s*	202.6 *s*
3	39.9 *t*	39.9 *t*	119.5 *d*	119.4 *d*
4	44.7 *s*	44.7 *s*	172.3 *s*	169.0 *s*
5	65.4 *d*	65.3 *d*	46.7 d	45.4 *d*
6	200.7 s	200.6 *s*	55.7 *s*	53.5 *s*
7	128.4 *d*	128.5 *d*	42.2 *t*	40.7 *t*
8	164.8 *s*	164.4 *s*	26.8 *q*	26.2 *q*
9	58.6 *d*	57.4 *d*	22.4 *q*	22.0 *q*
10	45.4 *s*	45.4 *s*	70.4 *t*	69.8 *t*
11	22.5 *t*	22.7 *t*		
12	45.7 *t*	42.7 *t*		
13	74.2 *s*	139.1 *s*		
14	146.2 *d*	125.9 *d*		
15	112.4 *t*	59.4 *t*		
16	27.8 *q*	16.3 *q*		
17	22.8 *q*	22.8 *q*		
18	29.6 *q*	29.6 *q*		
19	175.9 *s*	175.8 *s*		
20	15.5 *q*	15.6 *q*		
1′	95.8 *d*	95.8 *d*	104.0 *d*	104.3 *d*
2′	74.2 *d*	74.2 *d*	75.0 *d*	75.1 *d*
3′	78.1 *d*	78.2 *d*	78.0 *d*	78.5 *d*
4′	71.1 *d*	71.1 *d*	71.6 *d*	71.7 *d*
5′	79.0 *d*	79.0 *d*	78.1 *d*	77.3 *d*
6′	62.5 *t*	62.5 *t*	62.7 *t*	69.0 *t*

[a] ^{13}C NMR data (δ_C) were measured in C_5D_5N for **4**.
[b] Data of the apiofuranosyl unit of **4**, δ_C 111.2 (*d*, C-1″), 77.8 (*d*, C-2″), 80.5 (*s*, C-3″), 75.0 (*t*, C-4″), 65.5 (*t*, C-5″).

Cathargyroside A (**1**) was obtained as colorless solid, and its molecular formula was deduced to be $C_{26}H_{40}O_9$ by negative ion HRESIMS (calcd for $C_{26}H_{39}O_9$ 495.2594; found 495.2595), requiring 7° of unsaturation. The IR spectrum suggested the presence of hydroxy (3375 cm^{-1}), ester (1749 cm^{-1}) and α,*β*-unsaturated ketone (1677 cm^{-1}) groups. Observed in the ^{1}H and ^{13}C NMR spectra (Tables 2 and 3) were four tertiary methyl groups [δ_H 0.96 (3H, s), 1.28

Table 3
^{1}H NMR spectroscopic data for compounds **1**–**4** in CD_3OD.

No.	**1** δ_H (*J* in Hz)	**2** δ_H (*J* in Hz)	**3** δ_H (*J* in Hz)	**4**[a,b] δ_H (*J* in Hz)
1	1.88 *overlapped*	1.87 *m*	2.60 *m*	2.64 *m*
	1.34 *overlapped*	1.34 *overlapped*		
2	1.49 *overlapped*	1.51 *m*		
3	2.22 *overlapped*	2.23 *m*	6.06 *bs*	6.44 *bs*
	1.34 *overlapped*	1.34 *overlapped*		
5	2.43 *s*	2.45 *s*	2.53 *m*	2.33 *t* (5.7)
7a	5.82 *bs*	5.85 *bs*	2.92 *dt* (9.3, 5.5)	2.56 *dt* (9.0, 5.7)
7b			2.11 *d* (9.3)	1.89 *d* (9.0)
8			1.53 *s*	1.21 *s*
9	2.21 *overlapped*	2.29 *m*	1.01 *s*	0.85 *s*
10			4.53 *dd* (17.6, 1.7)	4.97 *dd* (17.1, 1.7)
			4.39 *dd* (17.6, 1.7)	4.51 *dd* (17.1, 1.7)
11	1.57 *overlapped*	1.66 *m*		
	1.55 *overlapped*	1.59 *m*		
12	1.86 *overlapped*	2.35 *m*		
	1.62 *overlapped*	2.13 *m*		
14	5.93 *dd* (17.4, 10.8)	5.42 *m*		
15a	5.22 *dd* (17.4, 1.4)	4.10 *d* (6.8)		
15b	5.06 *dd* (10.8, 1.4)			
16	1.28 *s*	1.72 *s*		
17	2.01 *s*	2.04 *s*		
18	1.43 *s*	1.43 *s*		
20	0.96 *s*	0.96 *s*		
1′	5.40 *d* (8.2)	5.40 *d* (8.2)	4.33 *d* (7.7)	4.88 *d* (7.7)
2′	3.30 *overlapped*	3.30 *overlapped*	3.45 *m*	4.08 *overlapped*
3′	3.41 *m*	3.41 *m*	3.30 *m*	4.21 *overlapped*
4′	3.32 *overlapped*	3.32 *overlapped*	3.50 *overlapped*	4.07 *overlapped*
5′	3.34 *overlapped*	3.34 *overlapped*	3.50 *overlapped*	4.10 *overlapped*
6′a	3.85 *dd* (12.1, 1.6)	3.85 *dd* (12.1, 1.5)	3.70 *m*	4.74 *d* (12.0)
6′b	3.67 *dd* (12.1, 4.8)	3.67 *dd* (12.1, 4.8)	3.65 *m*	4.17 *overlapped*

[a] ^{1}H NMR data (δ_H) were measured in C_5D_5N for **4**.
[b] Data of the apiofuranosyl unit of **4**, δ_H 5.78 (1H, *d*, 2.5, H-1″), 4.75 (1H, *bs*, H-2″), 4.59 (1H, *d*, 9.3, H-4″a), 4.34 (1H, *d*, 9.3, H-4″b), 4.14 (2H, *s*, H-5″).

(3H, s), 1.43 (3H, s), 2.01 (3H, s), δ_C 15.5 (q), 22.8 (q), 27.8 (q), 29.6 (q)], an α,β-unsaturated ketone [δ_H 5.82 (1H, bs), δ_C 128.4 (d), 164.8 (s), 200.7 (s)], an ABM coupling system of three olefinic protons at δ_H 5.06 (1H, dd, J = 10.8, 1.4 Hz), 5.22 (1H, dd, J = 17.4, 1.4 Hz), 5.93 (1H, dd, J = 17.4, 10.8 Hz), δ_C 146.2 (d), 112.4 (t), an ester carbonyl group [δ_C 175.9 (s)], and an oxygenated carbon [δ_C 74.2 (s)]. Thus, compound **1** had a bicyclic-labdane diterpenoid skeleton. Comparison of the NMR spectroscopic data of **1** with those of 6-oxo-isomanol (Zdero et al., 1991) established that they were quite similar, except for a moiety on ring A. The presence of the partial structures $-CH_2CH_2CH_2-$ (C-1 to C-3) and –CH(C-9)–CH_2(C-11)–CH_2(C-12)– was inferred from the ^{1}H–^{1}H COSY and HSQC spectra of **1**. Through careful analysis of the HMBC spectrum (Figure S1, Supplementary information), the partial structures were correlated to constitute a labdane on the basis of the cross-peaks of: H-5 (δ_H 2.43, 1H, s) with C-4, C-6, C-9, C-10, C -18, C -19 and C -20; H-14 (δ_H 5.93, 1H, dd, J = 17.4, 10.8 Hz) with C-12, C-13, C-16; H-17 (δ_H 2.01, 3H, s) with C-7, C-8, C-9; H-18 (δ_H 1.43, 3H, s) with C-3, C-4, C-5 and C-19; H-20 (δ_H 0.96, 3H, s) with C-1, C-5, C-9 and C-10, respectively. The signals at δ_C 95.8 (d), 79.0 (d), 78.1 (d), 74.2 (d), 71.1 (d) and 62.5 (t) in the ^{13}C NMR spectra of **1**, established that the sugar was a glucose moiety, with the β-configuration of the glucose determined from the coupling constant (8.2 Hz) of the anomeric proton signal in the ^{1}H NMR spectrum. The configuration of the sugar unit was confirmed as D-glucose, after acid hydrolysis of **1** by analysis of its R_f and its specific rotation. The correlation between H-1′ of the glucose and C-19 in the HMBC spectrum suggested that the β-D-glucopyranosyl moiety was located at C-19. The ROESY correlations of Me-20 to H-11, but not to H-5 and from H-5 to H-9, Me-18, but not to Me-20 indicated a *trans* fusion of the A and B rings, a *trans* orientation of H-9 and Me-20, as well as a *cis* orientation of H-5 and Me-18 (Figure S1, Supplementary information). The shifts of the resonances for the C-12, C-13, C-14, C-15, and C-16 carbons were similar to those of the corresponding carbons in the co-occurring 13-epicupressic acid methyl ester (**31**) and 7β,13*S*-dihydroxylabda-8(17),14-dien-19-oic acid (Wang et al., 2008), indicating that the configuration of the C-13 position is *S*. Therefore, structure **1** was established as 13*S*-hydroxy-labda-7,14-dien-6-oxo-19-oic acid β-D-glucopyranoside.

HRESIMS of **2** gave a molecular formula of $C_{26}H_{40}O_9$, the same as compound **1**. Its ^{1}H and ^{13}C NMR spectroscopic data were similar to those of **1**, with the significant difference being the absence of the partial structure –C(CH_3)(OH)–CH=CH_2 (C-13 to C-16) in **2** and the presence of the partial structure –C(CH_3)=$CHCH_2$(OH) (C-13 to C-16) which was supported by the measurement of 2D NMR spectroscopic data in **2** as follows. Observations of HMBC cross-peaks of H-15 (δ_H 4.10, 2H, d, J = 6.8 Hz) with C-13, C-14, and of H-16 (δ_H 1.72, 3H, s) with C-12, C-13 and C-14 supported this assignment. Chemical shifts at C-13, C-14, C-15, and C-16 in **2** were similar to those of 7α-hydroxyisocupressic acid (Lin and Rosazza, 1998), further supporting the presence of the partial structure –C(CH_3)=$CHCH_2$(OH) (C-13 to C-16) and the *E*-geometry of the double bond at C-13. Comprehensive 2D NMR (HSQC, HMBC and ROESY) correlation analysis further established the full structure **2** as 15-hydroxy-labda-7,13*E*-dien-6-oxo-19-oic acid β-D-glucopyranoside, named cathargyroside B.

The IR spectrum of vervenone-10-*O*-β-D-glucopyranoside (**3**) indicated the presence of hydroxy (3426 cm^{-1}) and an α,β-unsaturated ketone (1672 cm^{-1}) which was confirmed by analysis of the UV spectrum with the absorption maximum at 251 nm. Compound **3** exhibited a quasi-molecular ion peak at m/z 329 in the positive FABMS. Its positive HRESIMS had the same molecular formula as that of vervenone-8-*O*-β-D-glucopyranoside (Shikishima et al., 2001). The ^{1}H and ^{13}C NMR spectroscopic data of **3** were also similar to vervenone-8-*O*-β-D-glucopyranoside, the difference being in the location of the β-D-glucopyranose which was determined as

follows. Long-range correlations were observed from H-10a, 10b (δ_H 4.53, 4.39) to C-1′ of glc (δ_C 104.0) as well as H-1′ (δ_H 4.33) to C-10 (δ_C 70.4) in the HMBC experiment (Figure S1, Supplementary information), which indicated the point of attachment of the β-glucopyranose to C-10. ROESY (Figure S1, Supplementary information) correlations of H-8 to H-1, H-5, and of H-9 to H-7 implied that C-7 possessed β-orientation. Acid hydrolysis of **3** released D-glucose, with an $[\alpha]_D^{18}$ + 45.3 (*c* 0.21, H_2O). Full ^1H and ^{13}C NMR spectroscopic assignments of **3** were carried out by detailed 2D analyses (HSQC, HMBC and ROESY) (Tables 2 and 3).

Compound **4** was obtained as colorless solid, and its molecular formula was confirmed to be $C_{21}H_{32}O_{11}$ from the sodiated ion peak at *m*/*z* 483.1851 $[M + Na]^+$ (calcd for $C_{21}H_{32}O_{11}Na$, 483.1842) on the basis of positive HRESIMS. The ^1H and ^{13}C NMR spectra of the aglycone portion of **4** were similar to those of **3**, with compound **4** having five carbon atoms more than **3**. The molecular weight of **4** was 132 amu more than that of **3**. A consideration of the NMR signals of the five carbon atoms of **4** and its molecular weight indicated that the five carbon atom portion of **4** was a sugar. Comparison of chemical shifts of the sugars of **4** with those of plucheoside C (Uchiyama et al., 1991) indicated that **4** had the same sugar moieties. HMBC experiment showed the presence of a long-range correlation between H-1″ and C-6′, which indicated that the sugar moiety of **4** was β-D-apiofuranosyl(1″→6′)-β-D-glucopyranose. The relative configuration of the aglycone was the same as that of **3** in the ROESY spectrum. Finally, structure **4** was elucidated as vervenone-10-*O*-β-D-apiofuranosyl-(1″→6′)-β-D-glucopyranoside.

Compound **5** was established to have a molecular formula of $C_{25}H_{32}O_9$ by a HRESIMS analysis in the positive ion mode. A consideration of the NMR spectra of **5** as discussed below and comparison with the co-occurring massonianoside B (**9**) (Bi et al., 2001) indicated that its aglycone portion was that of a dihydrobenzofuranoid lignan. Two pairs of ABX protons in the aromatic region were confirmed by the ^{13}C NMR spectra, which gave clear evidence of the presence of two 1,2,4-substituted benzene rings. The furthest upfield signals at δ_C 32.6 and 35.9 were assigned to the benzylic methylene carbon (C-7′) and the central methylene carbon (C-8′) of the *n*-propanol chain, respectively. The methine resonance at δ_C 55.3 was ascribed to the C-8 benzofuran methine. The downfield signal at δ_C 88.0 was assigned to the aryl-substituted benzofuranmethine (C-7). The upfield resonances at δ_C 65.3 and 62.2 were ascribed to the two hydroxymethylene (C-9, C-9′). 2D NMR studies confirmed that the aglycone was dihydrobenzofuranoid lignan. In addition, one methoxy group was clearly recognized from its signal at 3.79 ppm. This methoxy group correlated with the aromatic quaternary carbon at δ_C 152.1 (C-3) in the HMBC spectrum which indicated the position of this group to be at C-3. This connection was confirmed by a ROESY correlation of H-2 to OMe-3. The chemical shifts of the sugar portion matched the literature values for the sugar portion of **9** which was confirmed by basic hydrolysis of **5** yielding L-rhamnose with $[\alpha]_D^{18}$ + 12.5 (*c* 0.10, H_2O). The L-rhamnose was linked to the 4-position of the aglycone from a strong HMBC correlation of H-1″ to C-4. This connection was also confirmed by a ROESY correlation of H-1″ to H-5. Strong NOE correlations were observed between protons H-7 and H-9a, but only weak correlations between H-7 and H-8. The value of the coupling constant $J_{7,8}$ (5.8 Hz) was also in agreement with those for *trans*-substituted dihydrobenzofuranoid lignan glycosides, **9** (Bi et al., 2001), **10** (Machida et al., 2002), **11** (Machida et al., 2002), (2*S*,3*R*)-3,3′-didemethoxydihydrodehydrodiconiferyl alcohol-4-*O*-β-D-glucopyranoside (Xu et al., 2006a), i.e., 5.8, 5.7, 5.8 and 5.6 Hz. On the basis of the above evidence, the orientation of the substituents at C-7, 8 was *trans*. Thus, structure **5** was assigned as the new compound cedrusinin-4-*O*-α-L-rhamnopyranoside.

The negative ion FABMS of **6** displayed a quasi-molecular ion peak at *m*/*z* 507 $[M - H]^-$, indicating a molecular weight of 508. A molecular formula of $C_{25}H_{32}O_{11}$ was established for **6** through high-resolution ESI-TOF-MS measurement of the ion at *m*/*z* 507.1858 $[M - H]^-$ (calcd: 507.1866). The existence of a sugar unit was indicated by the observation of an anomeric proton at δ_H 4.27 (1H, d, *J* = 7.5 Hz) and the corresponding anomeric carbon at δ_C 105.6. The ^{13}C NMR spectroscopic data of the sugar unit were consistent with those of xylose. The large coupling constant for the anomeric proton indicated a β-configuration for the xylopyranosyl moiety. Acid hydrolysis of **6**, with 2 N HCl at 70 °C for 6 h, produced D-xylose ($[\alpha]_D^{18}$ + 21.3 (*c* 0.10, H_2O)). The chemical shifts of the aglycone moiety were similar to those of (+)-cyclo-olivil (**27**) (Sugiyama et al., 1993), suggesting that compound **6** was an arylnaphthalene lignan glycoside. This coincided with the fact that the molecular weight of **6** was 132 amu more than that of **27**. This was supported by acidic hydrolysis of **6**, providing compound **27** which was identified by co-TLC ($CHCl_3$:CH_3OH, 9:1, R_f 0.5), co-HPLC [MeCN–H_2O (2:8)] with t_R of 25.3 min with corresponding authentic sample. Also, the relative configuration was corroborated by ROESY experiment as discussed below. In the ROESY spectrum, the correlations between H-8 and H-2, H-6, H-9b were observed, but there was no correlation from H-8 to H-7. The observation of long-range correlations in the HMBC spectrum between δ_H 4.27 (Xyl 1″) and δ_C 76.8 (Agly 9′) proved the sugar unit to be attached at C-9′ of aglycone. This attachment was confirmed by observation of a reverse correlation between δ_H 3.61, 4.10 (Agly 9′) and δ_C 105.6 (Xyl 1″). Accordingly, structure **6** was determined as (+)-cyclo-olivil-9′-*O*-β-D-xylopyranoside.

In the Supplementary information, a comparison of the chemical constituents of the genera *Cathaya*, *Picea* and *Pinus* is provided. This analysis indicated that serratane-type triterpenes exist only in *Cathaya* (**33**–**40**), *Picea* (**33**–**40**) and *Pinus* (**34**–**36**) of the Pinaceae. Lignans (**9**–**14**, **23**–**29**), phenylpropanoids (**15**–**17**), aromatic compounds (**20**–**21**), diterpenoids (**30**–**32**) and flavonoids (**18**, **41**–**42**) isolated from *Cathaya* had been also isolated from *Picea* or *Pinus* species or both. Therefore, the chemical constituents of these three genera are very similar, which confirmed that they are closely related in taxonomy and phylogeny.

3. Concluding remarks

There is only one species in *Cathaya*, i.e., *C. argyrophylla*. Herein a systematic chemical study on *C. argyrophylla* led to 45 compounds isolated, including six new compounds and five biologically active compounds, which extends the compound diversity of this plant. The results of careful comparison of chemical constituents of the genera *Cathaya*, *Picea* and *Pinus* of Pinaceae indicated that they are closely related in taxonomy and phylogeny, which is consistent with the results of molecular phylogeny.

4. Experimental

4.1. General experimental procedures

IR spectra were performed on a Bio-Rad FTS-135 spectrophotometer (KBr pellets), whereas UV spectra were obtained using a Shimadzu 2401PC spectrophotometer. Optical rotations were recorded on a Horbia SEAP-300 polarimeter. MS spectra were carried out on VG Autospec-3000 spectrometer and API Qstar Pulsar instrument. NMR spectra were measured with Bruker AM-400 and DRX-500 spectrometers (TMS, internal standard). Semipreparative HPLC was carried out on an Agilent 1100 apparatus with a UV detector at a flow rate of 2 mL/min. Samples were loaded on a YMC-Pack ODS-A (YMC, 10 mm × 15 cm) column. Sephedex LH-20 (25–100 μm, Pharmacia Fine Chemical Co., Ltd., Sweden), silica gel (10–40 μm, 100–200 mesh and 200–300 mesh, Qingdao Marine

Chemical, Inc., China), Lichroprep RP-8 (40–63 μm, Merck, Darmstadt, Germany), and MCI gel (75–150 μm, Mitsubishi Chemical Corporation, Japan) were used for column chromatography (CC). TLC was carried out using precoated silica gel GF_{254} glass plates, with plates visualized using UV light or by spraying with 10% H_2SO_4 in 95% EtOH and heating.

4.2. Material

Twigs and leaves of *C. argyrophylla* were collected from the National Nature Reserve of Huaping, Guangxi Zhuang Autonomous Region, China, in June 2006. Authenticity of the plant was confirmed by Prof. Zhong-Shu Yue (Kunming Institute of Botany, Chinese Academy of Sciences). A voucher sample (No. 0010442) is held at the herbarium of Kunming Institute of Botany, Chinese Academy of Sciences. Cancer cell lines, i.e., K562, HT-29, BEL-7402, SGC-7901, B16, BGC-823, U251 and A549, were bought from the Cell Culture Center of Institute of Basic Medical, Chinese Academy of Medical Sciences, Beijing, China. Bacteria *S. aureus* and *C. albicans* were purchased from the China General Microbiological Culture Collection Center, Beijing, China.

4.3. Extraction and isolation

Air-dried twigs and leaves (5 kg) were powdered and extracted with MeOH (3 × 10 L, each 7 days) at room-temperature, and a residue (650 g) was obtained. The latter was suspended in H_2O and partitioned in sequence with petroleum ether (60–80 °C, unless otherwise noted), EtOAc, and *n*-BuOH to afford a petroleum ether-soluble fraction (100 g), an EtOAc-soluble fraction (130 g), a *n*-BuOH-soluble fraction (210 g), and a H_2O-soluble fraction (120 g).

The *n*-BuOH extract (210 g) was subjected to polyamide CC eluting with H_2O to obtain Fraction 1 (20 g). Fraction 1 was subjected to silica gel CC eluting with a gradient of $CHCl_3$ and MeOH, and three fractions were obtained. Fraction 1.1 was submitted to RP-8 using a H_2O–MeOH gradient (100:0 → 0:100), yielding three fractions 1.11–1.13. Fraction 1.11 was repurified on MCI, eluting with a MeOH–H_2O gradient, to yield nine fractions 1.111–1.119. Fraction 1.111 was passed through a Sephadex LH-20 column (MeOH) and then loaded on semipreparative HPLC [MeOH–H_2O (18:82)] to give **21** (6 mg). Fraction 1.116 was passed through a Sephadex LH-20 column (MeOH) and then purified by semipreparative HPLC [MeCN–H_2O (16:84)] to give **7** (12 mg) and **3** (9 mg). Fraction 1.12 was purified by semipreparative HPLC [MeOH–H_2O (40:60)] to give **5** (2 mg). Fraction 1.13 was subjected to semipreparative HPLC [MeOH–H_2O (57:43)] to give **1** (24 mg) and **2** (2 mg). Fraction 1.2 was applied to an MCI column, eluting with a MeOH–H_2O gradient, to yield six fractions 1.21–1.26. Fraction 1.21 was passed through a Sephadex LH-20 column (MeOH) and then loaded on a semipreparative HPLC [MeOH–H_2O (24:76)] to yield compound **8** (9 mg). Fraction 1.22 was submitted to RP-8 using a H_2O–MeOH gradient (100:0 → 0:100), yielding four fractions 1.221–1.224. Fraction 1.221 was passed through a Sephadex LH-20 column (MeOH) and then purified by semipreparative HPLC [MeCN–H_2O (15:85)] to give **16** (30 mg) and **19** (73 mg). Fraction 1.223 was passed through a Sephadex LH-20 column (MeOH) and then loaded on semipreparative HPLC [MeCN–H_2O (10:90)] to give **6** (7 mg), **9** (37 mg) and a mixture of compounds **12**, **13** and **14** (100 mg). Compounds **12**, **13** and **14** were further purified by semipreparative HPLC [MeOH–H_2O (25:75)] using a ZORBAX Eclipse XDB C18 (Agilent, 9.4 mm × 25.0 cm) column at a flow rate of 2 mL/min to afford **12** (8 mg) and a mixture of **13** and **14** (52 mg). Fraction 1.23 was submitted to RP-8 using a H_2O–MeOH gradient (100:0 → 0:100), yielding two fractions 1.231–1.232. Fraction 1.231 (0.8 g) was passed through a Sephadex LH-20 column (MeOH) and then purified by semipreparative HPLC [MeCN–H_2O (24:76)] to yield compound **20** (8 mg). Fraction 1.3 was applied to an MCI column, eluting with a MeOH–H_2O gradient, to yield 4 fractions 1.31–1.34. Fraction 1.31 (0.8 g) was submitted to RP-8 using a H_2O–MeOH gradient (100:0 → 0:100), yielding two fractions 1.311–1.312. Fraction 1.311 was purified by semipreparative HPLC [MeCN–H_2O (11:89)] to give **17** (30 mg), a mixture of **4** and **18** (67 mg). Compounds **4** and **18** were further purified by semipreparative HPLC using a H_2O–MeOH gradient (20:80 → 50:50) to afford **4** (5 mg) and **18** (8 mg). Fraction 1.312 was purified by semipreparative HPLC [MeCN–H_2O (13:87)] to yield compounds **10** (4 mg), **11** (1 mg) and **15** (3 mg).

The EtOAc extract (130 g) was subjected to silica gel CC eluting with a gradient of $CHCl_3$ and MeOH to afford eight fractions. Fraction 3 was submitted to silica gel CC using a petroleum ether and Me_2CO gradient, to yield five fractions. Fraction 3.2 was passed through a Sephadex LH-20 column (MeOH) and then loaded on an RP-8 column using a H_2O–MeOH gradient (100:0 → 0:100) to give **22** (33 mg) and **25** (15 mg). Fraction 4 was subjected to silica gel CC eluting with a gradient of $CHCl_3$ and MeOH to obtain six fractions. Fraction 4.3 was passed through a Sephadex LH-20 column (MeOH) to afford four fractions. Fraction 4.33 was purified by semipreparative HPLC using a MeCN–H_2O gradient (30:70 → 90:10) to afford **23** (20 mg), **24** (15 mg) and **26** (10 mg). Fraction 4.34 was submitted to an RP-8 column using a H_2O–MeOH gradient (100:0 → 0:100) and then purified by semipreparative HPLC using a MeCN–H_2O gradient (30:70 → 90:10) to afford **28** (16 mg), **29** (5 mg) and **41** (20 mg). Fraction 5 was submitted to semipreparative HPLC using a H_2O–MeOH gradient (70:30 → 0:100) to obtain **27** (60 mg) and **42** (60 mg).

The petroleum ether extract (100 g) was subjected to silica gel CC eluting with petroleum ether–EtOAc (100:0; 10:1; 5:1; 1:1; 1:2; 0:100) to obtain nine fractions. Fraction 2 was subjected to silica gel CC using a petroleum ether and EtOAc gradient, to yield **30** (210 mg) and **33** (70 mg). Fraction 3 was applied to silica gel CC using a gradient of petroleum ether and EtOAc and then passed through a Sephadex LH-20 column [$CHCl_3$–MeOH (1:1)] to obtain **37** (10 mg) and **39** (40 mg). Fraction 5 was subjected to silica gel CC eluting with a gradient of petroleum ether and EtOAc to give **32** (10 mg) and **45** (20 mg). Fraction 6 was applied to silica gel CC using a gradient of petroleum ether and EtOAc and then passed through a Sephadex LH-20 column [$CHCl_3$–MeOH (1:1)] to obtain **35** (60 mg), **38** (500 mg) and **40** (620 mg). Fraction 7 was submitted to silica gel CC using a petroleum ether and Me_2CO gradient to obtain four fractions. Fraction 7.2 was passed through a Sephadex LH-20 column [$CHCl_3$–MeOH (1:1)] to obtain **44** (50 mg). Fraction 7.3 was subjected to silica gel CC using a gradient of petroleum ether and EtOAc to give **31** (230 mg) and **34** (120 mg). Fraction 8 was applied to silica gel CC eluting with a gradient of petroleum ether and EtOAc and then passed through a Sephadex LH-20 column [$CHCl_3$–MeOH (1:1)] to obtain **36** (1 g) and **43** (30 mg).

4.3.1. Cathargyroside A (*1*)

Colorless solid; $[\alpha]_D^{27}$ − 3.9 (*c* 0.60, MeOH); UV λ_{max} (MeOH) nm (log ε) 240 (3.75); IR ν_{max}^{KBr} cm^{-1}: 3375, 3265, 2974, 2940, 1749, 1677, 1467, 1434, 1400, 1208, 1138, 1079, 840, 802, 724; For ^1H NMR (CD_3OD, 400 MHz) and ^{13}C NMR (CD_3OD, 100 MHz) spectroscopic data, see Tables 2 and 3; FABMS (negative) *m*/*z* (%): 495 $[M - H]^-$ (20); HRESIMS (negative) *m*/*z* 495.2595 $[M - H]^-$, (calcd for $C_{26}H_{39}O_9$, 495.2594).

4.3.2. Cathargyroside B (*2*)

Colorless solid; $[\alpha]_D^{25}$ − 23.8 (*c* 0.04, MeOH); UV λ_{max} (MeOH) nm (log ε) 201 (3.82), 230 (3.67) nm; IR ν_{max}^{KBr} cm^{-1}: 3440, 1679, 1639, 1511, 1437, 1206, 1137, 1075, 1036, 840, 802, 723; For ^1H NMR (CD_3OD, 400 MHz) and ^{13}C NMR (CD_3OD, 100 MHz) spectroscopic

Table 4
^{1}H NMR and ^{13}C NMR spectroscopic data for compounds **5–6** in CD_3OD.

No.	**5** δ_H (*J* in Hz)	**6** δ_H (*J* in Hz)	**5** δ_C	**6** δ_C
1			139.1 *s*	138.3 *s*
2	7.01 *overlapped*	6.70 *d* (1.5)	111.2 *d*	114.1 *d*
3			152.1 *s*	149.1 *s*
4			146.5 *s*	146.1 *s*
5	7.07 *d* (8.5)	6.75 *d* (8.0)	119.6 *d*	116.0 *d*
6	6.90 *dd* (8.5, 1.8)	6.66 *dd* (8.0, 1.5)	119.1 *d*	123.6 *d*
7	5.51 *d* (5.8)	4.07 *d* (10.1)	88.0 *d*	44.7 *d*
8	3.44 *m*	2.02 *m*	55.3 *d*	47.6 *d*
9a	3.86 *m*	3.85 *overlapped*	65.3 *t*	60.8 *t*
9b	3.75 *m*	3.51 *overlapped*		
3-O*Me*	3.79 *s*	3.78 *s*	56.4 *q*	56.4 *q*
1′			135.8 *s*	126.5 *s*
2′	7.08 *bs*	6.62 *s*	125.9 *d*	112.9 *d*
3′			128.5 *s*	147.5 *s*
4′			159.4 *s*	145.2 *s*
5′	6.74 *d* (8.2)	6.18 *s*	109.8 *d*	117.3 *d*
6′	7.02 *overlapped*		129.8 *d*	133.5 *s*
7′a	2.62 *t* (7.7)	3.26 *d* (16.8)	32.6 *t*	40.0 *t*
7′b		2.76 *d* (16.8)		
8′	1.80 *m*		35.9 *t*	74.8 *s*
9′a	3.56 *t* (6.5)	4.10 *d* (9.4)	62.2 *t*	76.8 *t*
9′b		3.61 *d* (9.4)		
3′-O*Me*		3.80 *s*		56.4 *q*
1″	5.33 *d* (1.2)	4.27 *d* (7.5)	101.4 *d*	105.6 *d*
2″	3.86 *m*	3.20 *overlapped*	72.2 *d*	75.0 *d*
3″	4.05 *m*	3.34 *m*	72.1 *d*	77.8 *d*
4″	3.44 *m*	3.51 *overlapped*	73.8 *d*	71.2 *d*
5″a	3.72 *m*	3.87 *overlapped*	70.8 *d*	67.1 *t*
5″b		3.22 *overlapped*		
6″	1.22 *d* (6.2)		18.0 *q*	

data, see Tables 2 and 3; FABMS (negative) *m/z* (%): 495 $[M-H]^-$ (3); HRESIMS (negative) *m/z* 495.2588 $[M-H]^-$, (calcd for $C_{26}H_{39}O_9$, 495.2594).

4.3.3. Vervenone-10-O-β-D-glucopyranoside (**3**)

Colorless solid; $[\alpha]_D^{28}$ − 94.0 (*c* 0.25, MeOH); UV λ_{max} (MeOH) nm (log ε) 202 (3.44), 251 (3.59) nm; IR ν_{max}^{KBr} cm^{-1}: 3426, 2925, 2877, 1672, 1204, 1078, 1036, 896, 869; For ^{1}H NMR (CD_3OD, 400 MHz) and ^{13}C NMR (CD_3OD, 100 MHz) spectroscopic data, see Tables 2 and 3; FABMS (positive) *m/z* (%) 329 $[M+H]^+$ (100); HRESIMS (positive) *m/z* 329.1595 $[M+H]^+$, (calcd for $C_{16}H_{25}O_7$, 329.1600).

4.3.4. Vervenone-10-O-β-D-apiofuranosyl-(1″→6′)-β-D-glucopyranoside (**4**)

Colorless solid; $[\alpha]_D^{20}$ − 72.0 (*c* 0.25, MeOH); UV λ_{max} (MeOH) nm (log ε) 202 (3.93), 252 (3.6) nm; IR ν_{max}^{KBr} cm^{-1}: 3428, 2925, 1645, 1419, 1062, 875, 592; For ^{1}H NMR (C_5D_5N, 400 MHz) and ^{13}C NMR (C_5D_5N, 100 MHz) spectroscopic data, see Tables 2 and 3; ESIMS (positive) *m/z* (%) 483 $[M+Na]^+$ (100); HRESIMS (positive) *m/z* 483.1851 $[M+Na]^+$, (calcd for $C_{21}H_{32}O_{11}Na$, 483.1842).

4.3.5. Cedrusinin-4-O-α-L-rhamnopyranoside (**5**)

Colorless solid; $[\alpha]_D^{23}$ − 50.0 (*c* 0.10, MeOH); UV λ_{max} (MeOH) nm (log ε) 207 (3.87), 225 (4.06), 283 (3.50) nm; IR ν_{max}^{KBr} cm^{-1}: 3424, 2939, 1680, 1513, 1489, 1401, 1207, 1139, 1066, 1026, 982, 838, 803, 724; For ^{1}H NMR (CD_3OD, 500 MHz) and ^{13}C NMR (CD_3OD, 100 MHz) spectroscopic data, see Table 4; FABMS (positive) *m/z* (%): 477 $[M+H]^+$ (15); HRESIMS (positive) *m/z* 499.1933 $[M+Na]^+$, (calcd for $C_{25}H_{32}O_9Na$, 499.1944).

4.3.6. (+)-Cyclo-olivil-9′-O-β-D-xylopyranoside (**6**)

Colorless solid; $[\alpha]_D^{20}$ + 26.7 (*c* 0.10, MeOH); UV λ_{max} (MeOH) nm (log ε) 203 (4.80), 284 (3.85) nm; IR ν_{max}^{KBr} cm^{-1}: 3426, 2925, 1618, 1513, 1449, 1430, 1375, 1275, 1255, 1042; For ^{1}H NMR (CD_3OD, 400 MHz) and ^{13}C NMR (CD_3OD, 100 MHz) spectroscopic data, see Table 4; FABMS (negative) *m/z* (%) 507 $[M-H]^-$ (8); HRESIMS (negative) *m/z* 507.1858 $[M-H]^-$, (calcd for $C_{25}H_{31}O_{11}$, 507.1866).

4.4. Acid hydrolysis of **1–4**, **6**

A solution of fraction 1.13 (**1** and **2**) (20 mg) and fraction 1.116 (**3** and **4**) (20 mg) in 2 N HCl (3 mL) was individually incubated at 70 °C for 6 h. After cooling, $NaHCO_3$ was added to neutralize the reaction mixture. The reaction mixture was extracted with $CHCl_3$. D-Glucose was confirmed in the aqueous layer by TLC comparison with an authentic sample [$CHCl_3$–MeOH (8:2)]. The aqueous solution was dried and subjected to silica gel chromatography [$CHCl_3$, $CHCl_3$–MeOH (8:2)] to give (3.8 mg, $[\alpha]_D^{18}$ + 43.5 (*c* 0.30, H_2O); 4.1 mg, $[\alpha]_D^{18}$ + 45.3 (*c* 0.21, H_2O)) respectively. The glucose was identified on the basis of their R_f and specific rotation.

Similar treatment to **6** (5 mg) gave D-xylose (0.7 mg, $[\alpha]_D^{18}$ + 21.3 (*c* 0.10, H_2O)).

4.5. Basic hydrolysis of **5**

A solution of compound **5** (1.5 mg) in 3% NaOH (5 mL) was incubated at room temperature for 2 h. To the resulting solution, it was adjusted to pH 7 by 2 N HCl, and extracted with $CHCl_3$. L-Rhamnose was confirmed in the aqueous layer by comparison with an authentic sample using $CHCl_3$–MeOH (8:2) as a developing system. The aqueous solution was dried and subjected to silica gel CC to give L-rhamnose (0.3 mg, $[\alpha]_D^{18}$ + 12.5 (*c* 0.10, H_2O)). The L-rhamnose was established using their R_f and specific rotation.

4.6. Cytotoxic assay

Cytotoxic activities of the compounds were measured with the sulphorhodamine B (SRB) assay (Han et al., 2008). Aliquots (90 μL) of cells cultured in RPMI 1640 medium (Sigma) were seeded in 96-well flat-bottomed microtiter plates (Greiner) at a density of 3.3–7.7 × 10^4 cells/mL. Twenty-four hours later, 10 μL compounds were added in triplicate with a final concentration of 10 μg/mL. Cells were maintained at 37 °C in 5% CO_2 for another 48 h, and fixed by adding 50% ice-cold CCl_3COOH. Cells were maintained at 4 °C for 1 h. Dye was removed, and SRB was added in 10 mM Tris buffer (100 μL), and the absorbance was read with a Plate Reader at 560 nm (Molecular Devices, SPECTRA MAX 340) after plates were air-dried. IC_{50} values (50% inhibitory concentration) were used for cell growth inhibition, which were measured by dose–response curves with serial fivefold compounds dilutions.

4.7. Antibacterial assay

The turbidimetric method has been used with some modification for antimicrobial experiment (Joslyn and Galbraith, 1950). *S. aureus* and *C. albicans* were maintained in media Mueller–Hinton Broth (Oxiod, CM0405, Hampshire, England) and Potato Dextrose Broth (PDB, lab made), respectively to McFarland 0.5 and diluted with the media at a density of 1 × 10^6 CFU/mL. Aliquots of 90 μL were filled in 96-well U-bottomed microplate. The isolated compounds, dissolved in DMSO and diluted with the medium (10 μL), were dispensed in triplicate in the wells at a final density of 10 μg/mL. Absorbance was read at 620 nm with the microplate reader mentioned above after 24 h. Inhibition was measured as the percentage of maximum absorbance (0.04% DMSO control).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytochem.2012.07.013.

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