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Triterpenoid saponins and anti-inflammatory activity of *Codonopsis lanceolata*

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The ethanolic root extract of *Codonopsis lanceolata* were evaluated for anti-inflammatory activity using the carrageenan induced rat hind paw edema model and displayed a significant activity of 51.82% inhibition at 200 mg/kg at 3 h ($p < 0.05$). Further isolation of the extract yielded two new triterpenoid saponins, named codonolaside I (**1**) and codonolaside II (**2**). The spectroscopic and chemical data revealed their structures to be 3-*O*-[β-D-xylopyranosyl (1→3)-(6′-*O*-methyl)-β-D-glucuronopyranosyl]-3β, 16α-dihydroxyolean-12-ene-28-oic acid 28-*O*-[β-D-xylopyranosyl (1→4)-α-L-rhamnpyranosyl (1→2)-α-L-arabinopyranosyl] ester (**1**), and 3β, 16α-dihydroxyolean-12-ene-28-oic acid 28-*O*- [β-D-xylopyranosyl (1→3)-β-D-xylopyranosyl (1→4)-α-L-rhamnpyranosyl (1→2)-α-L-arabinopyranosyl] ester (**2**).

1. Introduction

Codonopsis lanceolata (Sieb et Zucc) Bentham et Hooker (Campanulaceae) is a perennial, twining vine that occurs in the northeast districts of China. Its roots have been used as an herbal drug for the treatment of bronchitis, cough, spasm, and inflammation in China, and as a tonic crude drug and an edible plant in Korea (Jiangsu New Medical College 1977). Apart from medicinal folklore background of *C. lanceolata* as mentioned above, the discovery of anti-mutagenic effect (Han et al. 2004a) associated with inhibition of the micronucleus rate in the bone marrow cells in mice (Han et al. 2003), inducing caspase-dependent apoptosis in human acute promyelocytic leukemia HL-60 cells (Lee et al. 2005), hepatoprotective activity (Han et al. 2004b), and anti-oxidative effect has been reported (Han et al. 1999). Codonoposide (Lee et al. 2002), a triterpenoid saponin, was reported as characteristic constituent of *C. lanceolata* roots and considered to be partially responsible for the therapeutic effects of this crude drug. However, saponin constituents of *C. lanceolata* seemed not to be completely known and thus deserved further studies. Our current study led to purification of two new triterpenoid saponins from a saponins-rich fraction of the root ethanol extract. This paper deals with the structural elucidation of the new triterpenoid saponins on the basis of chemical and spectral means, especially 1D and 2D NMR spectroscopy. The anti-inflammatory activity of the root ethanol extract is also reported.

2. Investigations, results and discussion

A concentrated root ethanol extract of *Codonopsis lanceolata* was suspended in water and defatted with petroleum ether, then extracted with EtOAc. The remaining aqueous solution was passed through a HPD-100 absorbent resin column, and eluted gradiently with ethanol-H_2O. The 75% ethanol eluate fraction was subjected to repeated column chromatography over silica gel, as well as Sephadex LH-20 to afford compounds **1** and **2**.

Compound **1** was isolated as a colorless powder, and had a molecular formula of $C_{58}H_{92}O_{26}$ determined from its negative ion HR-ESI-MS (at m/z 1239.55940 $[M + Cl]^-$). Its IR spectrum indicated the presence of hydroxyl (3426 cm^{-1}) and ester carbonyl group (1736 cm^{-1}). The ^1H NMR spectrum of **1** showed signals for seven tertiary methyl groups at δ 1.80, 1.27, 1.14, 1.05, 1.00, 0.95 and 0.82 (each s), and a trisubstituted olefinic proton at δ 5.59 (brs), which were characteristics of the olean-12-ene skeleton. Furthermore, five anomeric proton signals were identified at δ 6.49 (brs), 5.69 (brs), 5.22 (d, J = 7.6 Hz), 5.18 (d, J = 7.3 Hz), and 4.98 (d, J = 7.8 Hz). The ^{13}C NMR spectrum of **1** showed the presence of 58 carbon atoms in the molecule, 30 carbon signals were due to the triterpenoid aglycone, a carbon signal at δ 52.08 was observed for a methoxyl group, the remaining 27 carbon signals, including five anomeric carbon resonating at δ 107.32, 106.23, 106.08, 101.03, and 93.47, were seen for the sugar moieties, indicating the presence of two hexose and three pentose units. Evaluation of spin-spin couplings and chemical shifts with the aid of $^1H-^1H$ COSY and NOESY experiments allowed the identification of one β-glucuronopyranosyl, one α-arabinopyranosyl, one α-rhamnopyranosyl and two β-xylopyranosyl units (Corea et al. 2004). Comparison of the ^{13}C NMR data of the aglycone moiety of **1** with those of echinocystic acid (Zhang et al. 1999) showed that the signals for C-3 (δ 89.20) and C-28 (δ 175.98) were significantly shifted owing to glycosidation. These were confirmed by the fact that the acid hydrolysis of **1** with 1 M HCl gave a triterpene (**1a**) which was identified as echinocystic acid by ^{13}C NMR data (Zhang et al.

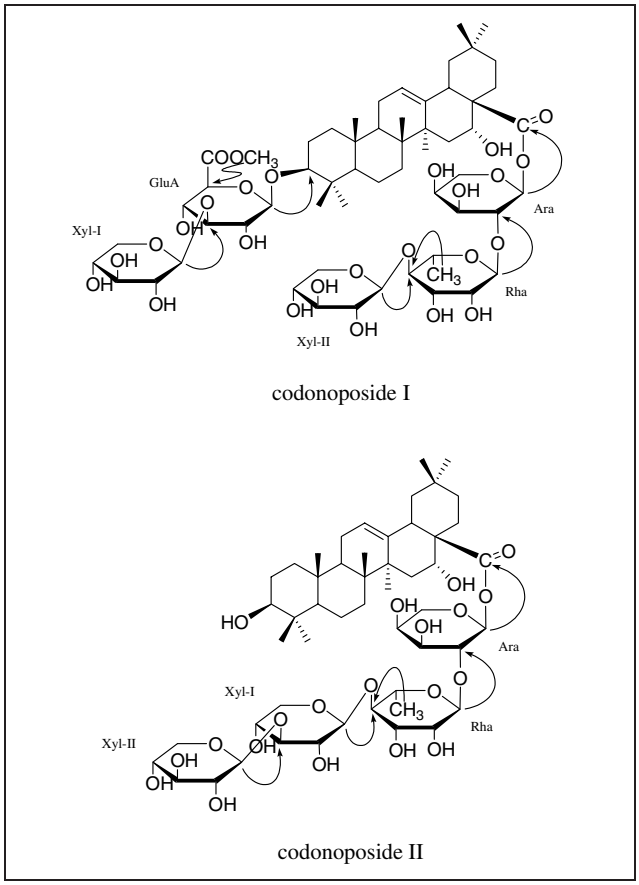

Fig.: Structures of codonoposide I (1) and II (2) and selected HMBC correlations

1999) and co-TLC with authentic sample, and the sugar moieties were shown to be glucuronic acid, xylose, rhamnose, and arabinose on TLC analysis. Alkaline hydrolysis of **1** furnished a prosapogenin (**1b**), identified as 3-*O*-β-D-glucuronopyranosyl echinocystic acid from its spectral data (Lee et al. 2002, 2005).

The sugar chain attached at C-3 of the aglycone was established from the HMBC correlations: the anomeric proton signal at δ 5.22 (Xyl-I) and C-3 of glucuronosyl at δ 87.11, the anomeric proton signal at δ 4.98 (GluA) and C-3 of the aglycone at δ 89.20. In addition, the HMBC correlation between the methoxyl protons at δ 3.72 and C-6 of the glucuronosyl at δ 170.90 indicated that the glucuronosyl moiety is a 6-methoxyl-derivative. The characteristic signals for the anomeric proton (δ 6.49) and carbon (δ 93.47) of the arabinosyl unit suggested that the arabinosyl should be directly attached to C-28 of the aglycone through an ester bond, which was confirmed by HMBC correlation of the anomeric proton at δ 6.49 (Ara) with C-28 of the aglycone at δ 175.98. Furthermore, the HMBC correlations between the anomeric proton signal at δ 5.17 (Xyl-II) and C-4 of rhamnosyl at δ 83.40, the anomeric proton signal at δ 5.69 (Rha) and C-2 of arabinosyl at δ 75.35 suggested the sugar sequence and linkage position of sugar chain attached at C-28 shown in Fig.1. Based on extensive analysis of its 2DNMR spectra (^{1}H−^{1}H COSY, NOESY, HSQC, and HMBC), the ^{1}H and ^{13}C NMR data for **1** were summarized in Table 1, which were found to be similar to those reported for codonoposide (Lee et al. 2002), However, **1** was characterized by the presence of a 6-methoxyl glucuronosyl unit instead of a glucuronosyl moiety. Besides, the terminal xylosyl (Xyl-II)

Table 1: ^{1}H and ^{13}C NMR data of codonoposide I (1) in pyridine-d_5[1]

Position	δ_H	δ_C	Position	δ_H	δ_C
Aglycone			Sugar at C-3		
1	1.45, 0.89 t (8.0)	38.79	GluA 1	4.98 d (7.8)	107.32
2	2.14, 1.85	26.67	2	4.08	75.46
3	3.36 dd-like	89.20	3	4.03	87.11
4		39.56	4	4.04	69.06
5	0.79 t (12.1)	55.91	5	4.60 d (9.7)	77.24
6	1.45	18.55	6		170.90
7	1.59 t (11.1), 1.43	33.49	−OMe	3.72 s	52.08
8		40.06	Xyl-I 1	5.22 d (7.6)	106.08
9	1.74 t (8.4)	47.19	2	4.02	74.96
10		37.02	3	4.13	78.23
11	1.89	23.84	4	4.16	70.97
12	5.59 brs	122.85	5	4.27, 3.67 t (10.5)	67.36
13		144.46	Sugar at C-28		
14		42.10	Ara 1	6.49 brs	93.47
15	2.42, 1.80	36.02	2	4.52	75.35
16	5.26 brs	74.11	3	4.52	75.27
17		49.59	4	4.54	69.53
18	3.58	41.29	5	4.53, 3.95	62.78
19	2.77 t (13.3), 1.35	47.19	Rha 1	5.69 brs	101.03
20		30.98	2	4.51	71.87
21	2.30, 1.30	36.20	3	4.55	72.73
22	2.30, 2.19	32.19	4	4.36	83.40
23	1.27 s	28.21	5	4.37	68.55
24	0.95 s	17.00	6	1.70 d (4.3)	18.41
25	0.82 s	15.68	Xyl-II 1	5.18 d (7.3)	106.23
26	1.05 s	17.61	2	4.47	73.24
27	1.80 s	27.23	3	4.26	77.94
28		175.98	4	4.16	70.97
29	1.00 s	33.33	5	4.17, 3.42	66.87
30	1.14 s	24.82			

[1] J values (in Hz) in parentheses

was assigned to attach to C-4 of the middle rhamnosyl rather than C-3 of the rhamnosyl, since both the anomeric proton signal at δ 5.17 (Xyl-II) and the secondary methyl signal at δ 1.70 (Rha) showed the long-range correlations with C-4 of rhamnosyl at δ 83.40 in the HMBC spectra. Accordingly, **1** was concluded to be 3-*O*-[β-D-xylopyranosyl (1→3)-(6′-*O*-methyl)-β-D-glucuronopyranosyl]-3β, 16α-dihydroxyolean-12-ene-28-oic acid 28-*O*-[β-D-xylopyranosyl (1→4)-α-L-rhamnpyranosyl (1→2)-α-L-arabinopyranosyl] ester, and named codonolaside I.

Compound **2** was obtained as a colorless powder. Its negative ion HR-ESI-MS showed a quasi-molecular ion peak at m/z 1013.53130 $[M-H]^-$), corresponding to a formula of $C_{51}H_{82}O_{20}$. A comparison of the ^1H and ^{13}C NMR data of **2** (Table 2) with those of codonolaside I (**1**) showed that they share the same aglycone. In addition, the ^1H and ^{13}C NMR data of the sugar moiety, including four anomeric proton signals at δ 6.53 (brs), 5.73 (brs), 5.25 (d, J = 7.6 Hz), and 5.20 (d, J = 7.0 Hz) correlated with anomeric carbons resonated at δ 93.49, 101.19, 106.08, and 106.26 respectively, indicated the presence of one α-arabinopyranosyl, one α-rhamnopyranosyl and two β-xylopyranosyl units. Furthermore, in the ^{13}C NMR spectrum of **2**, the C-3 and C-28 carbon signals were observed at δ 78.14, and δ 175.96 suggesting that no sugar linkage was formed at the C-3 hydroxyl group and that a tetrasaccharide was attached to the C-28 carboxyl group of the aglycone through an ester bond. The acid hydrolysis of **2** with 1 M HCl afforded sugars, which were identified as xylose, rhamnose, and arabinose by co-TLC with authentic samples.

The sugar chain attached at C-28 of the aglycone was established as shown in Fig. 1 from the HMBC correlations: the anomeric proton signal at δ 5.25 (Xyl-II) and C-3 of xylosyl (Xyl-I) at δ 87.11, the anomeric proton signal at δ 5.20 (Xyl-I) and C-4 of rhamnosyl at δ 83.44, the anomeric proton signal at δ 5.73 (Rha) and C-2 of arabinosyl at δ 75.35, the anomeric proton at δ 6.53 (Ara) and C-28 of the aglycone at δ 175.96. Thus, the structure of **3**

Table 2: ^1H and ^{13}C NMR data of codonoposide II (2) in pyridine-d_5[1]

Position	δ_H	δ_C	Position	δ_H	δ_C
Aglycone			Sugar at C-28		
1	1.61, 1.05	39.13	Ara 1	6.53 brs	93.49
2	1.83	28.18	2	4.56	75.35
3	3.47	78.14	3	4.56	75.30
4		39.42	4	4.57	69.60
5	0.90 d (11.9)	55.96	5	4.56,	62.83
6	1.58, 1.43	18.88		3.98 dd (3.9, 10.5)	
7	1.65, 1.47	33.58	Rha 1	5.73 brs	101.19
8		40.09	2	4.55	71.87
9	1.81	47.31	3	4.59	72.73
10		37.43	4	4.40	83.44
11	1.99	23.90	5	4.41	68.55
12	5.66 brs	122.89	6	1.75 d (4.7)	18.41
13		144.49	Xyl-I 1	5.20 d (7.0)	106.26
14		42.13	2	4.04	75.35
15	2.44, 1.83	36.00	3	4.05	87.11
16	5.29 brs	74.11	4	4.06	69.05
17		49.61	5	4.21, 3.46	66.86
18	3.61	41.32	Xyl-II 1	5.25 d (7.6)	106.08
19	2.80 t (13.6), 1.41	47.16	2	4.04	74.96
20		30.96	3	4.16	78.22
21	2.35, 1.33	36.21	4	4.18	70.96
22	2.35, 2.22	32.17	5	4.30 dd (4.5, 11.1),	67.35
23	1.23 s	28.80		3.68 t (10.4)	
24	1.05 s	16.60			
25	0.95 s	15.79			
26	1.13 s	17.65			
27	1.81 s	27.21			
28		175.96			
29	1.02 s	33.29			
30	1.17 s	24.80			

[1] J values (in Hz) in parentheses

Table 3: Effects of the roots ethanol extract of *Codonopsis lanceolata* (REE) and acetylsalicylic acid (ASA) on carrageenan-induced rat paw edema[1]

Treatment	Dose mg/kg	Diameter (in mm) at each measure time (h)			
		1.0	2.0	3.0	4.0
Control (normal saline)		1.78 ± 0.08	2.16 ± 0.07	2.47 ± 0.04	2.85 ± 0.06
REE	50	1.76 ± 0.11 (1.12)	1.79 ± 0.08 (17.13)	1.53 ± 0.09 (38.06)	1.98 ± 0.08 (30.53)
	100	1.43 ± 0.07 (19.66)	1.40 ± 0.06 (35.19)	1.39 ± 0.06 (43.72)	1.70 ± 0.04 (40.35)
	200	1.41 ± 0.02 (20.79)	1.32 ± 0.03 (38.89)	1.19 ± 0.11 (51.82)*	1.53 ± 0.10 (46.32)*
ASA	100	1.08 ± 0.02 (39.33)	0.79 ± 0.33 (63.43)**	0.52 ± 0.07 (78.95)**	0.82 ± 0.08 (71.23)**

[1] Each value represents the mean ± S.E.M. of six observations; percentage inhibitions in parentheses are indicated as %
* indicates significant activity compared to control ($P < 0.05$)
** indicates significant activity compared to control ($P < 0.01$)

was established as 3β,16α-dihydroxyolean-28-oic acid 28-*O*-[β-D-xylopyranosyl (1→3)-β-D-xylopyranosyl (1→4)-α-L-rhamnpyranosyl (1→2)-α-L-arabinopyranosyl] ester, named codonolaside II.

The anti-inflammatory effect of the ethanolic root extract of *Codonopsis lanceolata* was evaluated using the carrageenan induced hind paw edema of healthy adult albino rats. The oral pre-treatment with 100 mg/kg of the extract gave a mild activity (Table 3). It exhibited a significant ($p < 0.05$, $n = 6$) anti-inflammatory activity with the highest percentage of inhibition of 51.82% at 200 mg/kg dose level at 3 h, while the reference acetylsalicylic acid (ASA) exhibited an activity of 78.95% inhibition at 100 mg/kg dose level at 3 h. The low yields of codonolaside I and II did not permit their biological evaluation in the animal model employed in the present study. According to the literature (Navarro et al. 2001), some oleanane-type triterpenoid saponins have shown anti-inflammatory activity *in vivo*, so further chemical and pharmacological studies are necessary for the correlation between the isolated saponins and the traditional use of *Codonopsis lanceolata* in the treatment of inflammatory-based diseases.

3. Experimental

3.1. Equipment

Optical rotations were obtained on a Perkin-Elmer 241MC polarimeter. IR spectra were recorded in KBr discs using a Bruker IFS-55 spectrometer. NMR spectra were recorded on a Bruker ARX-600 (600 MHz) instrument with TMS as internal standard. ESI-MS were obtained on an Angilent LCQ mass spectrometer. For column chromatography, HPD-100 absorbent resin (Cangzhou Bon Chemical Co. Ltd, China), silica gel (200–300 mesh, Marine Chemical Industry Factory, Qingdao, China), ODS C_{18} (75 μm, YMC Co. Ltd, Japan), Sephadex LH-20 (Merck, German) were employed in the separations. TLC was performed with silica gel G (Marine Chemical Industry Factory, Qingdao, China), and developed by spraying with 10% ethanolic H_2SO_4 reagent followed by heating.

3.2. Plant material

The roots of *Codonopsis lanceolata* were collected in Liaoyang, Liaoning province, China, on May 2004. The plant material was identified by Professor Wei-Chun Wu of Shenyang Phamaceutical University, Shenyang, China. A voucher specimen (NO. 20041007) is preserved in School of Traditional Chinese Materia Medica, Shenyang Pharmaceutical University.

3.3. Animals

Animals used in this study were male Wistar rats with a weight of 150–180 g. Animals were allowed free access to food and water, except for the day of experiment.

3.4. Extraction and isolation

The dried underground parts (5.0 kg) of *C. lanceolata* were extracted twice with hot 70% ethanol. After concentration *in vacuo*, the ethanol extract (REE, 834 g) was suspended in water, and defatted with petroleum ether, then extracted with EtOAc. The remaining aqueous solution was passed through a HPD-100 absorbent resin column, and eluted gradiently with ethanol-H_2O. The 75% ethanol eluate fraction, in which triterpenoid saponins were enriched, was subjected to column chromatography over silica gel, eluting gradiently with $CHCl_3$–MeOH. The fraction eluted by $CHCl_3$–MeOH (85 : 15) contained **1** and **2**, and was repeatedly chromatographed over Sephadex LH-20, as well as octadecylsilanized (ODS) silica gel, to yield compounds **1** (70 mg), compounds **2** (30 mg).

3.5. Compounds isolated

3.5.1. Codonolaside I (**1**)

Colorless amorphous solid, $[\alpha]_D^{20} - 62.0°$ (C, 0.685, MeOH). IR (ν_{max}, cm^{-1}) 3426 (OH), 2925, 1736 (ester carbonyl), 1630, 1385, 1211, 1043, 616. Positive ion ESI-MS m/z: 1227 $[M + Na]^+$, 1095 $[M + Na\text{-xylose}]^+$; negative ion ESI-MS m/z: 1240 $[M + Cl]^-$, 471. Negative ion HR-ESI-MS m/z: 1239.55940 $[M + Cl]^-$ (calcd for $C_{58}H_{92}O_{26}Cl$ 1239.55648). ^1H NMR (600 MHz, TMS, pyridine-d_5) and ^{13}C NMR (150 MHz, pyridine-d_5) δ: see Table 1.

3.5.2. Codonolaside II (**2**)

Colorless amorphous solid, $[\alpha]_D^{20} - 33.3°$ (C, 0.300, MeOH). IR (ν_{max}, cm^{-1}): 3428 (OH), 2926, 1733 (C=O), 1633, 1384, 1043, 618. Positive ion ESI-MS m/z: 1037 $[M + Na]^+$, 471; negative ion ESI-MS m/z: 1013 $[M-H]^-$. Negative ion HR-ESI-MS m/z: 1013.53130 $[M-H]^-$ (calcd for $C_{51}H_{81}O_{20}$ 1013.53207). ^1H NMR (600 MHz, pyridine-d_5) and ^{13}C NMR (150 MHz, pyridine-d_5) δ: see Table 2.

3.6. Acid hydrolysis of 1 and 2

Compound **1** (15 mg) in 3 mL of 1 M HCl (MeOH : H_2O, v : v = 2 : 8) was refluxed at 100° for 6 h, and then neutralized with NH_4OH. The solution was extracted with EtOAc (1 mL × 2). The EtOAc and water layer were evaporated *in vacuo*. The EtOAc layer contained the aglycone (**1a**) and the water layer contained sugars. The aglycone (**1a**) was identified as echinocystic acid through comparison with its reported spectral data (Zhang et al. 1999) and by co-TLC ($CHCl_3$–MeOH, v : v = 12 : 1). Glucuronic acid, xylose, rhamnose, and arabinose in the water layer were revealed by co-TLC (EtOAc : MeOH : H_2O : AcOH, v : v = 15 : 5 : 3 : 3). By the same method, compound **2** was acid-hydrolyzed and yielded xylose, rhamnose, and arabinose from the water layer.

3.7. Alkaline hydrolysis of 1

Compound **1** (15 mg) in 3 mL of 1 M NaOH (MeOH : H_2O, v : v = 2 : 8) was refluxed at 90° for 1.5 h, and then neutralized with 1 M HCl. The solution was extracted with EtOAc (1 mL × 2). The EtOAc layer was evaporated *in vacuo* and afforded a prosapogenin (**1b**), which was identified as 3-*O*-β-D-glucuronopyranosyl echinocystic acid through comparison with its reported spectral data (Lee et al. 2002, 2005).

3.8. Anti-inflammatory activity evaluation

The carrageenan-induced rat hind paw edema was used as a model of acute inflammation (Mascolo et al. 1997). Animals were divided into groups of six each and pretreated as follows: control group (physiological saline solution, 0.9%, 10 ml/kg, p.o.), acetylsalicylic acid (ASA) group (100 mg/kg, p.o.), the roots ethanol extract of *Codonopsis lanceolata* (REE) group I (200 mg/kg, p.o.), REE group II (100 mg/kg, p.o.), and REE group III (50 mg/kg, p.o.). Sixty minutes after treatment, 1 ml of 0.1% (w/v) carrageenan (Sigma products) suspension in normal saline was injected into the right hind paw of each rat. The linear diameter of the injected paw was measured using a micrometer screw gauge for 4 h at 60 min interval after the administration of the phlogistic agent. The percentage inhibition of the inflammation was calculated from Eq. (1)

$$\% \text{ Inhibition} = D_0 - D_t/D_0 \times 100 \quad (1)$$

where D_0 is the average linear diameter of the injected paw of the control group rats at a given time; and D_t is the average linear diameter of the injected paw of the drug (i.e. REE or reference ASA) treated rats at the same time (Moody et al. 2006).

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