SHORT REPORT

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Natural Glycosides from *Indigofera stachyoides radix*

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Abstract: Six natural polar glycosides were isolated and identified from *indigofera stachyoides radix*, they were identified as β-sitosterol-D-glucoside (**1**), schizandriside (**2**), kaempferol-7-O-β-D-glucopyranoside (**3**), 3,4,5-trimethoxyphenyl-O-β-D-glucopyranoside (**4**), 2-methoxy-4-(2'-hydroxyethyl)–phenol-1-O-β-D-glucopyrano-side (**5**) and 2-(3-hydroxy-4-methoxyphenyl) ethyl 1-O-β-D-glucopyranoside (**6**). Meanwhile, four flavonoids including 7, 4'-dihydroxyl-3'-methoxyisoflavone (**7**), calycosin (**8**), 7-hydroxyl-4'-methoxyflavanone (**9**), maackiain (**10**) and one steroid compound, stigmasterol (**11**) were also reported herein.

Key words: *Indigofera stachyoides radix*; glycoside; schizandriside; kaempferol-7-O-β-D-glucopyranoside, natural medicine.

1. Plant Source

Indigofera stachyoides radix, the dry roots of *Indigofera stachyoides* Lindl. were collected in May 2016 from Liuzhi Special District of Guizhou Province, the southwest China, and were authenticated by Professor Deyuan Chen, Guiyang College of Traditional Chinese Medicine. A sample (No. 201605A) was deposited in Herbarium of Chinese Materia Medica and Ethnomedicines, School of Pharmacy, Guizhou Medical University.

2. Previous Studies

Indigofera stachyoides radix, named Xuerenshen in pinyin as Chinese medicine in the southwest China has been clinically proven to stimulating blood circulation, eliminating dampness & phlegm and relieving exterior syndrome based on traditional Chinese medicine theory (TCM). It has been used for treatment of cold, fever, headache, ulcer, women's abdominal pain & massive vaginal bleeding [1,2]. *Indigofera stachyoides radix* as the assistant herbal medicine in Qijiao Shengbai Capsule □ a kind of Chinese proprietary medicines plays an important therapeutic role. Our group has carried out systematic research on its medicinal substances, found its hepatoprotective activity [3-6]. In the actual research process, most compounds in this

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medicinal plant showed relatively large polarity and even unstable. In this work, we further had enriched chemical constituents of *Indigofera stachyoides radix* to provide a clear material basis for its new drug development and modernization of TCM.

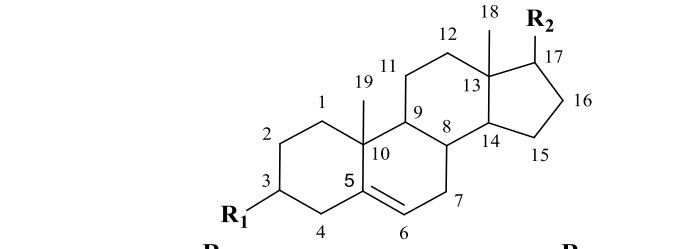
3. Present Study

The dry root of *Indigofera stachyoides* Lindl. 20.0 kg were pulverized to crude powder (80 mesh), then extracted three times with 95% ethanol under reflux (4h, 4h, 3h). After the extraction solution was filtered, the residue was further under reflux extraction using 50% ethanol for 2h. All filtration solution was merged, concentrated under reduced pressure to obtain the crude extract which was suspended in water and partitioned successively with petroleum ether, ethyl acetate, and n-butyl alcohol, respectively. After evaporation of the solvent under reduced pressure, the ethyl acetate extract 496 g was obtained, then subjected to silica-gel (200-300 mesh, Qingdao Marine Chemical, China) column chromatography with chloroform-methanol (19:1~1:1) as the gradient eluent to yield five fractions (Fr1~Fr5).

The Fr1 (12.1 g) was chromatographed on a silica gel column using a gradient solvent petroleum ether-acetone (200:1~1:1) to give seven subfractions (Fr1-1~Fr1-7). Compound **11** (100.0 mg) was obtained by recrystallization from Fr1-2. Fr1-3 was further purified by LH-20 column (GE Healthcare, Sweden) eluted with acetone to obtain subfractions, compound **6** (9.0 mg) and compound **10** (25.0 mg) were afforded by silica gel column chromatography with petroleum ether-ethyl acetate (20:1, 10:1). Fr1-4 was purified by LH-20 column eluted with dichloromethane-methanol (1:1), the subfractions were subjected to HPLC C18 column chromatography using the mobile phase of acetonitrile : water (23:77) to get compounds **7** (2.3 mg), **8** (3.0 mg) and **9** (2.5 mg). Fr4 (145 g) was chromatographed on MCI gel column (75-150 μm, Mitsubishi Chemical, Japan) eluting with methanol : water (5:95) to afford (Fr4.1~Fr4-5). Fr4-1 was further purified by LH-20 column eluted with methanol : water (1:1), and then Semi-preparative HPLC C18 column chromatography (Welch Ultimate XB-C18, 4.6 mm×250 mm, 10 μm) using the mobile phase of acetonitrile : water (7:93) to get compounds **4** (8.0 mg) and **5** (9.0 mg). Compound **1** (50.0 mg) was obtained by recrystallization from Fr4-2. Fr4-4 was purified to afford compound **3** (10 mg) by LH-20 column eluted with methanol, the eluted residue was finally recrystallized to get compound **2** (200 mg). All NMR spectra were recorded on Bruker AV-600 NMR, DRX-500 NMR spectrometers. ESI-MS spectra were recorded on Shimadzu LC-IT-TOF, Thermo TSQ Endura LC/MS/MS.

*Compound **1*** was obtained as a white powder. ^{1}H NMR (500 MHz, DMSO-*d*6) δ: 5.32 (1H, d, *J* = 4.8 Hz, H-1'), 4.90 (3H, m, H-6, 4'-OH, 2'-OH), 4.46 (1H, t, *J* = 5.8 Hz, 3'-OH), 4.21 (1H, d, *J* = 7.8 Hz, 6'-OH), 3.64 (1H, dd, *J* = 11.4, 7.2 Hz, H-2'), 3.09 (5H, m, H-3', 4', 5', 6', 6'), 2.89 (1H, td, *J* = 8.6, 4.8 Hz, H-3), 2.36 (1H, dd, *J* = 12.4, 3.5 Hz, H-4), 2.12 (1H, t, *J* = 12.3 Hz, H-7), 1.93 (2H, m, H-4, 7), 1.79 (2H, m, H-15, 16), 1.51 (7H, m, H-2, 11, 12, 15, 16, 28, 28), 1.27 – 0.98 (16H, m, H-1, 1, 2, 8, 9, 11, 12, 14, 17, 20, 22, 22, 23, 23, 24, 25), 0.81 (18H, m, 18, 19, 21, 26, 27, 29-CH_3). ^{13}C NMR (126 MHz, DMSO-*d*6) δ: 140.51 (C-5), 121.31 (C-6), 100.83 (C-1'), 76.99 (C-5'), 76.80 (2C-3, 3'), 73.52 (C-2'), 70.14 (C-4'), 61.14 (C-6'), 56.25 (C-14), 55.48 (C-17), 49.67 (C-9), 45.19 (C-24), 41.92 (C-4), 41.81 (C-13), 38.36 (C-12), 36.90 (C-1), 36.28 (C-10), 35.56 (C-20), 33.40 (C-22), 31.48 (C-8), 31.44 (C-7), 29.32 (C-2), 28.75 (C-25), 27.88 (C-23), 25.46 (C-15), 23.94 (C-16), 22.66 (C-28), 20.67 (C-11), 19.80 (C-27), 19.18 (C-26), 19.00 (C-21), 18.69 (C-19), 11.85 (C-29), 11.74 (C-18). The above NMR data was consistent with the reference [7], this compound was eventually identified as β-sitosterol-D-glucoside (RN 474-58-8) (Figure 1).

*Compound **2*** was obtained as white needle-like crystals. Its ESI-MS peak at *m/z* 491.16 $[M-H]^-$. ^{1}H NMR (500 MHz, CD_3OD) δ: 6.73 (1H, d, *J* = 2.0 Hz, H-2'), 6.69 (1H, d, *J* = 8.1 Hz, H-5'), 6.60 (1H, s, H-2), 6.58 (1H, dd, *J* = 8.1, 2.0 Hz, H-6'), 6.12 (1H, s, H-5), 4.01 (2H, m, H-9), 3.93 (1H, dd, *J* = 9.8, 2.4 Hz, H-1''), 3.77 (1H, d, *J* = 5.6 Hz, H-7'), 3.76 (3H, s, 3'-OCH_3), 3.75 (3H, s, 3-OCH_3), 3.67 (2H, m, H-9'), 3.41 (2H, m, H-5''), 3.16 (2H, m, H-2'', 3''), 3.07 (1H, m, H-4''), 2.77 (2H, m, H-7), 2.02 (1H, m, H-8'), 1.80 (1H, t, *J* = 10.4 Hz, H-8). ^{13}C NMR (126 MHz, CD_3OD) δ: 148.92 (C-3'), 147.15 (C-3), 145.83 (C-4'), 145.13 (C-4), 138.63 (C-1'), 134.31 (C-6), 129.10 (C-1), 123.11 (C-6'), 117.37 (C-5), 116.07 (C-5'), 114.23 (C-2'), 112.35 (C-2), 105.82 (C-1''), 77.91 (C-3''), 75.00 (C-2''), 71.26 (C-4''), 69.38 (C-9'), 66.91 (C-5''), 65.09 (C-9), 56.43 (3'-OCH_3), 56.36 (3-OCH_3), 49.85, 47.92 (C-7'), 45.87 (C-8'), 39.58 (C-8), 33.85 (C-7). The above NMR data was consistent with the reference [8], this compound was eventually identified as schizandriside (RN 80734-72-1).



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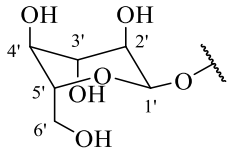
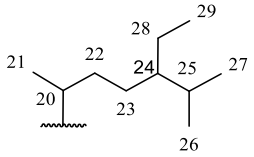
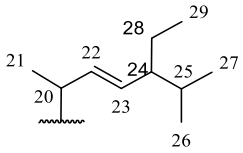
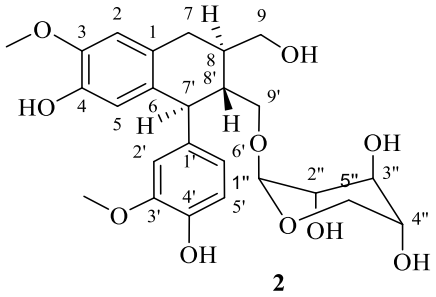
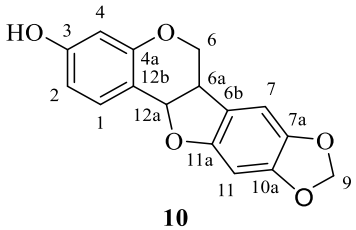
1		
11	OH	



2



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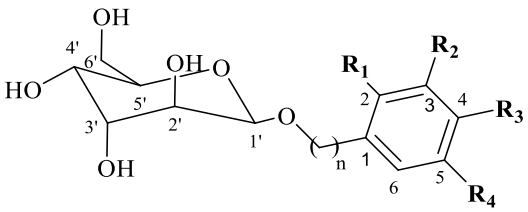
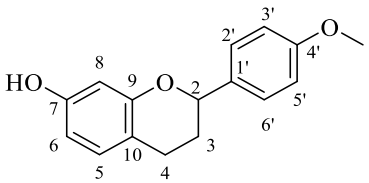
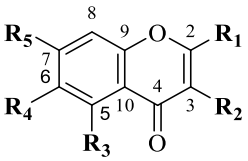




9

	n	R_1	R_2	R_3	R_4
4	0	H	OCH_3	OCH_3	OCH_3
5	0	OCH_3	H	CH_2CH_2OH	H
6	2	H	OH	OCH_3	H



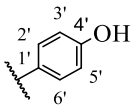
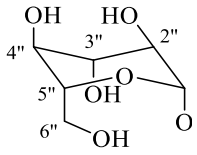
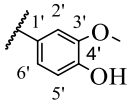
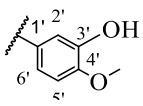
	R_1	R_2	R_3	R_4	R_5
3		OH	OH	H	
7	H		H	H	OH
8	H		H	H	OH

Figure 1. Structures of compounds **1-11**

*Compound **3*** was yellow needle-like crystals. Its ESI-MS peak at *m/z* 446.99 $[M-H]^-$. ^{1}H NMR (600 MHz, CD_3OD) δ: 8.12 (2H, d, *J* = 8.4 Hz, H-2', 6'), 6.90 (2H, d, *J* = 8.4 Hz, H-3', 5'), 6.76 (1H, s, H-8), 6.45 (1H, d, *J* = 2.2 Hz, H-6), 5.05 (1H, d, *J* = 6.8 Hz, H-1"), 3.93 (1H, dd, *J* = 12.2, 2.3 Hz, H-2"), 3.71 (1H, dd, *J* = 12.1, 5.9 Hz, H-3"), 3.54 (1H, ddd, *J* = 8.4, 5.9, 2.2 Hz, H-5"), 3.48 (2H, m, H-4", 6"), 3.40 (1H, t, *J* = 8.9 Hz, H-6"). ^{13}C NMR (151 MHz, CD_3OD) δ: 177.58 (C-4), 164.48 (C-7), 162.19 (C-5), 160.76 (C-4'), 157.76 (C-9), 148.81 (C-2), 137.61 (C-3), 130.89 (2C-2', 6'), 123.57 (C-1'), 116.33 (2C-3', 5'), 106.31 (C-10), 101.64 (C-1"), 100.23 (C-6), 95.57 (C-8), 78.37 (C-5"), 77.85 (C-3"), 74.75 (C-2"), 71.28 (C-4"), 62.46 (C-6"). The above NMR data was consistent with the reference [9], this compound was eventually identified as kaempferol-7-O-β-D-glucopyranoside (RN 16290-07-6).

Compound **4** was white powder, its NMR data in Table 1 was consistent with the reference[10], identified as 3,4,5-trimethoxyphenyl-O-β-D-glucopyranoside (RN 41514-64-1). Compound **5** was white powder, inferred as 2-methoxy-4-(2'-hydroxyethyl)-phenol-1-O-β-D-glucopyranoside[11]. Compound **6** was white powder, the ESI-MS peak at *m/z* 353 $[M+Na]^+$, 2-(3-hydroxy-4-methoxyphenyl) ethyl 1-O-β-D-glucopyranoside (RN 125180-72-5) was finally confirmed [12].

Table 1. ^{1}H NMR (600 MHz) and ^{13}C NMR (151 MHz) data of compound s **4-6** in CH_3OD (δ in ppm and *J* in Hz)

Position	**4**		**5**		**6**	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1	-	154.78	-	146.42	-	131.56
2	6.48 (s)	96.03		150.69	6.85 (d 2.0)	116.04
3	-	154.78	6.88 (d 2.0)	114.55	-	148.81
4	-	134.36		135.48	-	145.90
5	-	156.07	6.76 (dd 8.2,2.0)	122.51	6.68 (m)	113.71
6	6.48 (s)	96.03	7.08 (d 8.2)	118.26	6.68 (m)	122.38
7	-	-	3.72 (t 7.0)	64.27	2.84 (td 7.3,2.7)	71.99
8	-	-	2.76 (t 7.0)	39.81	3.86 (dd 11.9,2.7), 3.65 (dd 11.9,5.3)	36.75
9	-	-	-	-	-	-
10	-	-	-	-	-	-
1'	4.80 (d 7.5)	103.18	4.83 (d 7.4)	103.07	4.29 (d 7.8)	104.32
2'	4.80 (d 7.5)	74.93	4.83 (d 7.4)	74.93	4.05 (m)	75.12
3'	3.42 (m)	78.06	3.68 (dd 12.0,5.1)	77.82	3.71 (m)	78.09
4'	3.42 (m)	71.69	3.46 (m)	71.33	3.26 (m)	71.64
5'	3.42 (m)	78.43	3.46 (m)	78.17	3.26 (m)	77.95
6'	3.91 (dd 12.0,2.2), 3.65 (dd 12.0,6.7)	62.71	3.37 (dd 6.5,1.6)	62.49	3.34 (t 9.1), 3.18 (dd 9.1,8.0)	62.75
2-OCH_3	-	-	3.85 (s)	56.67	-	-
3-OCH_3	3.80 (s)	56.51 (s)	-	-	-	-
4-OCH_3	3.69 (s)	61.21 (s)	-	-	3.83 (s)	56.35
5-OCH_3	3.80 (s)	56.51 (s)	-	-	-	-

Compound **7** was pale yellow powder. Its ESI-MS peak at *m/z* 283.0611 $[M-H]^-$. The location of all carbons was finally determined by DEPT90 and DEPT135 data of this compound. The ^{1}H NMR spectrum of compound **7** displayed four ortho-coupling aromatic protons at δ_H 7.96, 6.82 (1H each, d, 8.9 Hz) and δ_H 6.94, 6.83 (1H each, d, 8.2 Hz), two intersite-coupling aromatic protons at δ_H 7.15 (1H, d, 2.0 Hz) and δ_H 6.67 (1H, d, 1.7 Hz) (Table 2). The HSQC spectrum showed that there were two COSY correlations of C-H. The HMBC cross-peaks from δ_H 8.08 (H-2) to C-3, C-4 and C-9, from δ_H 7.96 (H-5) to C-4, C-7 and C-9 together with the COSY correlation of H-5/H-6/H-8 indicated the assignment of these protons at C-2 and C-5 respectively. Besides, a methoxy group was observed at δ_H 3.88 (3H, s, 3'-OCH_3) corresponding to the carbon signal at δ_C 56.41 in the HMBC spectrum (Figure S31 in supporting information). The HMBC correlation from δ_H 3.88 (3'-OCH_3) to δ_C 148.76 demonstrated this methoxy group linked to C-3'. These above evidence revealed an isoflavone unit in the structure of compound **7**, the compound was eventually identified as a natural compound named 7, 4'-dihydroxyl-3'-methoxyisoflavone. Compound **8** was pale

yellow powder. Its ESI-MS peak at *m/z* 283 $[M-H]^-$, its structure was found to be similar with compound **7** except chemical substituents of C-3' and C-4', the NMR data was consistent with the reference [13], it was identified as calycosin (RN 20575-57-9). Compound **9** was pale yellow powder, this compound was eventually identified as 7-hydroxyl-4'-methoxyflavanone (RN 108837-20-3) whose NMR data was consistent with the reference [14]. Compounds **10**, **11** were maackiain (RN 2035-15-6) [15] and stigmasterol (RN 83-48-7) [16,17], the detailed spectral data were listed in supporting information of this article.

In this study, The compounds **4**, **5**, **6** and **9** were isolated from the family *Leguminosae* for the first time, the compounds **3**, **7** and **8** were first reported from the genus *Indigofera*.

Table 2. ^{1}H NMR (600 MHz) and ^{13}C NMR (151 MHz) data of compounds **7-9** in CH_3OD (δ in ppm and *J* in Hz)

Position	**7**		**8**		**9**	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
2	8.08 (s)	154.38	8.11 (1H, s)	154.75	5.37 (dd 13.2,2.9)	81.04
3	-	125.59	-	125.72	3.04 (dd 17.0,13.2), 2.68 (dd 17.0,2.9)	44.96
4	-	178.16	-	178.03	-	193.53
5	7.96 (d 8.9)	127.94	8.02 (d 8.8)	128.39	7.72 (d 8.8)	129.85
6	6.82 (dd 8.9,1.7)	118.96	6.90 (dd 8.8,2.3)	117.04	6.49 (dd 8.8,2.3)	111.83
7	-	169.82	-	165.90	-	167.01
8	6.67 (d 1.7)	103.71	6.80 (d 2.3)	103.35	6.34 (d 2.3)	103.84
9	-	160.52	-	159.93	-	165.60
10	-	116.01	-	117.70	-	114.90
1'	-	125.19	-	126.31	-	131.37
2'	7.15 (d 2.0)	114.15	7.03 (br s)	117.41	7.32 (d 8.6)	129.01
3'	-	148.76	-	147.42	6.81 (d 8.6)	116.30
4'	-	147.76	-	149.14	-	158.98
5'	6.83 (d 8.2)	116.14	6.96 (br s)	112.60	6.81 (d 8.6)	116.30
6'	6.94 (dd 8.2,2.0)	122.88	6.96 (br s)	121.60	7.32 (d 8.6)	129.01
6-OH	-	-	-	-	-	-
3'-OH	-	-	-	-	-	-
4'-OCH_3	3.88 (s)	56.41	3.87 (s)	56.40	3.87 (s)	56.45

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Supporting Information

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