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Extraction and isolation of major secondary metabolites in the roots of *Ceropegia driophila*

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ABSTRACT

To study the secondary metabolites in *Ceropegia driophila* Schneider. distributed in Hubei province of China, the grinded roots were extracted and partitioned by organic solvents or ionic liquid, and then isolated by column chromatography repeatedly. Seven compounds were obtained and their structures were successfully identified, which were found from the plant for the first time. According to the spectral analysis, they were determined as taraxasterol acetate (I), ursolic acid (II), 2α,3α-dihydroxy ursolic acid (III), luteolin-7-O-β-D-glucoside (IV), croymbosin (V), apigenin (VI) and trans-cinnamicacid (VII). Moreover, the various extraction methods were compared. The plant was supposed to be further utilized and developed as a meaningful natural resource in the genus of *Ceropegia* Linn.

KEYWORDS

Ceropegia driophila Schneider;
Secondary metabolites;
Ionic liquids;
Extraction;
Isolation.

INTRODUCTION

Ceropegia driophila Schneider. (abbreviated as 'CDS' in the following content) is a relatively rare scandent subshrubs unique to China, which is only distributed in a few regions of Hubei and Sichuan province. It is usually found in shrubbery at an altitude of over 600~900 meters, and its flower season is 6 months. In these areas, its roots are used by local people to treat venomous snake bite and carbuncle sore as an efficient herbal. Because of the existence of various meaningful secondary metabolites in natural resources, basic chemical research for them, discovery of bioactive constituents and the relationship between chemistry and biodiversity are always attractive tasks for chemists in related fields. Although many plants of Asclepiadaceae have been studied, there is no report about the secondary metabolites in the roots of CDS up to now. Only a few researches have been performed about the chemical constituents and bioactivity of plants in the genus of Ceropegia Linn, such as Ceropegia dolichpohylla Schltr[1] and Ceropegia juncea[2,3] see Figure 1. So it is necessary to discover more meaningful herbal resources.

In recent years, more and more ionic liquids (ILs) have been applied in extraction process of natural products as a green and useful solvent[4-6], which have attracted extensive attention from academic and industrial circles. They have shown great application poten-

Figure 1 : *Ceropegia dolichpohylla* Schltr. (L), *Ceropegia* juncea (M) and *Ceropegia driophila* Schneider.(R)

tial for target constituents with a wide range of polarity. In the strengthened mass transport process (e.g. ultrasonic wave or microwave assisted extraction), the extraction of ILs will be further improved. It is much-anticipated for the combination of green chemistry and natural product chemistry in daily laboratory work.

At present, the chemical research is needed for Ceropegia driophila Schneider, which can lay the foundation for its bioactivity study and resource exploitation. In order to explore major secondary metabolites in this rare herbal, the present paper introduced the isolation and structural elucidation of seven compounds see Figure 2 for the first time. Moreover, ILs is applied in the study and the comparison among different extraction conditions was investigated, which are wished to be meaningful and helpful for related researchers.

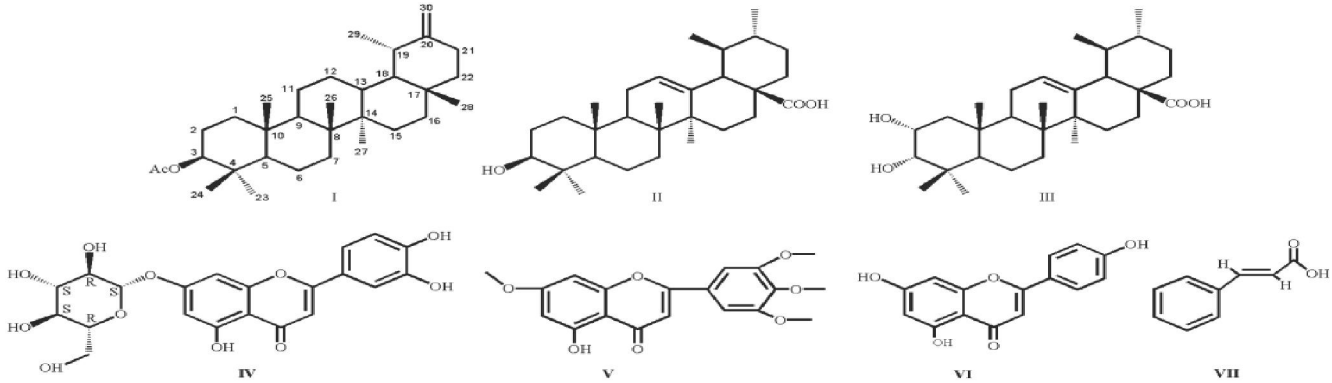

Figure 2 : Structure of seven bioactive constituents

MATERIALS AND METHODS

Instruments and reagents

The melting point was measured with an XRC-1 microscope melting-point apparatus (Sichuan University Instrumental factory, Chengdu, China) and was not corrected. WF-4000 microwave reactor was purchased from Jiyao Instrumental Co., Ltd. (Shanghai, China). Ultrasonic extractor was purchased from Kunshan Ultrasonic Instrumental Co., Ltd. (Jiangsu, China). IR (KBr-discs) spectra were recorded by AVATAR 360-FTIR spectrometer. NMR spectra were recorded at 300 K on Bruker, ACF-500 NMR instrument (^{1}H: 300 MHz, ^{13}C: 125 MHz), with TMS as internal standard. Mass spectra were obtained on MS Agilent 1100 Series LC/MSD Trap Mass spectrometer. HPLC analysis was performed with an LC-20AT pump, an SPD-M20A PDA detector (Shimadzu, Kyoto, Japan), a Waters symmetry C_{18} column, 5 μm, 3.9×150 mm i.d. (Waters, Massachusetts, USA), and an HCT-360 LC column cooler/heater (Hengao Tech & Dev, Tianjin,

FULL PAPER

China). A Class-VP workstation (Shimadzu, Kyoto, Japan) was used for data acquisition. Silica gel H (200-300 mesh, Ocean Chemical Co. Ltd, Qingdao, China) and Sephadex LH-20 (Pharmacia, Stockholm, Sweden) were used for column chromatography. Other reagents were of analytical purity. [HOEtMIm][BF_4] was purchased from Centre for Green Chemistry and Catalysis, LICP, CAS.

Plant material

The roots of Ceropegia driophila Schneider. were collected from Badong county, Hubei Province, China, which were identified by Professor Mingshan He of Hubei University of Traditional Chinese Medicine. The roots were dried, grinded and controlled in 40~60 mesh by passed through stainless steel sieve.

Extraction and isolation

The grinded roots (3.2 kg) of CDS were extracted with 30% [HOEtMIm][BF_4] aqueous solution under microwave for three times (40 min every time). After removal of the residue by filtration and appropriate concentration, the extracting solution was partitioned with the equal volume of n-hexane and ethyl acetate successively. The ethyl acetate soluble portion (71 g) was fractionated by a silica gel column (200~300 mesh), which was eluted with $CHCl_3$/MeOH (100:5, 100:10, 100:15, 100:20) to obtain four major fractions; each fraction was further subjected to repeated column chromatography with petroleum ether/EtOAc (10:1→1:1) or $CHCl_3$/MeOH (9:1→7:3) as eluents. Then key subfractions were purified by Sephadex LH-20 column and recrystallization to obtain seven pure compounds.

RESULTS AND DISCUSSION

Comparison of various extracting conditions

Most of ionic liquids applied in extraction process have the anion of [BF_4] or [PF_6], and the functional groups on their cations could be used to regulate and control the polarity of ILs. [HOEtMIm][BF_4] is a newer member in the family of methylimidazolium ionic liquids, which has been employed in some chemical reactions[7]. It can be expected that its anion and extra hydroxyl group on cation would make it have good extraction capability, which has been proved in our preliminary experiment. It shows higher extraction yield than those common imidazolium ionic liquids (e.g. [BMIm][BF_4], [BMIm][Br], [BMIm][PF_6]) in the extraction of CDS roots. Then the performance of [HOEtMIm][BF_4] aqueous solutions with various concentrations (0, 10, 30, 50, 70, 90%, V/V) under refluxing were investigated. It was found that the extraction efficiency was gradually improved with the increase of IL concentration in the range from 0 to 30%. When the volume percentage was above 50%, higher viscosity would result in poorer diffusivity and worse mass transfer. As the result, the yield of crude extracts began to decline. So 30% [HOEtMIm][BF_4] aqueous solution was selected in the following experiments.

Based on above results, four different extraction methods with various solvents (30%, methanol and ethanol aqueous solution) were compared, including percolation, refluxing under 100°C, extraction in ultrasonic wave (25 kHz) and microwave (500 W). In the comparison experiment, the weight of CDS roots (40~60 mesh) was 48 g, and the volume of extraction solvents was 480 mL. Extraction duration of four methods was set at 40 min (the flow rate of percolation was 12 mL/min). The results of extraction yield are shown in Fig.3, which further prove the excellent performance of [HOEtMIm][BF_4].

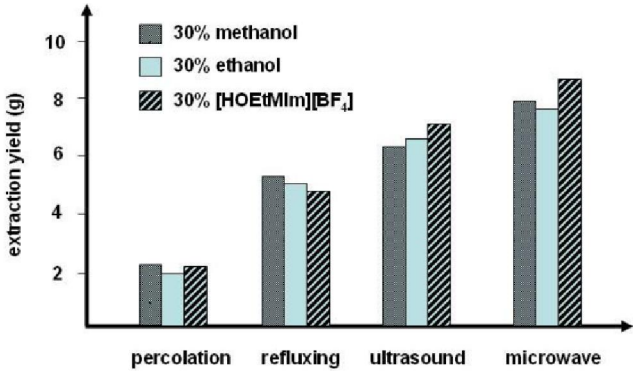

Figure 3 : Comparison of different extraction methods with various solvents

Structure identification

Compound I was isolated as colorless needle-like crystal (chloroform-methanol), which was positive (pink spot) in Liebermann-Burchard reaction. M.P.: 213-214 °C. ESI-MS m/z: 467 [M-H]$^-$; its molecular formula was determined as $C_{32}H_{52}O_2$ according to the data of

ESI-MS and NMR. ^{1}H NMR ($CDCl_3$, 300 MHz) showed seven signals of methyl groups from δ 0.82 to δ 1.02, which were 1.02 (3H, d, J=6.8 Hz, H-29), 1.01 (3H, s, H-26), 0.93 (3H, s, H-27), 0.86 (3H, s, H-25), 0.84 (3H, s, H-24), 0.83 (3H, s, H-28), 0.82 (3H, s, H-23), respectively. Moreover, two signals of terminal hydrogen were found in δ 4.61 (2H, m, H-30). The signal at δ 4.47 (1H, dd, J=10.2, 6.2 Hz, H-3α) was assigned to the proton on the carbon linked with oxygen and δ 2.05 (3H, s, $OCOCH_3$) was typical chemical shift of $–CH_3$ in acetyl group. ^{13}C NMR ($CDCl_3$, 125MHz) proved the existence of terminal double bond (δ 154.9 and 107.0) and acetyl group (171.2 ($O\underline{C}OCH_3$) and 21.4 ($OCO\underline{C}H_3$)). The other ^{13}C signals were at δ: 38.5 (C-1), 25.5 (C-2), 81.1 (C-3), 37.7 (C-4), 55.3 (C-5), 18.3 (C-6), 34.1 (C-7), 40.8 (C-8), 50.2 (C-9), 37.1 (C-10), 21.3 (C-11), 26.2 (C-12), 39.4 (C-13), 42.1 (C-14), 26.5 (C-15), 38.2 (C-16), 34.6 (C-17), 48.7 (C-18), 39.3 (C-19), 23.8 (C-21), 38.6 (C-22), 27.7 (C-23), 16.2 (C-24), 16.4 (C-25), 15.8 (C-26), 14.8 (C-27), 19.4 (C-28), 25.4 (C-29). Compared with the relative spectral data of the literature[8], compound I was identified as taraxasterol acetate.

Compound II was isolated as white powder (methanol), which was positive (pink spot) in Liebermann-Burchard reaction. M.P.: 285-287°C. ESI-MS m/z: 455 $[M-H]^-$. The molecular formula was determined as $C_{30}H_{48}O_3$ according to the data of ESI-MS and NMR. ^{1}H NMR(C_5D_5N, 300 MHz) showed seven signals of angular methyl groups, which were δ 1.23, 1.22, 1.07, 1.03, 0.92 (each 3H, s) and 1.02, 0.97 (each 3H, d, J=6.1 Hz), respectively. δ 5.50 (1H, t, J=2.9 Hz) was the signal of one and only unsaturated proton with allylic coupling. ^{13}C NMR (C_5D_5N, 125 MHz) showed two typical signals of olefinic carbon in α-amyrin type triterpene; δ 179.7 was the signal of carboxyl group on C-28. All of the ^{13}C NMR data were summarized in TABLE 1. Compared with the relative spectral data of the literature[9], compound II was identified as ursolic acid.

Compound III was isolated as white powder (chloroform-methanol), which was positive (pink spot) in Liebermann-Burchard reaction. M.P.: 284-285°C. ESI-MS m/z: 471 $[M-H]^-$. The molecular formula was determined as $C_{30}H_{48}O_4$ according to the data of ESI-MS and NMR. Above evidence indicated the similarity between it and compound I. ^{1}H NMR (C_5D_5N, 300 MHz) also showed seven signals of angular methyl groups and one unsaturated proton with allylic coupling, which were δ 1.28, 1.12, 1.01, 0.95, 0.87 (each 3H, s), 0.96 (3H, d, J=4.3 Hz), 0.92 (3H, d, J=6.1 Hz) and 5.46 (1H, br. s), respectively. ^{13}C NMR (C_5D_5N, 125 MHz) also showed three typical signals of olefinic carbon and C-28 carboxyl group (δ 125.7, 139.2 and 179.8). There were two signals of carbons connected with oxygen in δ 79.4 and 66.0. All of the ^{13}C NMR data were summarized in TABLE 1. Compared with the relative spectral data of the literature[10], compound III was identified as 2α,3α-dihydroxyursolic acid.

TABLE 1 : ^{13}C-NMR (C_5D_5N, 125 MHz) chemical shift of compound II and III

No.	II	III	No.	II	III
1	39.2	42.9	16	25.2	23.8
2	28.1	66.0	17	48.3	48.2
3	78.3	79.4	18	53.5	53.7
4	39.5	39.4	19	39.6	39.4
5	55.7	48.0	20	39.6	38.9
6	18.9	18.6	21	31.2	31.1
7	33.1	33.3	22	37.4	37.3
8	40.1	40.3	23	28.7	29.2
9	48.0	48.4	24	15.8	22.2
10	37.4	38.5	25	16.7	16.8
11	23.8	23.6	26	17.6	17.5
12	125.6	125.7	27	23.8	24.8
13	139.4	139.2	28	179.7	179.8
14	42.7	42.5	29	17.6	17.5
15	28.6	28.7	30	21.5	21.4

Compound IV was isolated as yellow powder (methanol). It was positive in Mg-HCl and Molish reactions, which indicated it could be flavonoid glycoside. M.P.: 179-181 °C. ESI–MS m/z: 449 $[M+H]^+$, and molecular formula is $C_{21}H_{20}O_{11}$. ^{1}H-NMR (300 MHz, DMSO-d_6) showed typical signals of C6-C3-C6 skeleton of flavonoids, which were 6.45 (1H, d, J=2.2 Hz, H-6), 6.72 (1H, s, H-3), 6.77 (1H, d, J=2.2 Hz, H-8), 6.87 (1H, d, J=8.1 Hz, H-5'), 7.42-7.47 (2H, m, H-2', 6'). Moreover, there were three active protons shifted down field in 9.49 (1H, br. s, OH-3'), 10.06 (1H, s, OH-4') and 12.95 (1H, s, OH-5) for

FULL PAPER

hydrogen bond. A double peak signal of terminal hydrogen in monosaccharide was found in 5.08 (1H, d, J=7.0 Hz, H-1''). ^{13}C-NMR (125 MHz, DMSO-d_6) δ: 164.7 (C-2), 103.2 (C-3), 182.1 (C-4), 161.4 (C-5), 99.6 (C-6), 163.2 (C-7), 95.0 (C-8), 157.2 (C-9), 105.4 (C-10), 121.6 (C-1'), 113.8 (C-2'), 146.1 (C-3'), 150.2 (C-4'), 116.1 (C-5'), 119.4 (C-6'), 100.2 (C-1''), 73.4 (C-2''), 76.5 (C-3''), 69.8 (C-4''), 77.2 (C-5''), 60.9 (C-6''). Compared with the relative spectral data of the literature[11], compound IV was identified as luteolin-7-O-β-D-glucoside.

Compound V was isolated as yellow needle-like crystal (chloroform-methanol). M.P.: 188-189 °C. It shows orange fluorescent light after sprayed with 1% $AlCl_3$-ethanol reagent on thin layer chromatography, which indicates it could be flavonoid. ESI-MS m/z: 359.0 $[M+H]^+$, and molecular formula is $C_{19}H_{18}O_7$. ^{1}H-NMR (300MHz, DMSO-d_6) showed a proton signal of phenolic hydroxyl group at δ 12.71, and five benzene proton signals at δ 7.10 (2H, d, J = 2.1 Hz, H-2' and 6'), 6.52 (1H, d, J =2.3 Hz, H-8), 6.38 (1H, d, J =2.3 Hz, H-6) and 6.61 (1H, s, H-3). Moreover, there were four methoxyl group signals at δ 3.97 (6H, s), 3.93 (3H, s) and 3.91 (3H, s), whose chemical shift indicated they were located on benzene ring. Compared with the relative spectral data of the literature[12], compound V was identified as croymbosin.

Compound VI was isolated as yellow needle-like crystal (methanol). M.P.: 347-348 °C. It was positive in Mg-HCl reaction, which indicated it could be flavonoid. ESI-MS m/z: 269.0 $[M-H]^-$, and molecular formula is $C_{15}H_{10}O_5$. In ^{1}H-NMR (300 MHz, DMSO-d_6), δ 6.25 (1H, d, J =2.1 Hz) and 6.55 (1H, d, J =2.1 Hz) are a pair of signals of m-coupling protons on benzene ring. Moreover, there was an AA'BB' coupling system at δ 7.04 (2H, d, J =8.8 Hz)? and 7.95 (2H, d, J = 8.8 Hz), which could prove the existence of p-substituted benzene ring. It also showed three -OH proton signals at δ 9.50 (1H, s), 9.52 (1H, s) and 13.03 (1H, s). Finally, a single peak without any coupling appeared at δ 6.61 (1H, s). Compared with the relative spectral data of the literature[13], compound VI was identified as apigenin.

Compound VII was obtained as white needle-like crystal (acetone), which appeared as red dark spot on GF_{254} thin layer chromatography. M.P.: 135-137 °C. ESI-MS m/z: 147 $[M-H]^+$, 103 $[M-45]^+$, 77, 51. ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 6.44 (1H, d, J=16.1 Hz, H-8), 7.42 (3H, m, H-3, 4, 5), 7.55 (2H, m, H-2, 6), 7.83 (1H, d, J=16.1 Hz, H-7). Coupling constant of 16.1 Hz could indicate the existence of trans-double bond. Compared with the relative spectral data of the literature[14], compound VII was identified as trans-cinnamic acid.

CONCLUSIONS

The results of this study indicated that Ceropegia driophila was supposed to be further utilized and developed as a meaningful natural resource in the genus. The ionic liquid [HOEtMIm][BF_4], as a newer member in the family of methylimidazolium ionic liquids, was first applied to the extraction of the plant to obtain seven compounds, which exhibited better extractive performance than the organic solvents in ultrasonic wave (25 kHz) and microwave (500 W). In addition, their structures were successfully identified by EMI-MS, ^{1}H NMR and ^{13}C NMR spectra. Above results and methods are expected to be helpful for related chemists.

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REFERENCES

[1] L.F.Wu, Y.S.Jing, Z.D.Zhang; Science and Technology of Food Industry, China, http://www.cnki.com.cn/Article/CJFDTotal-SPKJ201011012.htm, **11**, 78-80 **(2010)**.

[2] E.Sukumar, H.Gopal, P.B.Rao et al.; Fitoterapia, http://cat.inist.fr/?aModele=afficheN&cpsidt=2900775, **66**, 403-406 **(1995)**.

[3] N.A.Adibatti, P.Thirugnanasambantham, C.A.Kuilothungan; Phytochemistry, http://www.sciencedirect.com/science/article/pii/003194229183683C, **30**, 2449-2450 **(1991)**.

[4] B.K.Tang, W.T.Bi, M.L.Tian, H.R.Kyung; J.Chromatogr.B, http://www.sciencedirect.com/science/article/pii/S1570023212004308, **904**, 1-21 **(2012)**.

[5] Y.Yuan, Y.Wang, R.Xu, M.Huang, H.Zeng; Analyst, http://pubs.rsc.org/en/content/articlehtml/2011/an/c0an00864h, **136**, 2294 **(2011)**.

[6] J.Luo, H.Song, Y.W.Zhang, W.F.Luo, T.T.Li, S.Yao; NPAIJ, http://tsijournals.com/npaij/NatTOC.htm, **8**, 41-49 **(2012)**.

[7] X.D.Hou, W.Y.Lou, L.Q.Yan, M.H.Zong; Chem.J. Chinese Universities, http://en.cnki.com.cn/Article_en/CJFDTOTAL-GDXH201206021.htm, **33**, 1245 **(2012)**.

[8] L.M.Khalilov, A.Z.Khalilova, E.R.Shakurova, I.F.Nuriev, V.V.Kachala, A.S.Shashkov, U.M.Dzhemilev; Chem.Nat.Compd., http://link.springer.com/article/10.1023/A:1025478720459, **39**, 285-287 **(2003)**.

[9] W.Seebacher, N.Simic, R.Weis, R.Saf, O.Kunert; Magn.Reson.Chem., http://onlinelibrary.wiley.com/doi/10.1002/mrc.1214/full, **41**, 636-638 **(2003)**.

[10] E.N.Li, J.G.Kong; Phytochem.Anal., http://onlinelibrary.wiley.com/doi/10.1002/pca.1134/abstract, **20**, 338-343 **(2009)**.

[11] Y.R.Lu, L.Y.Foo; Phytochemistry, http://www.sciencedirect.com/science/article/pii/S0031942200003095, **55**, 263-267 **(2000)**.

[12] Z.P.Zheng, J.Y.Liang, L.H.Hu; Chin.J.Nat.Med., http://d.wanfangdata.com.cn/Periodical_zgtryw200405003.aspx, **2**, 272-275 **(2004)**.

[13] P.V.Loo, A.D.Bruyn; Magn.Reson.Chem., http://onlinelibrary.wiley.com/doi/10.1002/mrc.1260241007/abstract, **24**, 879-882 **(1986)**.

[14] S.Karthikeyan, V.Ramamurthy; J.Org.Chem., http://pubs.acs.org/doi/abs/10.1021/jo0617722, **72**, 452-458 **(2007)**.