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Chemical investigation of an antimalarial Chinese medicinal herb *Picrorhiza scrophulariiflora* ☆

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ABSTRACT

An antimalarial medicinal plant *Picrorhiza scrophulariiflora* was chemically investigated as part of our ongoing research in traditional chinese medicines (TCM). Mass directed fractionation of the active part of the crude extract led to the isolation of ten main components, three new compounds (**1**–**3**) and seven known compounds (**4**–**10**). Compound **10** inhibited the growth of the *Plasmodium falciparum* 3D7 malarial parasite line, with an IC_{50} value of 8.3 μM. This compound accounted for ~95% of *P. falciparum* growth inhibitory activity in the crude extract confirming, for this TCM, that a single compound was responsible for the antimalarial activity.

Malaria is one of the worlds' most significant human infectious diseases, with 216 million cases leading to an estimated 655,000 deaths in 2010.[1,2] Most morbidity and mortality is caused by parasites of the genus *Plasmodium*, which like other malaria parasites is transmitted by the bite of female Anopheles mosquitoes.[1] Although several drugs are currently available for the prevention and treatment of malaria, all are now susceptible to parasite drug resistance or reduced clinical efficacy, including the gold standard artemisinin combination therapies.[3] The increasing problem of drug-resistant *Plasmodium* strains means that new therapies are urgently needed to treat this devastating disease.[3]

Historically, natural products have played a major role in the treatment of malaria.[4–7] For centuries the indigenous people from South America have used the bark from the 'fever tree', *Cinchona succiruba* for the treatment of malaria. Similarly, the Chinese medicinal plant, *Artemisia annua*, commonly known as Qinghao, has been used in China as an antimalarial herbal remedy for hundreds of years.[4–7] Subsequent chemical investigations of *Cinchona succiruba* and *Artemisia annua* identified the major active metabolites to be quinine and artemisinin (Qinghaosu), respectively.[4–7] Artemisinin is considered one of the most important discoveries in contemporary herbal research.

Traditional Chinese medicine (TCM) has been regarded as a national heritage for centuries. Approximately 13,000 kinds of medicinal plants have been recorded in China.[8] Among them, over 300 are commonly used for antimalarial remedies. In our ongoing effort in understanding TCM and its action, a collaborative research project was established to investigate antimalarial traditional Chinese medicinal plants. The objective of the project was not only to identify antimalarial natural products, but also to investigate whether a TCM's action is solely associated with a single active component, an underlying philosophy of western medicine. One hundred antimalarial Chinese medicinal herbs recorded in the Chinese Pharmacopeia were selected and tested *in vitro* against the malaria parasite *Plasmodium falciparum* 3D7 for antimalarial activity. Of these, 82 extracts showed >50% inhibition at 10 mg/mL, while 35 had >90% inhibition at the same concentration. These results largely confirmed the traditional knowledge of the antimalarial activity of the herbal medicines recorded in the Chinese Pharmacopiea. The crude extract from the rhizomes of a Tibetan *Picrorhiza scrophulariiflora* Pennell showed potent activity and was chemically investigated. Flash chromatography of the crude extract followed by mass-directed fractionation of the active fraction led to the isolation of three new compounds, scrophuloside C–D (**1–2**), and hebitol III (**3**), along with seven known compounds, namely, hebitol II (**4**),[8] scrophuloside B (**5**),[9] scrophenoside B (**6**),[10] scroneoside A (**7**),[11] picroside-I (**8**),[12] 25-(acetyloxy)-2-(β-D-glucopyranosyloxy)-3,16,20-trihydroxy-9-methyl-19-norlanost-5-en-22-one (**9**),[13] and

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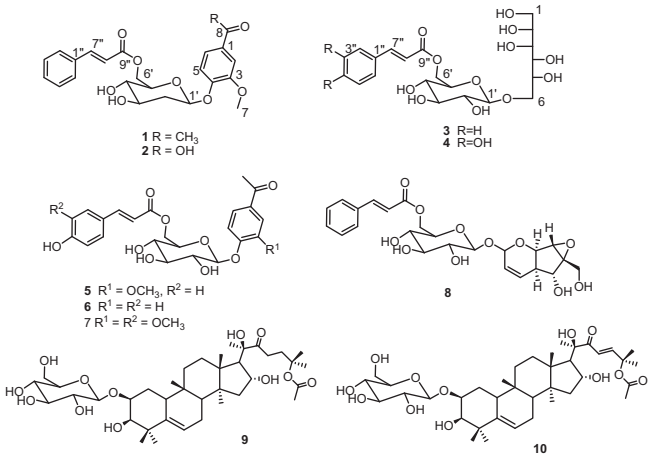
25-(acetyloxy)-2-(β-D-glucopyranosyloxy)-3,16-dihydroxy-9-methyl-19-norlanosta-5,23-dien-22-one (**10**).[13]

Picrohiza scrophulariiflora (Scrophulariaceae) is a perennial distributed throughout the high altitude region (over 4,400 m) in the southeast of Tibet and the northwest of Yunnan, China. The plant has been traditionally used for diarrhea, jaundice and malaria. The rihizomes of *Picrorhiza scrophulariiflora* (Scrophulariaceae) in this study collected from Tibet Autonomous Region, China, were purchased from Bozhou Herbal Medicine Market, Anhui Province in July, 2011. The dried and ground plant material (300 g) was exhaustively extracted by MeOH, and then partitioned between petroleum ether, EtOAc and *n*-BuOH. Silica gel flash chromatography of the EtOAc layer yielded one active fraction which was subjected to LC-MS analysis. Mass-directed purification by reverse phase C_{18} HPLC led to the isolation of nine major components, including three new metabolites, scrophuloside C (**1**, 4.4 mg, 0.0015% dry weight), scrophuloside D (**2**, 3.3 mg, 0.0011% dry weight) and hebitol III (**3,** 1.1 mg. 0.0004% dry weight), along with seven known compounds, namely, hebitol II (**4**, 3.2 mg, 0.0011% dry weight), scrophuloside B (**5**, 12.5 mg, 0.0042% dry weight), scrophenoside B (**6**, 13.3 mg, 0.0044% dry weight), scroneoside A (**7**, 3.5 mg, 0.0012% dry weight), picroside-I (**8**, 63.0 mg, 0.021% dry weight), 25-(acetyloxy)-2-(β-D-glucopyranosyloxy)-3,16,20-trihydroxy-9-methyl-19-norlanost-5-en-22-one (**9**, 435.0 mg, 0.145% dry weight), and 25-(acetyloxy)-2-(β-D-glucopyranosyloxy)-3,16-dihydroxy-9-methyl-19-norlanosta-5,23-dien-22-one (**10**, 485.3 mg, 0.167% dry weight).



Scrophuloside C (**1**)[14] was obtained as an optically active colorless solid with an $[\alpha]_D$ value of −74.0. The molecular formula of **1** was determined to be $C_{24}H_{26}O_9$ by HRESIMS (*m*/*z* 481.1461 $[M+Na]^+$), with twelve degrees of unsaturation. The ^1H NMR spectrum of **1** (Table 1) indicated the presence of a cinnamoyl functionality, a sugar moiety (δ_H 3.5–5.5), a 1, 2, 4-trisubstituted benzene ring, a methyl group (δ_H 2.34), and a methoxyl group (δ_H 3.83). The double bond in the cinnamoyl group was determined as *trans* based on its coupling constant (δ_H 7.64 and 6.63, *J* 16.0 Hz) (Table 1). Twenty three carbon resonances were deduced from the HSQC and HMBC correlation data. Nine carbon resonances (δ_C 166.4, 145.2, 134.4, 131.0 × 3, 128.9 × 2, and 118.6) were consistent with the presence of a cinnamoyl group. The sugar moiety was assigned as β-glucose based on its ^1H and ^{13}C NMR data and the coupling constant of the anomeric proton (δ_H 5.12, *J* 7.6 Hz). The formation of the acetyl group and its attachment on C-1 position of the tri-substituted benzene ring were established by the HMBC correlations from the methyl singlet (δ_H 2.34) to a ketone carbonyl (δ_C 196.7) and an aromatic carbon (δ_C 131.4). Further HMBC correlation was observed from the methoxyl singlet (δ_H 3.83) to a aromatic carbon (δ_C 149.2), indicating the methoxyl group was attached at C-3 position of the benzene ring. The connectivity was confirmed by the HMBC correlations from the aromatic protons (δ_H 7.17 and 7.45) to the aromatic carbons (δ_C 123.0, 131.4, 149.2 and 150.9). The HMBC correlation from the glucose H-6′ (δ_H 4.44 and 4.24) to the cinnamoyl carbonyl carbon (δ_C 166.4) suggested the formation of an ester bond between these two functional groups. The formation of an ether bond between the glucose moiety and the tri-substituted benzene ring was determined by the HMBC correlation from the anomeric proton (δ_H 5.12) to the C-4 aromatic carbon (δ_C 150.9). The planar structure of **1** was therefore elucidated as 1-*O*-phenyl-6-*O*-cinnamoyl-β-glucopyranoside.

The absolute configuration of the glucose moiety in **1** was determined by gas chromatography of sugar enantiomers as acetylated thiazolidine derivative.[15,16] Acid hydrolysis of **1** followed by derivatization with L-cysteine methyl ester gave a GCMS peak at 17.92 min, same as that of a standard D-glucose derivative, namely methyl 3-acetyl-2*R*-(1′*R*,2′*S*,3′*R*,4′*R*,5-pentaacetoxypenta-1-yl)-thiazolidine-4*R*-carboxylate (17.97 min, while L-glucose derivative methyl 3-acetyl-2*R*-(1′*S*, 2′*R*, 3′*S*, 4′*S*, 5-pentaacetoxypenta-1-yl)-thiazolidine-4*R*-carboxylate at 18.48 min), indicating a D-glucose in the molecule. Scrophuloside C (**1**) was therefore identified as 1-*O*-(2-methoxy-4-acetylphenyl)-6-*O*-(*E*-cinnamoyl)-ß-*D*-glucopyranoside.

Scrophuloside D (**2**)[17] was also isolated as an optically active colorless solid, with an $[\alpha]_D$ value of −17.6. Its molecular formula was determined as $C_{23}H_{24}O_{10}$ by HRESIMS (*m*/*z* 459.1286 $[M-H]^-$), indicating 2 mass unit more than that of **2**. The comparison of the ^1H and ^{13}C NMR data between **2** and **1** suggested that the two compounds possessed similar structural features, the differences being the absence of an acetyl methyl signal in **2** and a up-field shift of the carbonyl carbon (δ_C 196.7 in **1** to δ_C 167.8 in **2**). These changes suggested that the acetyl functionality in **1** was replaced by a carboxylic acid in **2**, which was supported by the molecular formula of **2**. Using the same GCMS method as that for compound **1**, the absolute configuration of the glucose in **2** was determined as β-D-glucose (with a retention time of 17.96 min). The structure of scrophuloside D (**2**) was therefore elucidated as 1-*O*-(2-methoxy-4-carboxylphenyl)-6-*O*-(*E*-cinnamoyl)-ß-D-glucopyranoside.

Hebitol III (**3**)[18] was isolated as an amorphous solid with a negative optical rotation ($[\alpha]_D$ −38). Its molecular formula, $C_{21}H_{30}O_{12}$, was deduced by HRESIMS, indicating seven degrees of unsaturation. The ^1H NMR spectrum of **3** (Table 1) displayed signals for a cinnamoyl group and sugar moieties (δ_H 3.04–4.41). The double bond in the cinnamoyl group was determined as *trans* based on its large coupling constant (δ_H 7.65 and 6.65, *J* 16.0 Hz). Twenty one carbon resonances were observed in ^{13}C NMR spectrum in combination with HSQC and HMBC correlation data. Nine carbon resonances (δ_C 166.7, 145.2, 134.5, 129.4 × 2, 129.1, 128.9 × 2, and 118.4) were consistent with the presence of a cinnamoyl group. Six carbon signals (δ_C 104.2, 74.1, 76.5, 70.4, 74.2 and 64.3) along with their corresponding ^1H NMR signal (δ_H 3.04–4.41) indicated the presence of a glucopyranoside unit. The large coupling constant of the anomeric proton (δ_H 4.23, *J* 8.0 Hz) indicated that the glucose unit had a ß-configuration. The remaining 6 carbon resonances (δ_C 73.4–64.3) were assigned to a mannitol functionality based on the absence of a low field anomeric carbon ($\sim\delta_C$ 100), and lack of double bond equivalence. Further analysis of the HSQC and HMBC correlation data confirmed the assignment. The HMBC correlations from the glucose H-6′ (δ_H 4.41 and 4.17) to the cinnamoyl C-9″ (δ_C 166.7) suggested the formation of an ester bond between the two functional groups. Further HMBC correlation from the glucose H-1′ (δ_H 4.23) to the mannityl C-6 (δ_C 73.4) indicated an ether linkage between the glucose and the mannityl sub-units. The comparison of the carbon chemical shifts between compound **3** and the known compound, hebitol II, confirmed the sugar moiety β-glucopyrannosyl-(1 → 6)mannitol.

Table 1
^{1}H and ^{13}C NMR Data for Compounds **1**–**3** in DMSO-d_6

	Compound **1**[a,b]			Compound **2**[b,c]			Compound **3**[a]		
Position	δ_C	δ_H (J in Hz)	HMBC	δ_C	δ_H (J in Hz)	HMBC	δ_C	δ_H (J in Hz)	HMBC
1	131.4			124.9			64.3	3.58, dd (11.5, 2.3) 3.36, dd (11.5, 5.8)	
2	111.4	7.45[d]	C-1, C-3, C-4, C-6	113.5	7.47, d (1.9)	C-3, C-4, C-6, C-8	71.7	3.43[d]	C-4
3	149.2			149.0			70.0	3.62, dd (3.8, 11.5)	C-2, C-5
4	150.9			150.6			70.1	3.52, d (8.9)	
5	114.7	7.17, d (8.0)	C-1, C-3	115.0	7.18, d (8.3)	C-1, C-3, C-4	70.1	3.53, d (8.9)	
6	123.0	7.45[d]	C-2, C-4	123.3	7.50, dd (8.3, 1.9)	C-2, C-4, C-8	73.4	4.03, dd (10.5, 1.3), 3.40[d]	C-1′
7	56.1	3.83, s	C-3	56.4	3.81, s	C-3			
8	196.7			167.8					
9	26.5	2.34, s	C-1, C-8						
1′	99.7	5.12, d (7.6)	C-4	99.7	5.12, d,(7.6)	C-4	104.2	4.23, d (8.0)	C-6
2′	73.5	3.38[d]		73.5	3.33[d]	C-3′	74.1	3.04, t (8.5)	C-1′, C-3′
3′	77.3	3.37[d]	C-2′, C-4′	77.2	3.34[d]	C-2′	76.5	3.18[d]	C-5′
4′	70.7	3.37[d]		70.5	3.23[d]		70.4	3.15[d]	C-6′
5′	74.3	3.76, m	C-1′	74.5	3.74 m		74.2	3.41[d]	C-1′
6′	63.9	4.44, dd (10.3,1.9) 4.24, dd (10.3, 6.1)	C-5′, C-9″	63.8	4.45, dd (11.8, 1.9) 4.19, dd (11.8, 6.7)	C-5′, C-9″	64.3	4.41, d (10.3) 4.17, dd (10.3, 6.1)	C-5′, C-9″
1″	134.4			134.5			134.5		
2″	128.9	7.72, m	C-3″, C-6″, C-7″	128.6	7.69, m	C-3″, C-4″	128.9	7.72[d]	C-1″,C-7″
3″	131.0	7.45[d]	C-1″, C-2″	129.6	7.43[d]	C-1″	129.4	7.41[d]	C-1″,C-2″
4″	131.0	7.45[d]		130.8	7.43[d]		129.1	7.40[d]	
5″	131.0	7.45[d]	C-1″, C-6″	129.6	7.43[d]	C-1″	129.4	7.41[d]	C-1″
6″	128.9	7.72, m	C-2″, C-4″, C-7″	128.6	7.69, m	C-4″, C-5″	128.9	7.72[d]	C-1″,C-7″
7″	145.2	7.64, d (16.0)	C-2″, C-8″, C-9″	145.6	7.61, d (16.0)	C-2″, C-8″, C-9″	145.2	7.65, d (16.0)	C-2″,C-8″, C-9″
8″	118.6	6.63, d (16.0)	C-1″, C-9″	118.5	6.60, d (16.0)	C-1″, C-9″	118.4	6.65, d (16.0)	C-1″, C-9″
9″	166.4			166.5			166.7		

[a] Spectra were recorded at 600 MHz for ^{1}H and 150 MHz for ^{13}C using d_6-DMSO as solvent at 30 °C.
[b] The ^{13}C NMR spectra were observed from the HSQC and HMBC experiments.
[c] Spectra were recorded at 500 MHz for ^{1}H and 125 MHz for ^{13}C using d_6-DMSO as solvent at 30 °C.
[d] Signals were overlapping.

The planar structure of compound **3** was therefore determined to be 1-*O*-mannityl-6-*O*-cinnamoyl-ß-glucopyranose.

The GCMS analysis of a peracetylated thiazolidine derivative of the hydrolyzed **3** established a D-glucose moiety (with a retention time of 17.93 min) as in **1** and **2**. The attempt to isolate the mannitol moiety form the hydrolyzed mixture, measure its optical rotation, and therefore determine the absolute configuration of mannitol was hindered by the small quantity of compound **3**. Hebitol III (**3**) was therefore determined as 1-*O*-mannityl-6-*O*-(*E*-cinnamoyl)-ß-D-glucopyranose.

Seven known compounds, namely hebitol II (**4**),[8] scrophuloside B (**5**),[9] scrophenoside B (**6**),[10] scroneoside A (**7**),[11] picroside-I (**8**),[12] 25-(acetyloxy)-2-(β-D-glucopyranosyloxy)-3,16,20-trihydroxy-9-methyl-19-norlanost-5-en-22-one (**9**),[13] and 25-(acetyloxy)-2-(β-D-glucopyranosyloxy)-3,16-dihydroxy-9-methyl-19-norlanosta-5, 23-dien-22-one (**10**),[13] were also isolated from the active fraction of *Picrorhiza scrophulariiflora*. These compounds were previously reported from the plants *Picrorhiza scrophulariiflora* and *Picrorhiza kurroa* and their ^{1}H and ^{13}C NMR data were identical to those reported in the literature.

The antimalarial activity of the crude extracts and the isolated pure compounds (**1**–**10**) were evaluated *in vitro* against *P. falciparum* 3D7 malaria parasites. Chloroquine was used as positive control which had an IC_{50} value of 0.018 ± 0.002 μM. Compounds **1**–**9** all had IC_{50} > 25 μM, while **10** had an IC_{50} value of 8.3 ± 0.6 μM, and an IC_{90} value of 17.3 ± 1.6 μM. Compounds **9** and **10** were the two main components in the active fraction, and the only structural difference is a double bond between C-23 and C-24. Accordingly, the presence of a α,ß-unsaturated carbonyl group in compound **10** may be important for its antimalarial activity. Since compound **10** has a Michael acceptor functionality,[19,20] we also tested its activity against a noncancer cell line, neonatal foreskin fibroblast (NFF). It showed no cytotoxicity to NFF cells at the concentration of up to 100 μM.

The crude extract of *Picrorhiza scrophulariiflora* had 95% inhibition against *P. falciparum* 3D7 malaria parasites at the concentration of 10 mg/mL. A calculated IC_{95} for compound **10** was obtained based on the activity of the crude extract, the percentage yield of compound **10** and its molecular weight. The results showed that the calculated IC_{95} (23.5 μM) of **10** was comparable with that of the experimental IC_{90} (17.3 μM), suggesting that the antimalarial activity of this TCM, *Picrorhiza scrophulariiflora*, was contributed mainly by a single component. Given artemisinin (Qinghaosu) was also the single major antimalarial component identified from *Artemisia annua*, perhaps the anti-parasitic activity of TCM is controlled by singlecompounds rather than multiple components in TCMs effective against other diseases. Further isolation and activity evaluation of antimalarial Chinese herbal medicines is currently ongoing. Results will not only lead to some interesting bioactive natural products, but also shed light on whether antimalarial TCMs contain single or multiple active constituents.

Acknowledgments

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Supplementary data

Supplementary data (Copy of ^{1}H, ^{13}C, COSY, HSQC and HMBC NMR spectra of **1**–**3**, GC–MS traces of sugar derivatives of hydro-

lyzed **1**–**3**, detailed experimental procedures including general experiment procedure, plant material, extraction and isolation procedure for compounds **1**–**10**, acid hydrolysis and derivatisation, GCMS analysis, antimalarial assay and cytotoxicity assay. This material can be found in the online version.) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2013.08.077.

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14. Scrophuloside C (**1**): colorless solid; $[\alpha]_D$−74.0 (*c* 0.09, CH_3OH); UV (CH_3OH) λ_{Max} (log ε) 270 (sh) (3.41) nm; ^1H and ^{13}C NMR data (DMSO-d_6) see Table 1; (+)-HRESIMS *m*/*z* 481.1461 $[M+Na]^+$ (calcd. for $C_{24}H_{26}O_9Na$, 481,1490).
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17. Scrophuloside D (**2**): colorless solid; $[\alpha]_D$−17.6 (*c* 0.125, CH_3OH); UV (CH_3OH) λ_{Max} (log ε) 229(3.59), 204 (sh) (3.81) nm; ^1H and ^{13}C NMR data (DMSO-d_6) see Table 1; (−)-HRESIMS *m*/*z* 459.1286 $[M-H]^-$ (calcd for $C_{23}H_{23}O_{10}$, 459.1291).
18. Hebitol III (**3**): colorless solid; $[\alpha]_D$−38.0 (*c* 0.1, CH_3OH); UV (CH_3OH) λ_{Max} (log ε) 259 (sh) (3.55), 204 (sh) (3.55) nm; ^1H and ^{13}C NMR data (DMSO-d_6) see Table 1; (+)-HRESIMS *m*/*z* 497.1643 $[M+Na]^+$ (calcd for $C_{21}H_{30}O_{12}Na$, 497.1629).
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