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Distinctive Features of Chemical Composition from *Bupleurum chinense* Applicable to Original Authentication

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Abstract: The original determination of plant-derived subjects in traditional herb medicines is often complicated by the lack of morphological features in the visual or microscopic inspection. Improved methods are urgently needed. In this paper, the hydrogen nuclear magnetic resonance (^{1}H NMR) and high performance liquid chromatography (HPLC) profiles of fractionated polar extracts from samples of *Bupleurum chinense* were recorded and analyzed by comparing them to each other and to those of the isolated compounds. The ^{1}H NMR spectra revealed distinct common features among samples of *B. chinense* collected, with the characteristic signals of their major constituents, saikosaponins, being exhibited explicitly and reproducibly. These features were further confirmed by the procedure of HPLC and online HPLC/MS (mass spectrometry) analysis. Also, thirty-five compounds, including twenty-seven saikosaponins and eight others, were isolated from the extracts of the roots of *B. chinense*. On the basis of chemical investigation, the signals and peaks in the fingerprints were unambiguously assigned to their corresponding compounds. The general features of ^{1}H NMR spectra coupled with HPLC profiles established for authentic samples of *B. chinense* gave out specific data from those special compounds and can be used for original authentication.

Keywords: *Bupleurum chinense* DC.; Apiaceae; saikosaponins; original identification; ^{1}H NMR; HPLC

Introduction

The original determination of plant-derived subjects, such as cut or sliced or powdered crude drugs in traditional Chinese medicines (TCM), is often complicated by the lack of morphological features in their visual or microscopic inspections. Improved methods are urgently needed. In our effort to develop a more scientific, convenient and accurate method as supplementary means for the simultaneous original authentication of plants and their systematic relationships, a methodological investigation has been conducted which established a standardized procedure for obtaining the applicable and characteristic plant extracts.[1-3] The detection of the specificities of these extracts was carried out by means of phytochemical analysis, and some famous crude drugs in TCM, which contained phthalide or chromone derivatives as the most important and accepted constituents in chemotaxonomy, have been investigated. This developed method is based on the idea of relatively and comprehensively exhibiting the characteristic constituents of plants examined, including their existence and relative compositions, with kinds of polar and non-polar extracts being concerned. It has been argued that, with the ^{1}H NMR and HPLC fingerprinting analyses of these extracts, the whole procedure is objective, convenient, repeatable and stable with time for the original and systematic authentication of medicinal plant species. Here, it should also be further pointed out that the extensive investigation on chemical constituents of the corresponding extract is necessary in fingerprinting analysis of medicinal plants. This kind of investigation is based on the consideration of clearly exhibiting the existence of the major characteristic constituents, preferably the bioactive ones, and unambiguously assigning the signals in the fingerprints to their corresponding compounds, which underlies authentication of systematic relationship of plants in the field of chemotaxonomy. In the present paper, we report

on the investigation of ^{1}H NMR and HPLC fingerprinting analyses of specific extracts on original authentication of *B. chinense*, which was one of the most famous genera in traditional herbal remedies and contained oleanane-type saponin derivatives as the most important and accepted constituents in pharmaceutics and chemotaxonomy.

Bupleurum chinense DC., belonging to the family Umbelliferae, is a well-known Chinese medicinal herb. The cut crude root of *B. chinense* exhibits various therapeutic functions in the definition of TCM, such as the activities of dispelling exogenous evils and invigorating splenic yang, and has often been used to treat fever and hypochondriasisare,[4] among others. About 40 Bupleurum species have been studied in the field of phytochemistry which indicated that derivatives of saikosaponins are their major native chemical constituents. In the meantime, a number of coumarins, flavonoids, lignans, steroids, polyacetylenes, and fatty acids have also been isolated from some Bupleurum species.[5]

Traditionally, the authentication of the roots of *B. chinense* was based on morphological and histological inspections by experts with visual or microscopic means. During the last two decades, chromatographic and spectroscopic profiles of metabolites began to be used as more objective means. The profiles reported up to date were mainly generated by the application of analytical techniques over simple water or alcoholic plant extracts, such as the utilization of HPLC and the online HPLC/MS.[6-8] Obviously, the analytical instrumentations were more concerned than the phytochemical procedures in the previous literatures. In support of the idea for determining plant-derived subjects with more scientific, exact, and convenient means, the polar and neutral extracts (extract-P) from the roots of *B. chinense* sourced from eight different regions and suppliers in China were obtained by a standardized procedure. The ^{1}H NMR spectra

and HPLC traces of these extracts were recorded, respectively, with optimized conditions. Meantime, as part of our ongoing project on the explanation of the signals in those fingerprinting, the chemical investigation were performed, with thirty-five compounds being isolated from the extract-P and the ethyl acetate (EtOAc) soluble fraction of an ethanol (EtOH) extract of *B. chinense* (Figure 1). The signals of the ^{1}H NMR and HPLC fingerprints were also analyzed and assigned by comparing them to each other and to the reference ^{1}H NMR spectra and HPLC profiles of the isolated compounds, as well as by online HPLC/MS analysis.

Materials and methods

Chemicals

Dimethyl sulfoxide-d_6 (99.9% atom D, containing 0.03% v/v Trimethylsilane) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile was in HPLC grade and was purchased from Fisher Scientific (Waltham, Massachusetts, USA). Silica gel was purchased from Qingdao Marine Chemical Factory (Qingdao, Shangdong province, China) and was used for column chromatography (CC). RP-18 (YMC-GEL, ODS-A, 12 nm, S-50 mm; YMC Co., Kyoto, Japan) were also used for CC. Sephadex LH-20 (Pharmacia Biotech AB, Uppsala, Sweden). Thin-layer chromatography (TLC) was carried out with glass plate precoated silica gel G. Spots were visualized under ultraviolet (UV) light and by spraying with 10% H_2SO_4 in 95% EtOH, followed by heating. Other solvents were of analytical grade and were purchased from Beijing Chemical Company (Beijing, China).

Plant materials

Samples of the roots of *B. chinense* employed were sourced by authors or by associate Prof. L Ma (a savant in plant systematics at Institute of Materia Medica, Chinese Academy of Medical

Sciences and Peking Union Medical College) from habitats of the *Bupleurum* species (Table 1). All samples were authenticated by associate Prof. L Ma according to their morphological and histological characteristics, and by comparing their appearances with those of voucher specimens in the Herbarium of Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College. Voucher specimens (ID-S-22154, ID-S-2372-2378) of some subjects were deposited in the Herbarium of Institute of Materia Medica, Chinese Academy of Medical Sciences, Beijing, P. R. China.

Standardized experimental procedure for the preparation of extract-P

Dried and pulverized roots (50 g) of *B. chinense* was extracted with 95% aq. EtOH (2 × 100 mL, 1 h, 0.5 h) under reflux. The combined EtOH extracts were evaporated in vacuum to yield a dark-brown residue, which was suspended in 80-90% aq. EtOH (100 mL). The resulting suspension was extracted with petroleum ether (3 × 100 mL) in separatory funnel. Evaporation of the aq. layer in vacuum yielded another dark-brown residue, which was re-dissolved in water (100 mL), and then extracted with EtOAc (2 × 100 mL) in separatory funnel. On finishing the extraction, 20 g of sodium chloride (NaCl) was added to the aq. phase, which was re-extracted once with EtOAc (100 mL). The water layer was then extracted with n-butyl alcohol (*n*-BuOH) (2 × 100 mL) in separatory funnel. The combined *n*-BuOH phase were washed with the solution of 5% sodium bicarbonate ($NaHCO_3$) in water (H_2O) (3 × 100 mL) and then H_2O (2 × 100 mL), respectively, to pH 7, and then dried over anhydrous sodium sulfate (Na_2SO_4). Complete removal of the solvent *n*-BuOH *in vacuo* and boiling water led to extract-P.

Extraction and isolation of chemical constituents

Dried and powdered roots (10.0 Kg) of *B. chinense* were extracted for three times with 95%

EtOH (2 h, 1 h, 1 h) under reflux. After evaporation of the EtOH solvent under vacuum, a dark-brown residue of 1.2 Kg was obtained. The residue was suspended in 80% EtOH (3000 mL) and extracted with petroleum ether (3 × 3000 mL). Evaporation of the aq. layer *in vacuo* yielded another dark-brown residue, which was re-dissolved in water (2000 mL), and then processed with the same procedure as described in '*Standardized experimental procedure for the preparation of extract-P*'. The EtOAc and *n*-BuOH soluble portions were evaporated to dryness to afford relevant brown residues, respectively.

The EtOAc fraction (93 g) was separated by CC over silica gel using a gradient of trichloromethane ($CHCl_3$)-methanol (MeOH) (100:0-1:1) as eluant to yield ten fractions (designated as F1 to F10) according to their TLC profiles. F1 (5 g) and F6 (11.0 g) were separated as above to yield saikochrome (40 mg), *α*-spinasterol (10 mg), anomalamide (7 mg), and quercetin (50 mg), *β*-sitosteryl-3-*O*-*β*-D-glucopyranoside (200 mg), respectively. Fraction 8 (9.7 g) was separated as above to yield kaempferol-7-rhamnoside (20 mg) and six further subfractions (F8-1 to F8-6). F8-4 (3.9 g) was submitted to a Sephadex LH-20 CC (MeOH) to give five further subfractions (F8-4-1 to F8-4-5). F8-4-2 (2.0 g) was further chromatographed over an ODS-A column with a gradient of MeOH-H_2O (60%-100%) as eluant, yielding seven further subfractions (F8-4-2-1 to F8-4-2-7). F8-4-2-3 (170 mg) and F8-4-2-6 (80 mg) were finally purified by preparative RP-18 [mobile phase: MeOH-H_2O (80:20); flow rate: 5 mL min^{-1}; UV detection at 203 and 210 nm simultaneously] to give prosaikogenin F (123 mg) and 3",6"-di-*O*-acetyl-saikosaponin b_2 (30 mg), respectively. F9 (12.0 g) was separated as the EtOAc fraction to yield three further subfractions (F9-1 to F9-3). F9-2 (5.0 g) was separated as F8-4 to yield three futher subfractions (F9-2-1 to F9-2-3). F9-2-2 (2.5 g) was chromatographed as F8-4-2,

yielding seven futher subfractions (F9-2-2-1 to F9-2-2-7). F9-2-2-2 (236 mg), F9-2-2-4 (450 mg), and F9-2-2-6 (200 mg) were purified by preparative RP-HPLC [mobile phase: acetonitrile (CH_3CN)-H_2O (45:55), (47:53), and (50:50), respectively; flow rate: 5 mL min^{-1}; UV detection at 203 and 210 nm simultaneously], yielding 6''-*O*-acetylsaikosaponin b_3 (36 mg), prosaikogenin D (104 mg), and 6''-*O*-acetylsaikosaponin b_1 (25 mg), respectively.

The *n*-BuOH fraction (173 g) was separated as the EtOAc fraction to yield thirteen fractions (F1 to F13) according to their TLC profiles. F1 (3.5 g) was submitted to a Sephadex LH-20 CC (MeOH) for further separation to yield three further subfractions (F1-1 to F1-3). F1-1(1.0 g) was separated by CC on silica gel with $CHCl_3$-MeOH-EtOAc-H_2O (2:2:4:1) as eluant to yield saikochromoside A (30 mg). F1-2 (2.5 g) was further chromatographed over an ODS-A column (MeOH: 65%-100%), yielding 6''-*O*-acetylsaikosaponin e (40 mg). F2 (6.2 g) was separated by CC on silica gel ($CHCl_3$-MeOH=25:1-1:1) to yield five further subfractions (F2-1 to F2-5). F2-3 (2.4 g) was further submitted to CC on silica gel ($CHCl_3$-MeOH-EtOAc-H_2O =3:2:5:1) to yield five subfractions (F2-3-1 to F2-3-5). F2-3-2 (329 mg) was finally subjected to an ODS-A column (MeOH: 70%-100%), yielding 6''-*O*-acetylsaikosaponin a (155 mg). F3 (10.8 g) was separated by CC on silica gel ($CHCl_3$-MeOH=15:1-1:1) to yield five further subfractions (F3-1 to F3-5). F3-2 (7.5 g) was further separated as F3 to yield five further subfractions (F3-2-1 to F3-2-5). F3-2-5 (1.8 g) was separated as F2-3 to yield four subfractions (F3-2-5-1 to F3-2-5-4). F3-2-5-2 (278 mg) was finally separated as F2-3-2, yielding saikosaponin e (40 mg). F4 (19.1 g) was separated by CC on silica gel ($CHCl_3$-MeOH =10:1-1:1) to yield five further subfractions (F4-1 to F4-5). F4-3 (2.4 g) was further chromatographed as F2-3, yielding 6''-*O*-acetylsaikosaponin b_2 (700 mg) and kaempferol-3,7-dirhamnoside (40 mg). F4-4 (7.0 g) was separated as F2-3 to yield

saikosaponin a (1.363 g). F4-5 (2.3 g) was finally purified by an ODS-A column (MeOH: 60%-100%), yielding saikosaponin b_1 (514 mg). F5 (10.3 g) was separated as F2-3 to yield six further subfractions (F5-1 to F5-6). Saikosaponin b_3 (536 mg) was isolated from F5-4 (2.5 g) by an ODS-A column (MeOH: 60%-100%) which also yielded eight further subfractions (F5-4-1 to F5-4-8). F5-4-7 (102 mg) and F5-4-2 (88 mg) were purified by preparative RP-18 [mobile phase: CH_3OH-H_2O (80:20) and (60:40), respectively; flow rate: 5 mL min^{-1}; UV detection at 203 and 210 nm simultaneously], yielding saikosaponin g (53 mg), and Bupleurosides Ⅲ (20 mg), ⅩⅢ (38 mg), respectively. F6 (6.6 g) was separated as F2-3 to yield saikosaponin b_2 (1.68 g). F9 (9.9 g) was separated by CC over silica gel ($CHCl_3$-MeOH-EtOAc-H_2O=3:2.1:4.9:1) to yield five further subfractions (F9-1 to F9-5). Saikosaponins c (522 mg), f (1.124 g), and h (425 mg) were directly obtained from F9-5 (1.5 g) by an ODS-A column (MeOH: 60%-100%), which also yielded five further subfractions (F9-5-1 to F9-5-5). F9-5-5 (300 mg) was finally purified by preparative RP-18 [mobile phase: CH_3CN-H_2O (25:75); flow rate: 5 mL min^{-1}; UV detection at 203 and 210 nm simultaneously], yielding saikosaponin w (10 mg) and saikosaponin l (33 mg). F10 (8.0 g) was separated as F9 to yield six further subfractions (F10-1 to F10-6). F10-5 (1.7 g) was submitted to Sephadex LH-20 CC (MeOH) to give three further subfractions (F10-5-1 to F10-5-3). F10-5-2 (0.94 g) was chromatographed over an ODS-A column (MeOH: 40%-100%), yielding five subfractions (F10-5-2-1 to F10-5-2-1-5). F10-5-2-4 (354 mg) was finally purified by preparative RP-18 [mobile phase: CH_3CN-H_2O (35:65); flow rate: 5 mL min^{-1}; UV detection at 203 and 210 nm simultaneously], yielding 21*β*-hydroxysaikosaponin b_2 (28 mg) and 2''-*O*-*β*-D-glucopyranosylsaikosaponin b_2 (35 mg). F13 (9.2 g) was separated by CC on silica gel ($CHCl_3$-MeOH-EtOAc-H_2O=2.8:2.2:5:1) to yield seven further subfractions (F13-1 to F13-7).

F13-6 (0.9 g) was chromatographed as F10-5 to give four further subfractions (F13-6-1 to F13-6-4). Elatoside A (15 mg) was obtained from F13-6-3 (0.35 g) by an ODS-A column (MeOH: 60%-100%). F13-4 (3.4 g) was chromatographed as F10-5 to give three further subfractions (F13-4-1 to F13-4-3). F13-4-2 (3.0 g) was chromatographed over an ODS-A column (MeOH: 30%-100%), yielding six subfractions (F13-4-2-1 to F13-4-2-6). F13-4-2-3 (0.62 g) was applied to an ODS-A column (MeOH: 50%-100%), yielding six subfractions (F13-4-2-3-1 to F13-4-2-3-6). F13-4-2-3-2 (70 mg) and F13-4-2-3-3 (120 mg) were purified by preparative RP-18 [mobile phase: CH_3CN-H_2O (22:78) and (20:80), respectively; flow rate: 5 mL min^{-1}; UV detection at 203 and 210 nm simultaneously] to give saikosaponin v (7 mg), saikosaponin q-1 (8 mg), and saikosaponin v-1 (10 mg) , respectively..

Experimental procedures for recording ^{1}H NMR spectra and HPLC traces of Extract-Ps

After finishing the preparative procedure, the ^{1}H NMR spectra of each extract-P were recorded directly on a Varian Mercury-400 MHz NMR spectrometer (Palo Alto, California, USA) operating at 400.13 MHz ^{1}H NMR frequency, with DMSO-d_6 as solvent and using VMR 6.2C NMR software package and Z-axis gradients.

Each spectrum consisted of 16 scans of 64 K complex data points, with a spectral width of 10000 Hz, an acquisition time of 3.17 s, and a recycle delay of 1 s per scan. The pulse angle was 90 º. The receiver gain was set at the same value for all samples. Spectra were Fourier transformed with 0.3 Hz line broadening, phase- and baseline-corrected using the Topspin software. Spectra were further transferred to a personal computer for data analysis.

Approximately 13 mg of each extract-P were dissolved in methanol, respectively, and all gave a total volume of 2.0 mL. The resulting solutions were cleansed on a protective 0.45 μm

Polytetrafluoroethylene (PTFE) syringe filter, and then were directly applied to an Agilent 1200 series HPLC instrument with Diode array detector (DAD) detector (Santa Clara, California, USA). The running time was 55 min. And then the trace of extract-P was recorded. About 3.0 mg of each compound were dissolved in methanol to give a total volume of 2.0 mL, respectively, and then injected into HPLC system after filtration with a 0.45 μm PTFE syringe filter under the identical HPLC conditions of extract-P. The profiles of each compound were also recorded.

The HPLC analysis was carried out with a Kromasil C_{18} column (250 mm×4.6 mm I.D., 5 μm particle size) at column temperature of 25 ℃. The mobile phase was consisted of acetonitrile (A) and pure water (B), using a gradient elution of 30~40% A at 0~20 min, 40~50% A at 20~35 min, 50~60% A at 35~45 min, and 60~80% A at 45~55 min. The flow rate was set at 1.0 mL min^{-1} and the wave of the DAD detector was set at 210 nm and 254 nm, respectively. The volume of injection was 20 μL.

HPLC-DAD/ESIMS analyses of chemical constituents in extract-Ps

For the online HPLC/DAD/ESIMS (electrospray ionization mass spectrometry) analyses, an Agilent 1100 Series liquid chromatography system was utilized equipped with a quaternary pump, degasser, column oven, autosampler, and diode array detector operating at 203 nm and 254 nm, which was coupled to the ion-trap mass spectrometer (Santa Clara, California, USA). The positive ion ESIMS experiments were conducted using conditions as follows: drying temperature, 325 ℃; drying gas, 6.0 L min^{-1}; nebulizer, 15 psi; skimmer, 40 V; syringe pump rate, 5.0μL min^{-1}. HPLC separation was performed under the same condition as experimental procedure for HPLC profiles of extract-P.

Results and Discussion

Structure characterization of monomers

The investigation on the chemical constituents showed that saikosaponins were the main constituents of extract-P, compared with the EtOAc soluble fraction, from the roots of *B. chinense*. The extract-P and EtOAc soluble fraction were submitted to multiple chromatographic steps, respectively, for isolating their chemical constituents as described in "Experimental". Twenty-two saikosaponins were isolated from the extract-P, and five ones from the EtOAc soluble fraction. In addition, eight others were also isolated, including two from the former and six from the latter. The structures of the known compounds were elucidated by means of comparing their nuclear magnetic resonance (NMR) and MS spectroscopic data to those of literatures, and the five new ones on the basis of detailed spectroscopic analysis and qualitative chemical methods. As a result, the names and structures of all saikosaponins are given in Figure 1, and the eight other compounds were identified as saikochrome, [9] saikochromoside A, [9] quercetin, [10] kaempferol-3,7-dirhamnoside, [11] kaempferol-7-rhamnoside, [11] *β*-sitosterol-3-*O*-β-D-glucopyranoside, [12] *α*-spinasterol, [13] and anomalamide, [14] respectively. The pseudomolecular ion peaks (*m/z*) of main compounds on the part of the HPLC profiles of extract-P were shown in Tables 2 and 3, and the structures of typical compounds were given in Figure 1. This kind of liquid chromatography/mass spectrometry (LC/MS) analysis unambiguously confirmed the existence of saikosaponins as the main constituents in extract-Ps from the subjects and was very useful for elucidating the known constituents stemming from the plant extracts without the need of isolating pure compounds.

^{1}H NMR fingerprinting analysis

On the one hand, the eight samples of *B. chinense* employed (see Table 1) showed significant

consistency to each other in terms of the appearances of their ^{1}H NMR spectroscopic characteristics. On the other, the ^{1}H NMR spectrum of extract-P of *B. chinense* exhibited significantly exclusive features in the composition of signals when compared to species beyond *Bupleurum* (data not shown for species outside *Bupleurum*). By comparing these spectra with those of the isolated saikosaponins, as well as with literature values, it was found that most signals from aliphatic, alkoxyl, and anomeric and olefinic regions of the spectrum could be recognized as those of saikosaponins. The ^{1}H NMR spectrum was conveniently divided into three regions according to the spectroscopic features, i.e. δ 0.54-2.36, 2.88-4.12, and 4.16-6.37. From the point of ^{1}H NMR spectroscopic investigation, for the most part, these signals could not be directly assigned to exact protons of one or the other of the compounds detected in *B. chinense*. They were regarded as the overlapped result of a wide range of compounds, because the extract-P was a mixture composed mainly of saikosaponins. In this point, the piled-up mountain-like profile at δ 0.54-2.36 was mainly due to the resonances of aliphatic methyls and methylenes from saikosaponins. The overlapped multiplets at δ 2.88-4.12 were mainly assigned to the protons of methineoxy and methyleneoxy groups in the structures of saikosaponins, including saccharide moieties and aglycones. In brief, these two regions were short of specificity for the original identification of *B. chinense*. Besides, the signals in relatively lower field were very complicated and showed poorer resolution because of the influence of labile protons from hydroxyls. When the labile protons were interchanged with deuterium from D_2O, the signals in the field region of δ 4.16-6.37 exhibited much better reproducibility and significant resolution. As such, the signals from anomeric protons of sugar moieties and from olefinic protons on C-11 and C-12 of the aglycones of main saikosaponins were unambiguously displayed in this region. Also, by

comparing the ^{1}H NMR spectrum of extract-P with those of saikosaponins, it was elucidated that the signals of the olefinic protons on C-11 and C-12 of saikosaponins a, b_2, and c, and 6''-*O*-acetylsaikosaponin b_2 and on C-12 of saikosaponin f were shown at the region of δ 5.13-6.39 as distinctive doublets or double doublets or broad singlets. These olefinic signals were conveniently assigned to the exact protons of their corresponding compounds, and the nearly same relative intensity of the signals at δ 6.37 (d, *J* = 10.2Hz, H-12 of 6''-*O*-acetylsaikosaponin b_2 and saikosaponin b_2), 5.77 (d, *J* = 10.5Hz, H-12 of saikosaponins a and c), 5.48 (d, *J* = 10.2Hz, H-11 of 6''-*O*-acetylsaikosaponin b_2 and saikosaponin b_2), and 5.25 (dd, *J* = 10.2, 2.2Hz, H-11 of saikosaponins a and c) was regarded as one of the most discriminable features in the original identification of *B. chinense*. The signal at δ 5.13 (br s, H-12 of saikosaponin f) was detected to be very weak, indicating trace content of saikosaponin f. The other resolvable signals at the region of δ 4.16-6.37 were assigned to the decided anomeric protons of the following saccharide units: the two glucose moieties of saikosaponin c and the two glucose moieties of saikosaponin f being overlapped together at δ 4.17 (d, *J* = 7.5Hz), the fucose moieties from 6''-*O*-acetylsaikosaponin b_2, saikosaponins a and b_2, respectively, at δ 4.23 (m), the glucose moieties from saikosaponins a and b_2, respectively, at δ 4.35 (d, *J* = 7.5Hz), the rhamnose moieties from saikosaponins c and f, respectively, at δ 4.70 (s), and the one glucose moiety of 6''-*O*-acetylsaikosaponin b_2 at δ 4.38 (d, *J* = 7.5Hz). The recognition and assignment of these olefinic and anomeric protons were indirectly but solidly confirmed by the subsequent HPLC and above HPLC/DAD/ESIMS analyses, which explicitly revealed the existence of saikosaponins a, b_2, c, and f, and 6''-*O*-acetylsaikosaponin b_2, inter alia., with saikosaponins a, c and b_2 predominated furthest in the extract-P of the roots of *B. chinense*. The above composite features of ^{1}H NMR spectrum demonstrated a fairly consistent

picture with the structures of saikosaponins presented in publications and in the above description of isolating chemical constituents.

HPLC fingerprinting analysis

HPLC procedure is a method now used to separate and analyze the chemical constituents of plants which combines column efficiency with speed of analysis. By means of HPLC procedure, both quantitative and qualitative data can be obtained on plant-derived components, since measurements of the area under the peaks shown on the HPLC trace are directly related to the concentration of different components in the original mixture, and the structure of each compound can be identified by comparing to authentic compounds. As a supplementary and expected means, HPLC procedure was applied to analyze and compare the specificity and consistence of the composition of saikosaponins within the extract-Ps of different specimens of the roots of *B. chinense* under the same HPLC analysis condition. As shown in Tables 2 and 3, and Figures 3 and 4, excluding three subjects (BCs 6-8), the HPLC trace of each other sample of *B. chinense* mainly displayed eight nearly identical peaks at 15.1 (peak 1), 16.4 (peak 2), 23.7 (peak 7), 24.3 (peak 8), 28.2 (peak 9), 30.7 (peak 10), 32.6 (peak 11), and 46.7 (peak 17) min when the wavelength of the DAD detector was set at 210 nm and four nearly identical peaks at 17.9 (peak 4), 24.3 (peak 6), 28.2 (peak 9), and 32.6 (peak 10) min at 254 nm, respectively, i.e. exhibiting consistencies of retention times and relative areas of each peak. Compounds isolated in the present work were measured as reference substances under the same HPLC condition as the analysis of extract-P, and their retention times were compared with those of the peaks in the HPLC trace of extract-P. As a result, the main three peaks were ascribed to their corresponding counterparts, that is, peaks 1, 7, and 8 corresponded to saikosaponins c, a, and b_2, respectively, for the former case. Three minor

peaks of 2, 9, and 11 were identified as saikosaponin f, 2''-*O*-*β*-D-glucopyranosylsaikosaponin b_2, and 6''-*O*-acetylsaikosaponin b_2, respectively, leaving two other minor peaks (10 and 17) at the same wavelength being unidentified. Peak 5 appearing in some samples was assigned to saikosaponin b_1. Besides, all three specimens of BCs 6-8 exhibited a more relatively higher peak at 39.7 (peak 15) min than other subjects at 210 nm and this peak was not indicated to be saikosaponins according to the HPLC-MS analysis, but which was obviously a peak consisted of several blended compounds. When the wavelength of the DAD detector was set at 254 nm, the general profiles was somewhat different from that at 210 nm, with the above mentioned four main peaks being detected in all of extract-Ps from samples of *B. chinense*. Save for other assigned peaks, peak 4 and 6 at 254 nm of detector was ascribed to saikosaponin h and b_2 by the same means as described above, respectively.

Conclusions

Whereas cut or sliced or powdered crude drugs are used in preparations of many traditional medicine systems, such as TCM, the project reported here aims at seeking a plant chemotaxonomic approach to distinguish between species and between plant origins by phytochemical procedure. In the case of this paper, the subjects containing oleanane-type saponin derivatives as the most important constituents were emphasized. Phytochemical investigation, as well as ^{1}H NMR and HPLC analyses were conducted over the fractionated polar extracts (extract-Ps) obtained by standardized phytochemical procedures from eight subjects of *B. chinense*. As one of the results, thirty-five compounds were isolated and identified from one of them, including twenty-two saikosaponins from extract-P, indicating its peculiarity in chemical constituents. Another underlined point reported here illustrates the significant similarities of

chemical composition of the extract-Ps from the eight *B. chinense* samples on the part of the ^{1}H NMR and HPLC traces, i.e., the fingerprints of the samples were found to be consistent among themselves. For the most part, the basic signals on the ^{1}H NMR and HPLC traces were unambiguously assigned by means of extensive investigation on chemical constituents of the extract-Ps. Hence, it was concluded that, with the ^{1}H NMR and HPLC fingerprinting analyses, the extract-P obtained by standardized procedure of this paper can be used for the original authentication of *B. chinense*.

Acknowledgements

We thank associate Professor L Ma for the sample collection and authentication. This work was supported by grants from National Science and Technology Project of China (2011ZX09307-002-01) and from the National Key Project for Basic Research of Science and Technology (2007FY130100).

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Figure and Table Legends:

A. Figure 1: Chemical structures of the saikosaponins isolated or detected from the extract-Ps of the roots of *B. chinense*.

B. Figure 2: ^{1}H NMR imaging profile of the extract-P of *B. chinense* (400 MHz in DMSO-d_6+D_2O).

C. Figure 3: HPLC profiles of extract-Ps of *B. chinense* at 210 nm.

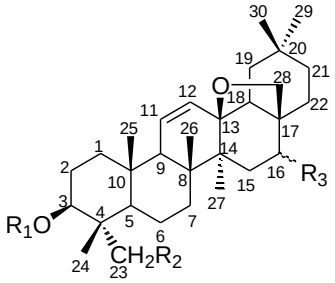
D. Figure 4: HPLC profiles of extract-Ps of *B. chinense* at 254 nm.

E. Table 1: Subjects employed

F. Table 2: Basic HPLC data of extract-Ps of the roots of *B. chinense* with the relative peak areas (PA) of saikosaponin a as reference at 210 nm.

G. Table 3: Basic HPLC data of extract-Ps of the roots of *B. chinense* with the relative peak areas (PA) of saikosaponin b_2 as reference at 254 nm.

H. Table 4: Basic ^{1}H NMR spectroscopic data of extract-Ps of the roots of *B. chinense* (Anomeric and olifinic protons; 400 MHz, DMSO-d_6+D_2O).



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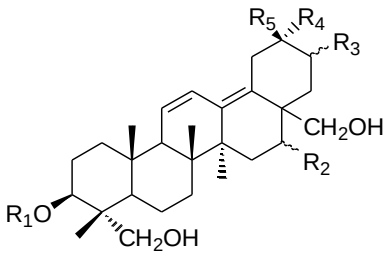

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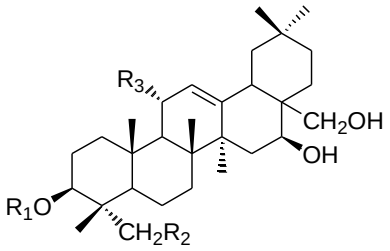

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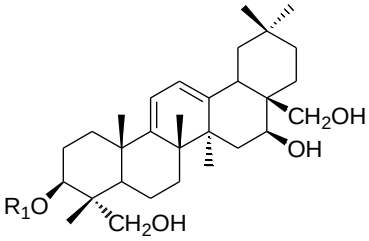

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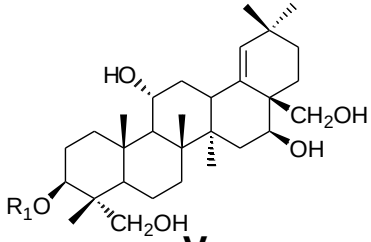

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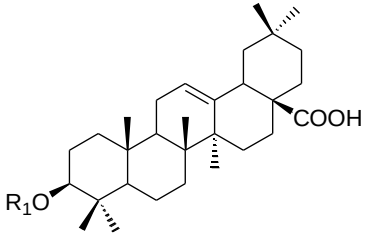

VI

Names of compounds	aglycones	R_1	R_2	R_3	R_4	R_5	reference
6"-*O*-Ac[a]-SS[b] a[c]	Ⅰ	F[d](3→1)G[e]-6"-*O*-Ac	OH	β-OH			15
SS a[c]	Ⅰ	F(3→1)G	OH	β-OH			15
SS d[cf]	Ⅰ	F(3→1)G	OH	α-OH			16
SS c[c]	Ⅰ	G[(4→1)R[g]](6→1) G	H	β-OH			16
SS e	Ⅰ	F (3→1) G	H	β-OH			15
SS F	Ⅰ	Fuc	OH	β-OH			16
6"-*O*-Ac-SS b_2[c]	Ⅱ	F (3→1) G -6"-*O*-Ac	α-OH	H	Me	Me	17
SS b_1[c]	Ⅱ	F(3→1) G	β-OH	H	Me	Me	15
SS b_2[c]	Ⅱ	F(3→1) G	α-OH	H	Me	Me	15
SS h[c]	Ⅱ	G [(4→1)R] (6→1) G	β-OH	H	Me	Me	16

2"-*O*-β-D-G-SS b_2[c]	Ⅱ	F(3→1)G (2→1)-G	α-OH	H	Me	Me	17
SS l	Ⅱ	F(3→1) G	α-OH	H	Me	CH_2OH	18
3",6"-*O*-diAc-SS b_2	Ⅱ	F(3→1)G -3",6"-*O*-diAc	α-OH	H	Me	Me	17
prosaikogenin D	Ⅱ	F	α-OH	H	Me	Me	16
SS q-1	Ⅱ	G [(4→1)R] (6→1) G	α-OH	H	Me	CH_2OH	19
SS v-1	Ⅱ	F(3→1) G	α-OH	H	Me	COO-(1)X[h]	20
SS v	Ⅱ	F(3→1) G	α-OH	H	Me	COO-Y[i]	18
SS b_3[c]	Ⅲ	F(3→1) G	OH	OMe			15
SS f[c]	Ⅲ	G [(4→1)R](6→1) G	H	H			15
bupleuroside Ⅲ	Ⅲ	F(3→1) G	OH	OH			21
SS g	Ⅳ	F(3→1) G					16
bupleuroside ⅩⅢ	Ⅴ	F(3→1) G					21
elatoside A	Ⅵ	GA[j] [(2→1)Xl[k]](3→1) G					22
SS w	Ⅲ	F(3→1) G	OH	$NHCONH_2$			23
21*β*-hydroxy-SS b_2	Ⅱ	F(3→1) G	α-OH	β-OH	Me	Me	23
6"-*O*-Ac-SS e	Ⅰ	F(3→1) G -6"-*O*-Ac	H	β-OH			23
6"-*O*-Ac-SS b_1	Ⅱ	F(3→1) G -6"-*O*-Ac	β-OH	H	Me	Me	23
6"-*O*-Ac-SS b_3	Ⅲ	F(3→1) G -6"-*O*-Ac	OH	OMe			23

[a]Ac: acetyl; [b]SS: saikosaponin; [c]detected from extract-Ps in HPLC/DAD/ESIMS analyses; [d]F: fucopyranosyl; [e]G: glucopyranosyl; [f]Not obtained in the isolating work but detected in HPLC/DAD/ESIMS analyses; [g]R: rahmnopyronosyl; [h]X: xylitol ; [i]Y: (6) glucopyranosyl(1→1)xylitol; GA[j]: glucuronic acid; Xl[k]:xylopyranosyl,.

Figure 1 Chemical structures of the saikosaponins isolated or detected from the extract-Ps of the roots of *B. chinense*.

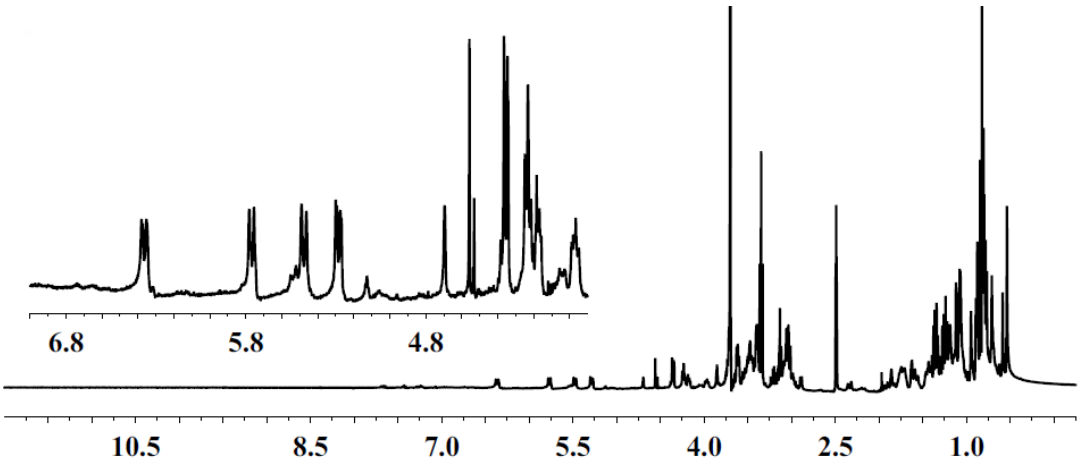

Figure 2 ^{1}H NMR imaging profile of the extract-P of *B. chinense* (400MHz in DMSO-d_6+D_2O).

Axis X represents chemical shift (δ), given in ppm using TMS as an internal standard (δ 0.00).

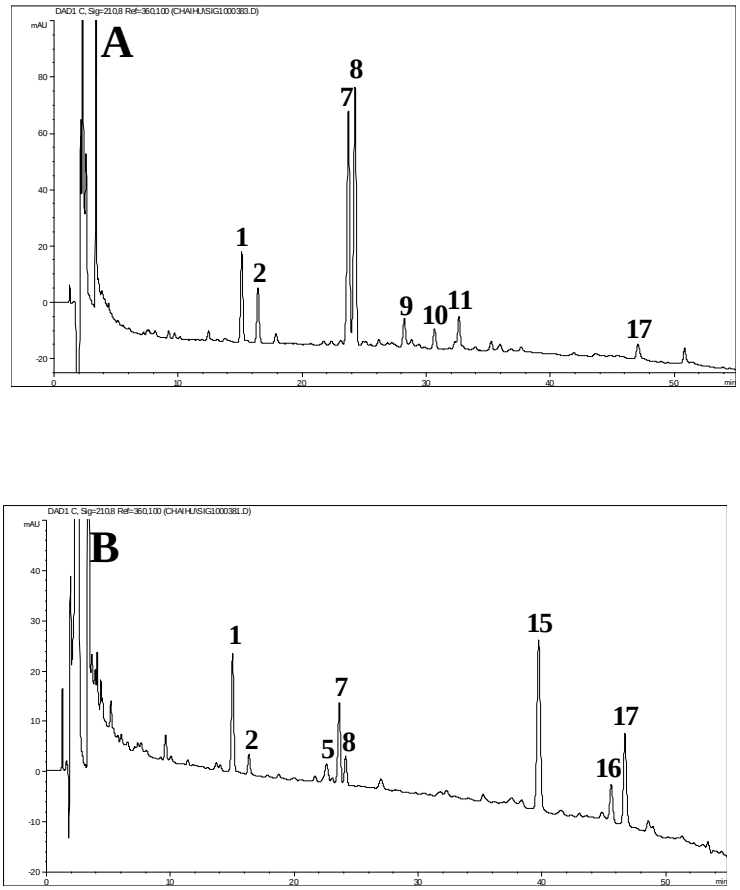

Figure 3 HPLC profiles of extract-Ps of *B. chinense* at 210 nm.

A. *B. chinense* (BC1); B. *B. chinense* (BC6).

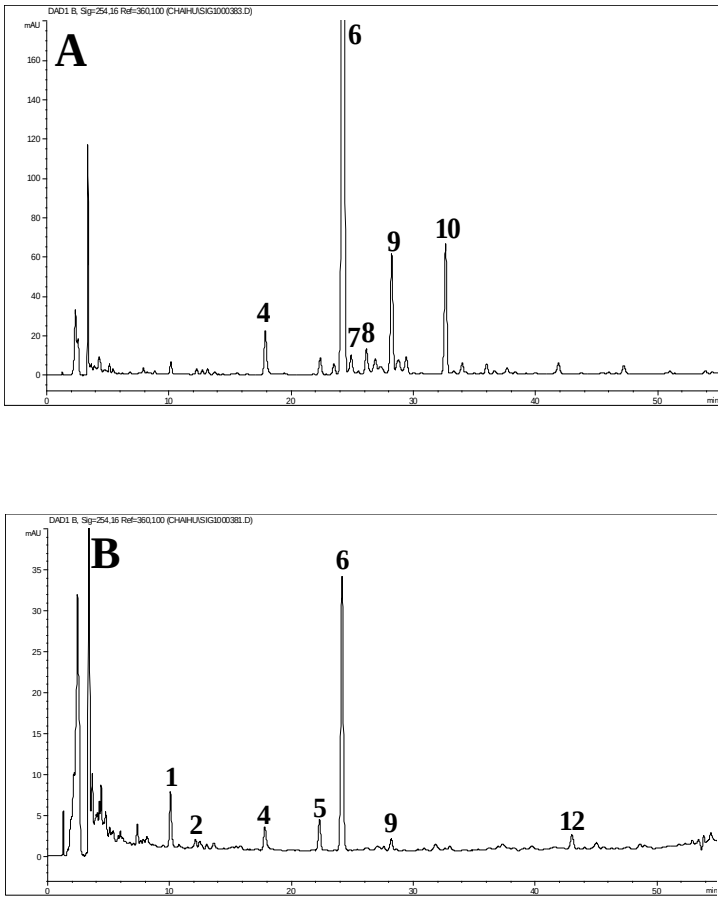

Figure 4 HPLC profiles of extract-Ps of *B. chinense* at 254 nm.

A. *B. chinense* (BC1); B. *B. chinense* (BC6).

Table 1 Subjects employed.

	species	sources	type
BC1	*B. chinense* DC.	Songxian, Henan Province	dried ecotype
BC2	*B. chinense* DC.	Xixia, Henan Province	dried ecotype
BC3	*B. chinense* DC.	Tongbaishan, Henan Province	dried ecotype
BC4	*B. chinense* DC.	Taihangshan, Henan Province	dried ecotype
BC5	*B. chinense* DC.	Xinglong, Hebei Province	dried ecotype
BC6	*B. chinense* DC.	Jixian, Tianjin City	dried ecotype
BC7	*B. chinense* DC.	Baoze, Shanxi Province	dried ecotype
BC8	*B. chinense* DC.	Zuoquan, Shanxi Province	dried ecotype

Tabel 2 Basic HPLC data of extract-Ps of the roots of *B. chinense* with the relative peak areas (PA) of saikosaponin a as reference at 210 nm.

No.	Peaks (t_R min)	PA	BC1	BC2	BC3	BC4	BC5	BC6	BC7	BC8	$[M+Na]^+$ (*m/z*)
1	15.1	0.14-2.23	+[a]	+	+	+	+	+	+	+	949
2	16.4	0.16-0.59	+	+	+	+	+	+	0.84	+	951
3	18.1	0.03-0.09	+	+	+	+	+	+	+	+	1023
4	21.8		-[c]	-	-	-	-	±	±	±	965[d]
5	22.4	0.03-0.35	±	±	±	±	±	+	+	+	965[d]
6	23.5		-	-	-	-	-	-	-	-	935[e]
7	23.7 (R)	1.00	+	+	+	+	+	+	+	+	803[f]
8	24.3	0.70-1.09	+	+	+	+	+	0.46	+	+	803[f]
9	28.2	0.12-0.28	+	±	+	+	+	±	±	±	803[f]
10	30.7	0.02-0.19	+	+	+	+	+	-	-	-	787
11	32.6	0.05-0.34	+	+	+	+	+	+	+	+	845
12	34.2		-	-	-	-	-	-	-	-	935[e]
13	35.7	0.04-0.37	±	+	+	±	+	±	+	+	803[f]
14	36.7	0.02-0.17	±	±	+	±	±	±	0.26	±	801
15	39.7		-	-	-	-	-	2.73	5.13	0.68	887
16	45.6	0.14-0.55	-	-	-	-	-	+	1.22	+	997
17	46.7	0.09-0.37	+	+	+	+	+	1.29	2.11	+	857

[a](+) The peak listed in the first column was detected. [b](±) The peak listed in the first column was detected faintly. [c](-) The peak listed in the first column wasn't detected. [d,e,f] Some saikosaponins possess the same molecular weight. For example, saikosaponins a, d, b_1, and b_2 have the same molecular weight of 780, and the same quasimolecular ion peaks at *m/z* 803$[M+Na]^+$ were shown in the positive-mode ESI-MS spectra. So did saikosaponins S and N, and bupleuroside I, etc., with their quasimolecular ion peaks shown at *m/z* 965$[M+Na]^+$, and saikosaponins k and S_3, and buddlejasaponin Ⅲ, etc., with their quasimolecular ion peaks shown at *m/z* 935$[M+Na]^+$.

Table 3 Basic HPLC data of extract-Ps of the roots of *B. chinense* with the relative peak areas (PA) of saikosaponin b_2 as reference at 254 nm.

No.	Peaks (t_R min)	PA	BC1	BC2	BC3	BC4	BC5	BC6	BC7	BC8	$[M+Na]^+$ (m/z)
1	10.1	0.05-0.11	±[a]	+[b]	+	+	+	+	0.25	-[c]	965[c]
2	12.0	0.04-0.05	-	-	-	-	-	±	+	+	1023
3	13.1	0.09-0.10	±	+	±	±	+	±	±	±	965[d]
4	17.9	0.01-0.10	+	+	+	+	+	+	+	+	949
5	22.4	0.01-0.15	+	+	±	+	+	+	+	+	965[d]
6	24.3 (R)	1.00	+	+	+	+	+	+	+	+	803[e]
7	24.9	0.01-0.03	+	+	+	+	+	-	-	-	833[f]
8	26.2	0.01-0.03	+	+	±	±	+	-	+	+	833[f]
9	28.2	0.11-0.23	+	+	+	+	±	±	±	+	803[e]
10	32.6	0.08-0.26	+	±	+	+	±	±	+	0.53	845
11	39.1		-	-	-	-	-	-	-	-	613
12	43.2	0.06-0.08	-	-	-	-	-	+	+	±	1029

[a](±) The peak listed in the first column was detected faintly. [b](+) The peak listed in the first column was detected. [c](-) The peak listed in the first column wasn't detected. [d,e,f] Some saikosaponins which have the same molecular weight are existent in *B. chinense*.

Table 4 Basic ^{1}H NMR spectroscopic data of extract-Ps of the roots of *B. chinense* (Anomeric and olifinic protons; 400 MHz, DMSO-d_6+D_2O).

Data of fingerprint (*J* in Hz)	BC1	BC2	BC3	BC4	BC5	BC6	BC7	BC8
6.37 d(10.2)	++[ab]	++[b]	++[b]	++[b]	++[b]	+	+	++[b]
5.77 d(10.5)	++[b]	++[b]	++[b]	++[b]	++[b]	++	++	++[b]
5.48 d(10.2)	++[b]	++[b]	++[b]	++[b]	++[b]	+	+	++[b]
5.25 dd(10.2, 2.2)	++[b]	++[b]	++[b]	++[b]	++[b]	++	++	++[b]
5.13 br s	+	+	+	+	+	+	+	+
4.70 s	++	++	++	++	++	++	++	++
4.38 d(7.5)	++	++	++	++	++	++	++	++
4.35 d(7.5)	++	++	++	++	++	++	++	++
4.24 d(6.8)	++	++	++	++	++	++	++	++
4.17 d(7.5)	++	++	++	++	++	++	++	++

[a](++) The preceding signal was detected strongly. [b] Signals with the same superscript in the same column showed nearly the same relative intensity. [c](+) The preceding signal was detected. [d](-) The preceding signal wasn't detected.

Table of Contents Entry

Distinctive Features of Chemical Composition from Bupleurum chinense Applicable to Original Authentication

Jin-Qian Yu[1], An-Jun Deng[2], Hai-Lin Qin[2,]*

The features of ^{1}H NMR and HPLC for *B. chinense* samples showed specific data from saikosaponins and can apply to original authentication.