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Analytical Methods

Authentication of the anti-tumor herb Baihuasheshecao with bioactive marker compounds and molecular sequences

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ABSTRACT

Baihuasheshecao (*Hedyotis diffusa*), a Chinese herb for cancer treatment, is frequently adulterated by a related species *Hedyotis corymbosa*. DNA sequencing of the complete internal transcribed spacer region was applied to differentiate *H. diffusa* from *H. corymbosa* and other closely related species. The molecular data showed that four out of seven herb samples of Baihuasheshecao were adulterants. Chemical analyses by TLC and HPLC were used to authenticate *H. diffusa* and *H. corymbosa*. Two marker compounds were identified exclusively in *H. diffusa*: 6-*O*-(*E*)-*p*-coumaroyl scandoside methyl ester (compound **1**) and 10(*S*)-hydroxypheophytin a (compound **2**). Both compounds showed moderate anti-proliferation effect on PC3 human androgen-independent prostate cancer cells, while compound **2** also showed strong anti-proliferation effect on LNCaP human androgen-sensitive prostate cancer cells. Accordingly, these bioactive marker compounds could be applied to verify the authenticity and assess the quality of Baihuasheshecao.

1. Introduction

Baihuasheshecao is an herb derived from the whole plant of *Hedyotis diffusa* Willd. in the family Rubiaceae. The Pharmacopoeia of the People's Republic of China (2005) chose to use the name *Oldenlandia diffusa* (Willd.) Roxb., which is regarded as a synonym by *Flora Republicae Popularis Sinicae* (Ko, 1999). It is commonly used in the Orient and tropical Asia (Ahmad et al., 2005; Perry, 1980), for making teas and botanicals for the relief of 'heat', removal of 'toxins' and promotion of diuresis to eliminate 'wetness-evil'. It is used also in the West (Kane, Kane, & Jain, 1995). A more direct application is for the treatment of prostate cancer and other tumors (Liang, 2004). The anti-cancer properties of Baihuasheshecao have generated not a few studies (Gupta, Zhang, Yi, & Shao, 2004; Willimott, Barker, Jones, & Opara, 2007). Its anti-cancer activities are related to immuno-stimulating activity on the immune system (Shan, Zhang, Du, & Li, 2001), superoxide burst and caspase activation (Yadav & Lee, 2006).

In the market, Baihuasheshecao is frequently adulterated by a related species *Hedyotis corymbosa* (L.) Lam (Zhao & Li, 2005). They share similar morphological characters but the functions and efficacies of these two herbs are not quite the same (Lin, Ng, & Yang, 2004). In order to ensure effective and correct use of Baihuasheshecao, it is necessary to develop efficient methods and reliable markers for the authentication and quality control of Baihuasheshecao. Various approaches have been applied to differentiate the genuine Baihuasheshecao derived from *H. diffusa* from the adulterant derived from *H. corymbosa* and related species. The two species look very much alike even when fresh, differing only by the variable number of flowers, length of pedicels and shape of stems (Ko, 1999). These characters become unapparent when dried and dispensed in short fragments. Various attempts were made using chemical methods to authenticate Baihuasheshecao by thin layer chromatography (TLC) (Xie, Zhang, & Lu, 1997) and high performance liquid chromatography (HPLC) (Liang, He, Fong, Jiang, & Zhao, 2008; Liang, Jiang, Leung, & Zhao, 2006b). However, these studies relied on unidentified spots or markers with no reference to biological functions. DNA sequences of the internal transcribed

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spacer 1 (ITS-1) and internal transcribed spacer 2 (ITS-2) regions were separately proposed to distinguish Baihuasheshecao derived from *H. diffusa* from *H. corymbos*a (Hao, Liu, & Wang, 2004; Liu & Hao, 2005; Liu, Hao, & Wang, 2004). The accuracy of these sequences, however, has not been confirmed. Therefore, it is necessary to overhaul the molecular and chemical analyses for authentication of Baihuasheshecao.

In this study, we applied molecular authentication of Baihuasheshecao using the whole region of internal transcribed spacer (ITS) to distinguish *H. diffus*a from *H. corymbosa* and other *Hedyotis* species. Bioactive chemical markers with anti-proliferation effects on prostate cancer cells were identified from *H. diffusa*. These markers could be used for identification and quality assessment of Baihuasheshecao.

2. Materials and methods

2.1. Samples studied

A total of 21 voucher specimens, consisting of 14 *Hedyotis* species, and seven herb samples of Baihuasheshecao retailed in the market were included in this study. Leaves were picked from 14 voucher specimens deposited in Hong Kong Herbarium, Agriculture, Fisheries and Conservation Department, for molecular study. The names of these specimens are listed here together with the collectors' number and the corresponding NCBI Genbank accession number of the ITS region in a bracket: *Hedyotis auricularia* L. (Y.W. Lam 1545, EF570980; Y.W. Lam 1018, EF570979), *Hedyotis biflora* (L.) Lam. (Y.W. Lam 1478, EF570993), *Hedyotis bracteosa* Hance (Y.S. Lau 3195, EF570998; K.L. Yip 4084, EF570997), *Hedyotis consanguinea* Hance (B. Walden, EF570995), *Hedyotis costata* (Roxb.) Kurz (Y.W. Lam 1248, EF570982), *Hedyotis effusa* Hance (Y.W. Lam 1241, EF570994), *Hedyotis pinifolia* Wall. ex G. Don (F. Yip, EF570989), *Hedyotis shiuyingiae* T. Chen (Y.W. Lam 245, EF570999; F.W. Xing 6937, EF571000), *Hedyotis tenelliflora* Blume (Y.W. Lam 898, EF570990), *Hedyotis verticillata* (L.) Lam (Y.W. Lam 878, EF570991; Y.W. Lam 909, EF570992). Seven fresh plant samples were collected in the field in Hong Kong and their voucher specimens were deposited in the Herbarium of the Department of Biology, The Chinese University of Hong Kong (CUHK): *Hedyotis acutangula* Champ. (S.Y. Hu & P. But 24053, EF570996), *Hedyotis corymbosa* (L.) Lam. (S.Y. Hu & P. But 24052, EF570974; W.L. Chu 005, EF570975), *Hedyotis diffusa* Willd. (M. Li 041, EF570985; M. Li 035, EF570986; M. Li 042, EF570988), and *Hedyotis hedyotidea* (DC.) Merr. (M. Li 044, EF570981). The identities of the samples of *H. diffusa* and *H. corymbosa* were double confirmed by Hong Kong Herbarium. Seven dried herb samples purchased from Guangzhou (China), Hong Kong (China) and Boston (USA) were deposited in the Museum of Chinese Medicine, Institute of Chinese Medicine, The Chinese University of Hong Kong. They are listed here together with their accession number in the Museum and source placed in a bracket: Baihuasheshecao 1 (2005–2686; Guangzhou), Baihuasheshecao 2 (2005–2687; Guangzhou), Baihuasheshecao 3 (2005–2688; Guangzhou), Baihuasheshecao 4 (2005–2683; Hong Kong), Baihuasheshecao 5 (2005–2684; Hong Kong), Baihuasheshecao 6 (2005–2685; Hong Kong) and Baihuasheshecao 7 (2005–2686; Boston).

2.2. Molecular authentication by DNA sequencing

Fresh materials, herbarium specimens and herb samples were subjected to total DNA extraction using a modified DNA extraction method as previously described (Poon, Shaw, Simmons, & But, 2007). Complete ITS regions were amplified in a 50 μl reaction containing 27.5 μl distilled water, 5 μl 10× buffer solution (750 mM Tris–HCl, pH 8.8, 200 mM $(NH_4)_2SO_4$, 0.1% Tween 20), 4 μl 25 mM $MgCl_2$, 4 μl 2.5 mM dNTPs, 2 μl 10 mM forward primer Sol-18d (5′-GAG GAA GGA GAA GTC GTA ACA AG-3′), 2 μl 10 mM reverse primer Sol-28 cc (5′-GGT AGT CCC GCC TGA CCT GG-3′), 1 unit *Taq* polymerase and 3 μl total DNA extract. Polymerase chain reaction (PCR) was performed in a thermocycler through 35 cycles of 95 °C for 30 s; 60 °C for 20 s; 72 °C for 1 min. The PCR products were resolved in a 1.7% agarose gel and then purified with Gel-M™ Gel Extraction System (Viogene). Purified PCR products were

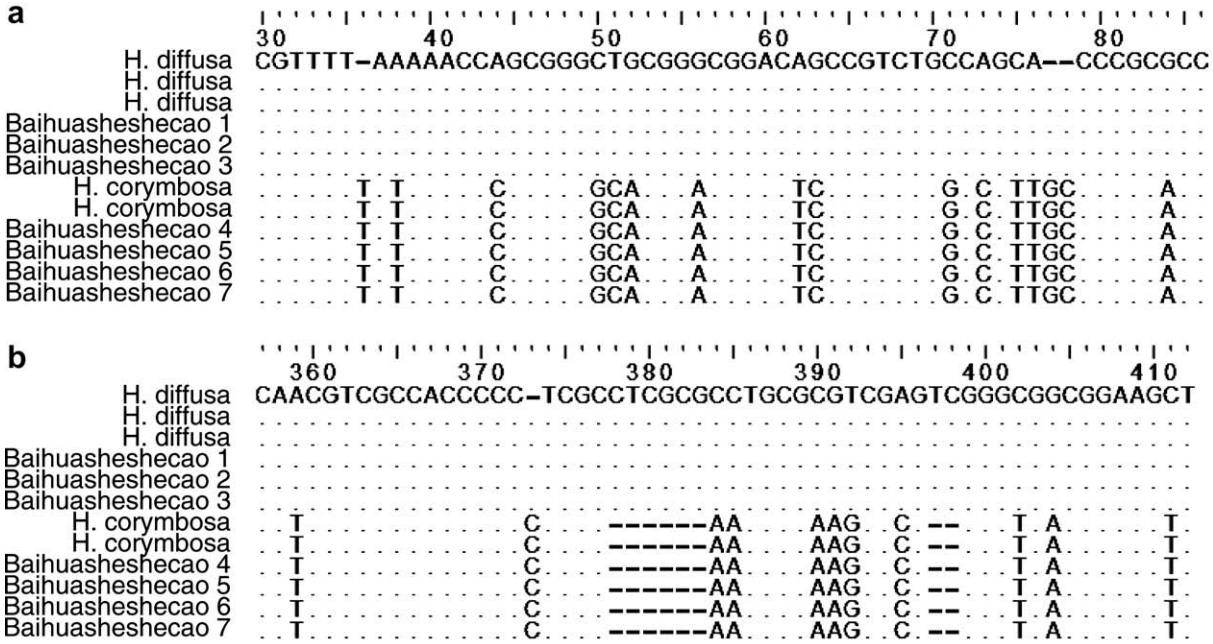

Fig. 1. Molecular identification of *H. diffusa*, *H. corymbosa* and other *Hedyotis* species using complete ITS region. Partial alignment of the complete ITS regions showing notable base polymorphisms between *H. diffusa* and *H. corymbosa* in (a) ITS-1 and (b) ITS-2 regions. The numbers on the top line represent the base numbers in sequence alignment. '.' represents the base being identical to the first sequence. '–' represents gap.