Short communication

Application of novel loop-mediated isothermal amplification (LAMP) for rapid authentication of the herbal tea ingredient *Hedyotis diffusa* Willd.

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1. Introduction

*Hedyotis diffusa* Willd. (Rubiacceae, common name: Baihuasheshcao) is an ingredient of popular herbal teas in the Orient and tropical Asia (Perry, 1980). It has been commonly consumed for health maintenance and also as a dietary medicine for the treatment of prostate cancer (Liang, 2004). Scientific studies also showed that *H. diffusa* possessed anti-cancer properties (Gupta, Zhang, Yi, & Shao, 2004; Willimott, Barker, Jones, & Opara, 2007), and its effects were related to the stimulation of the immune system, activation of caspase and burst of superoxide (Shan, Zhang, Du, & Li, 2001; Yadav & Lee, 2006). Along with the commercialization of this herb, adulteration by a related species, *Hedyotis corymbosa* (Lam), has been more frequent in the market (Zhao et al., 2005). The chemical components and therapeutic value of these two herbs are quite different. For example, two anti-prostate tumor chemicals, 6-O-(E)-p-coumaroyl scandoside methyl ester and 10(S)-hydroxylpheophytin a, were found only in *H. diffusa* but undetectable in *H. corymbosa* (Li et al., 2010). Although these two *Hedyotis* species are not closely related sister groups from the phylogenetic point of view (Guo, Simmons, But, Shaw, & Wang, 2011), they look very much alike even in fresh. They differ only by the shape of stems, number of flowers and size of pedicels (Ko, 1999), and these characters were difficult to observe when the materials are dried, cut or pulverized. Thin layer chromatography, high performance liquid chromatography, and DNA sequencing have been developed to differentiate *H. diffusa* from *H. corymbosa*, with materials being dried, cut or pulverized. Thin layer chromatography, high performance liquid chromatography, and DNA sequencing have been developed to differentiate *H. diffusa* from *H. corymbosa* (Lau et al., 2012; Li et al., 2010; Yu et al., 2012). These methods, however, may be interfered by chemical variation among samples or time consuming. A rapid molecular authentication technique is needed for better quality assurance of this herbal tea ingredient. Loop-mediated isothermal amplification (LAMP) is a cutting-edge molecular technique which enables amplification of DNA to a large amount under constant operation temperature (Nagamine, Hase, & Notomi, 2002; Notomi et al., 2000). This technique has been applied for the identification of *Escherichia coli* in food, detection of genetically modified food, and authentication of Chinese medicinal materials (Chaudhary, Hemant, Mohsin, & Ahmad, 2012; Chen, Guo, Wang, Kai, & Yang, 2011; Liu et al., 2008; Sasaki, Komatsu, & Nagumo, 2008; Wang, Jiang, & Ge, 2012). The objective of this study is to develop LAMP technique to distinguish *H. diffusa* from its adulterant *H. corymbosa*. Internal control was developed to ensure the quality of the concerned DNA region for LAMP.
2. Materials and methods

2.1. Samples studied

Three authentic samples of the herbal materials of *H. diffusa* were collected from Jiangxi (Hd-1), Jiangsu (Hd-2) and Guangxi (Hd-3). Another three samples of the adulterants *H. corymbosa* were collected from Guangxi (Hc-1) and Hong Kong (Hc-2 and Hc-3). These materials were identified by Shenzhen Institute For Drug Control, Shenzhen, China. The samples were stored in the Institute of Chinese Medicine, The Chinese University of Hong Kong.

2.2. DNA extraction and quantification

Approximately 5 mg of herbal materials were used for DNA extraction using modified cetyltrimethylammonium bromide (CTAB) method as described previously (Li et al., 2012). DNA was quantified by absorbance at 260 nm using NanoDrop (Thermo Scientific, US) and stored at −20 °C.

2.3. Primer design

The DNA sequences of ITS region of *H. diffusa* (EF570985, EF570986, and EF570988) and *H. corymbosa* (EF570974 and EF570975) were retrieved from GenBank, National Center for Biotechnology Information. Multiple sequence alignment and identification of polymorphic sites were performed using the sequence analysis software Bioedit 7.0 (Hall, 1999). Four common primers were designed from six loci in ITS region, including the forward outer primer F3 (5′-TTG TCG AAT CCT GCA AA-3′), forward inner primers FIP (5′-TTG AGT AGT CCT TGG CGC TTA CCG CCA ACA CGT TTT-3′), backward inner primer BIP (5′-ACC ACG ACT CTC GGC AAC TGA TTC CCG TTG CTT C-3′) and backward outer primer B3 (5′-AGC GGA TTC TGG AAT TCA CA-3′), using the PrimerExplorerV4 (http://primerexplorer.jp) and OligoAnalyzer 3.1 (Integrated DNA technologies, US). Specific forward outer primer S_F3 (5′-TAA AAA CCA GCG GGC TG-3′) and specific forward inner primer S_FIP (5′-GGA CGA TCC GTT TGA ACA GCC GTC TGC TAC GTT CTT-3′) were also designed for LAMP specific to *H. diffusa* (Fig. 1).

2.4. Loop-mediated isothermal amplification (LAMP)

LAMP reaction was carried out using an Isothermal Mastermix amplification kit (OptiGene, UK) accordingly to the manufacturer’s instruction. In brief, a 25 µl reaction mixture containing 0.2 µM of each outer primer, 2 µM of each inner primer, 1 µl DNA template and 1× reagent mix was subjected to isothermal amplification at 63 °C. Once the reaction started, the temperature was held constant at 63 °C until a negative result was obtained. LAMP products were confirmed by gel electrophoresis on 1.5% agarose gel using SYBR Gold staining.
65 °C for 60 min followed by inactivation at 98 °C for 5 min. Primers F3, FIP, BIP and B3 were used for internal control. Primers S_F3, S_FIP, BIP and B3 were used for specific LAMP. LAMP was real-time monitored using the portable Genie II LAMP detector (OptiGene, UK). The LAMP products were visualized by 1% agarose gel electrophoresis stained with ethidium bromide.

### 3. Results and discussion

_H. diffusa_ is an herbal tea ingredient for daily consumption to maintain health. This anti-tumor herb has been adulterated by _H. corymbosa_ which showed different chemical constituents and medicinal values (Li et al., 2010). In this study, we have applied LAMP for rapid differentiation of these two _Hedyotis_ species. The design of LAMP primers was based on the ITS region, which is a supplementary DNA barcode commonly used in the molecular identification of herbal materials including _H. diffusa_ (Li et al., 2010, 2012). Four internal control primers (F3, FIP, BIP and B3) were designed from six loci in the ITS region (Fig. 1). These primers contain minimum amount of polymorphic sites so that both _H. diffusa_ and _H. corymbosa_ were detected by LAMP started from 30 to 40 min and saturated from 40 to 60 min (Fig. 2). In contrast, two forward primers specific to _H. diffusa_ (S_F3 and S_FIP) were designed based on the ITS region and many sites polymorphic to _H. corymbosa_ were included at the 3' ends (Fig. 1). The use of these two specific primers together with the two backward primers (BIP and B3) led to specific LAMP of _H. diffusa_ started from 20 to 30 min and saturated from 30 to 50 min (Fig. 2). LAMP is a highly sensitive amplification technique which could amplify targets with as low as six copies of DNA template (Notomi et al., 2000). Our data suggested that LAMP was detectable with 30 ng to 3 pg of DNA template for both internal control and specific LAMP (Fig. 3).

In the molecular authentication of herbal materials and other processed samples such as food products and Chinese medicinal materials, poor DNA quantity, integrity and the presence of inhibitors are the major concerns of successful identification (Li, Cao, But, & Shaw, 2011). For example, LAMP was applied to distinguish the herbal material Ginseng Radix (_Panax ginseng_ C. A. Mey., Araliaceae) from Panacis Japonici Rhizoma (_Parribacus japonicus_ C. A. Mey., Araliaceae) and Glycyrrhizae Radix (_Glycyrrhiza uralensis_ Fisch. ex DC., Fabaceae) (Sasaki et al., 2008). Although DNA purity and quantity were considered, the problem of DNA degradation in samples of Panacis Japonici Rhizoma and Glycyrrhizae Radix was not addressed and this may lead to false negative identification results. In fact, it is quite difficult to determine the degree of DNA integrity in small amount of DNA. Alternatively, the introduction of internal control can serve as an indication of sufficient DNA template, intact DNA region of concern and the absence of inhibitors. The specific primers, either forward, backward or both, must be designed in the region flanked by the outer primers of the internal control (F3 and B3) so that DNA integrity of the concerned region in specific LAMP could be assured (Fig. 2).

Correct use of ingredient is important for quality assurance of food and drinks. In this study, we have successfully applied LAMP technique for the identification of the herbal tea ingredient _H. diffusa_ and differentiated it from its adulterant _H. corymbosa_. Internal control was developed to avoid false identification conclusion drawn by negative LAMP results. This study provided a novel

![Fig. 2. Differentiation of _H. diffusa_ from _H. corymbosa_ using LAMP. (A) Internal control (left panel) and specific LAMP (right panel) was real-time monitored by Genie II. Both _H. diffusa_ and _H. corymbosa_ were detected using the internal control primers, while only _H. diffusa_ was detected using the specific primers. LAMP without DNA template was applied as the negative control (neg). (B) End-point detection of internal control and specific LAMP by 1% agarose gel electrophoresis stained with ethidium bromide. ‘M’ represents 100 bp DNA ladder, the size of 800 bp is indicated for reference. ‘Hd’ represents _H. diffusa_ and ‘Hc’ represents _H. corymbosa_.](image)
molecular technique for rapid identification of *H. diffusa* and the strategy employed may be extended to other medicinal materials.

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**References**


