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Species identification of Dyers woad leaf by DNA sequence of 5S-rRNA spacer domain and random amplified polymorphic DNA (RAPD) analysis

KJ Zhao[1], TTX Dong[1], YC Wong[1], PF Tu[2] and KWK Tsim[1,*]

[1]Department of Biology, The Hong Kong University of Science and Technology, Clear Water bay Road, Hong Kong, SAR, China; [2]College of Pharmacy, Beijing University, University Road, Beijing, 100083, China

SUMMARY

Dyers woad leaf (Daqingye) is a traditional Chinese medicine commonly used as anti-pyretic, anti-bacterial and anti-viral agent against infectious diseases. The Chinese Pharmacopoeia (2005) records that Dyers woad leaf should be derived from the leaves of *Isatis indigotica* Fort., but the leaves of *Polygonum tinctorium* Ait., *Baphicacanthus cusia* (Nees) Bremek. and *Clerodendron cyrtophyllum* Turcz. have also been used as substitutes of Dyers woad leaf in different regions of China. The leaf morphologies of these four species show a close resemblance, and based on their morphological appearance, it is difficult to identify them. Here, molecular genetic methods were developed as a target to identify different members of Dyers woad leaf. The 5S-rRNA spacer domain was amplified by polymerase chain reaction from genomic DNAs isolated from *I. indigotica*, *P. tinctorium*, *B. cusia* and *C. cyrtophyllum*, and the nucleotide sequences showed a great diversity. In addition, random amplification of polymorphic DNA analysis was also used to distinguish the members of Dyers woad leaf. These molecular methods could be used as a tool in authentic identification of Dyers woad leaf.

Key words: *Isatis indigotica; Polygonum tinctorium; Baphicacanthus cusia; Clerodendron cyrtophyllum;* Species identification; 5S-rRNA; RAPD

INTRODUCTION

Traditional Chinese medicinal herbs provide an alternative form of medication and are extensively used in Orient today. There are about 11,146 species of medicinal plants in China. It is rather common that a single medicinal herb could be derived from different plant species. In contrast, different medicinal herbs could carry the same name, or the same medicinal herb is named differently. This problem confuses the origins and usages of herbal medicines on the current market. Therefore, correct identification of herb is a prerequisite for chemical and pharmacological investigations of traditional Chinese medicines and also for their clinical applications.

Dyers woad leaf is a traditional Chinese medicine commonly used as anti-pyretic, anti-bacterial and anti-viral agent against infectious diseases such as measles, laryngitis, mumps, erysipelas and carbuncle (Tang and Eisenbrand, 1992); however, its nomenclature has been chaotic for hundreds of years. The Chinese Pharmacopoeia (Zheng, 2005) records that Dyers woad leaf is the dry leaf *of Isatis indigotica* Fort. (Folium Isatidis). The first description of Dyers woad leaf was in the first medicinal book in China

*Correspondence: KWK Tsim, Department of Biology, The Hong Kong University of Science and Technology, Clear Water Bay Road, Hong Kong, SAR, China. Tel: +852-2358-7332; Fax: +852-2358-1559; E-mail: botsim@ust.hk

"*Shen Nong Bencao Jing*" in ~100 B.C.. The species of Dyers woad leaf as listed in that book was possibly describing the leaves of *Clerodendron cyrtophyllum* Turcz. However, three different plants were subsequently recorded as Dyers woad leaf throughout the history in China, which could be exampled by the description of *Polygonum tinctorium* Ait. and *I. indigotica* in "Xin Xiu Bencao" in 659 A.D. and *Baphicacanthus cusia* (Nees) Bremek. in "*Ben Cao Gang Mu*" in 1596 A.D.. Today, the leaves of *I. indigotica*, *P. tinctorium*, *B. cusia* and *C. cyrtophyllum* are all being used either as Dyers woad leaf or its substitutes (Lou and Qin, 1995). According to recent studies, these four species are different in their chemical compositions; however, the differences are in their levels of active constituents, which could be varied according to the source of framing and the time of harvest (Ma and Xia, 2000). For instance, the active constituents including indirubin, indigo and tryptanthrin have been isolated from *P. tinctorium*, *I. indigotica* and *B. cusia* (Tang and Eisenbrand, 1992), but not in *C. cyrtophyllum* (Ma and Xia, 2000). Clinically, leaves from the four species also have different pharmacological properties and clinical efficacies (Gan *et al.*, 1985).

Because of the differences in the active constituents and pharmacological actions, the usages of the leaves from *I. indigotica*, *P. tinctorium*, *B. cusia* and *C. cyrtophyllum* should be differentiated. Chemically, these plant species are hard to distinguish because of its chemical composition might change under different environmental factors. Here, we targeted to identify molecular genetic markers of *I. indigotica*, *P. tinctorium*, *B. cusia* and *C. cyrtophyllum*, which could distinguish different members of Dyers woad leaf. Having molecular marker in species identification has several advantages over classical morphological and chemical analyses, which requires genotype instead of the phenotype; the genotype does not change according to the variation of environment. Moreover, most of the traditional Chinese medicinal herbs require processes such as drying, heating or long-term storage. Under some circumstances, the herbs have to be grounded into powder before medication. This could make morphological identification impossible. The identification of herbs by using genetic sequences is faster and reliable, which meets the needs of the current market. Here, two approaches were used for identification, which included the determination of 5S-rRNA spacer domains and the DNA fringerprints after random amplification of polymorphic DNA (RAPD) analysis.

MATERIALS AND METHODS

Acquisition of plant materials

Fresh plants and dried herbs were collected from agricultural farms in China. The voucher specimens were deposited in the Department of Biology, The Hong Kong University of Science and Technology, Hong Kong. Fresh plants of *I. indigotica* (00-07-21) and *P. tinctorium* (00-07-24) were obtained from Hebei, while *B. cusia* (00-06-01) was collected from Guangdong and *C. cyrtophyllum* (00-08-10) from Jiangsu. Samples were kept in silica gel during field collection. All the plant materials were collected in October of 2000 and dried under vacuum. The botanical origins of all the materials were identified morphologically. The dried herbs of different species were collected from the same region as the fresh materials. In general, over 10 individual plants of the same species were tested genetically.

Extraction of genomic DNA

DNA extraction was followed by a standard DNA precipitation method (Sambrook and Russell, 1989; Cui *et al.*, 1999). The leaves (freshly collected) and dried herbs were frozen with liquid nitrogen and grounded into a fine powder. Genomic DNA was extracted from the grounded powder by using DNA extraction buffer consisting of 25 mM Tris-HCl, pH 8.0, 50 mM EDTA, 0.5% SDS, 10 μg/ml RNase, 0.2% -mercaptoethanol (Sigma, St Louis, MO). The mixed solution was incubated for 15 minutes at 58°C, then centrifuged at 5,000 × g. The

supernatant was extracted by equal volumes of water-saturated phenol: chloroform (1 : 1), mixed and then centrifuged. The DNA was ethanol precipitated, and the resulting pellet was collected after centrifugation and dissolved in 10 mM Tris-HCl pH 8.0, 5 mM EDTA (Tris-EDTA buffer).

Polymerase chain reaction (PCR) amplification

Fifty μl PCR reaction mix consisted of 5 μl 10X reaction buffer, 50 ng genomic DNA, 1 μl of 10 mM dNTPs stock, 2.5 μl (0.5 M) forward and reverse primers (synthesized by Invitrogen Life Technologies, Carlsbad, CA) and 1 unit of *Taq* polymerase (Roche, Indianapolis, IN). The primers used for amplification of 5S-rRNA spacer were 5S-P1 (5'-GTG CTT GGG CGA GAC TAG TA-3') and 5S-P2 reverse primer (5'-TTA GTG CTG GTA TGA TCG CA-3'; Gerlach and Dyer, 1980; Cai *et al.*, 1999; Dong *et al.*, 2003). Cycling condition consisted of an initial 5 minutes at 94°C following by 1 minute at 94°C, 2 minutes at 50°C and 2 minutes at 72°C, repeated for 30 cycles and with 10 minutes extension at 72°C in a Gene Amp^{R} PCR System 9700 (Applied Biosystem, Foster City, CA). In RAPD PCR, two 10-base oligonucleotides (Operon Technologies, Inc., Alameda, CA) were used: A2 (5'-TGC CGA GCT G-3') and Z8 (5'-GGG TGG GTA A-3'). The RAPD PCR was done with 2 l of genomic DNA (~50 ng) to a PCR mix containg 10 μM 10-base oligonucleotide and 0.5 unit of Taq DNA polymerase, in a total volume of 20 μl according to Cui *et al.* (2003). The products were subject to 1.2% agarose gel electrophoresis (Cai *et al.*, 1999) and visualized by ethidium bromide staining under UV. The DNAs were purified by Concert^{TM} Rapid gel extraction system (Invitrogen Life Technologies).

Sequencing and phylogenetic analysis

The PCR products were subcloned into pGET-T easy vector (Promega, Madison, WI) and transformed in *E. Coli* JM 109 cells (Sambrook and Russell, 1989). The correct DNA inserts were verified by restriction analysis. The plasmid DNAs from verified colonies were isolated with the Wizard

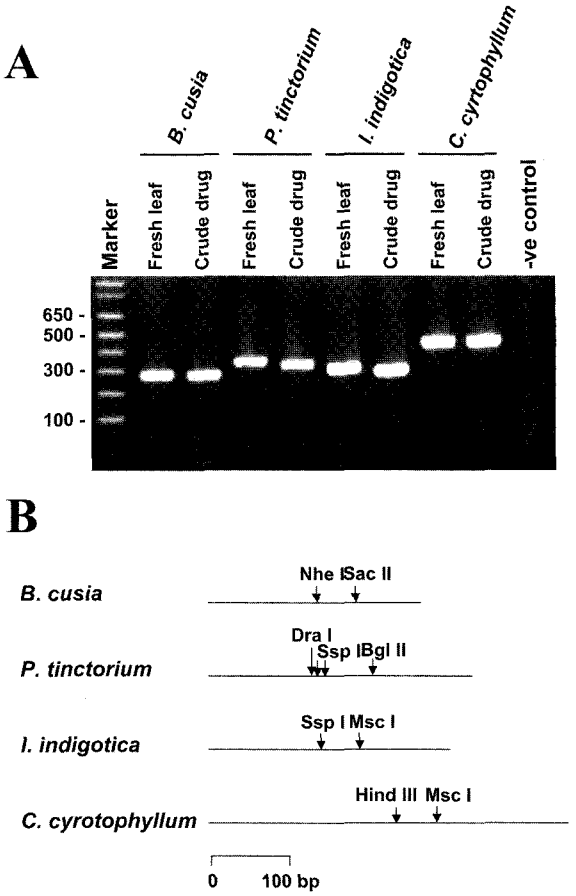

Fig. 1. PCR products generated by primers (5S-P1 and 5S-P2) flanking the spacer domain of 5S-rRNA using DNAs isolated from *I. indigotica*, *P. tinctorium*, *B. cusia* and *C. cyrtophyllum* as templates. (**A**): The PCR products were separated in a 1% agarose gel and visualized by ethidium bromide staining. PCR products having the same size were obtained from ten individual tested samples (N=10). The negative control does not contain genomic DNA. DNA markers in bp are indicated. (**B**): The restriction enzyme mapping of the spacer domains of 5S-rRNA genes was shown; the digested sites were predicted by sequence search of using MacVector. Bgl II, Dra I, Hind III, Msc I, Nhe I, Sac II and Ssp I restriction enzymes are shown in the map. Arrows indicate the restriction sites. Only the distinct sites are shown here. Bar; size marker.

Minipreps DNA Extraction system (Promega). ABI Prism^{TM}, BigDye^{TM} Terminator and Cycle Sequencing Ready Reaction Kit were used for sequence reaction with T7 or SP6 primers. Sequence was detected by ABI Prism^{TM} 310 Genetic Analyzer

(Applied Biosystems). Both strands of DNA clones were sequenced at least twice and the sequences were aligned by MacVector software (Kodak, New Haven, CT). The entire sequences of the tested samples were aligned by a software Clustal X having a gap opening of 10 and gap extension of 0.05. A phylogenetic relationship based on the hierarchical clustering of the alignments of the 5S-rRNA spacer was assessed by neighbor-joining (UPGMA) method (Dong *et al.*, 2003).

RESULTS AND DISCUSSION

Primers flanking the spacer domain of 5S-rRNA were used in PCR analyses of genomic DNAs isolated from *I. indigotica*, *P. tinctorium*, *B. cusia* and

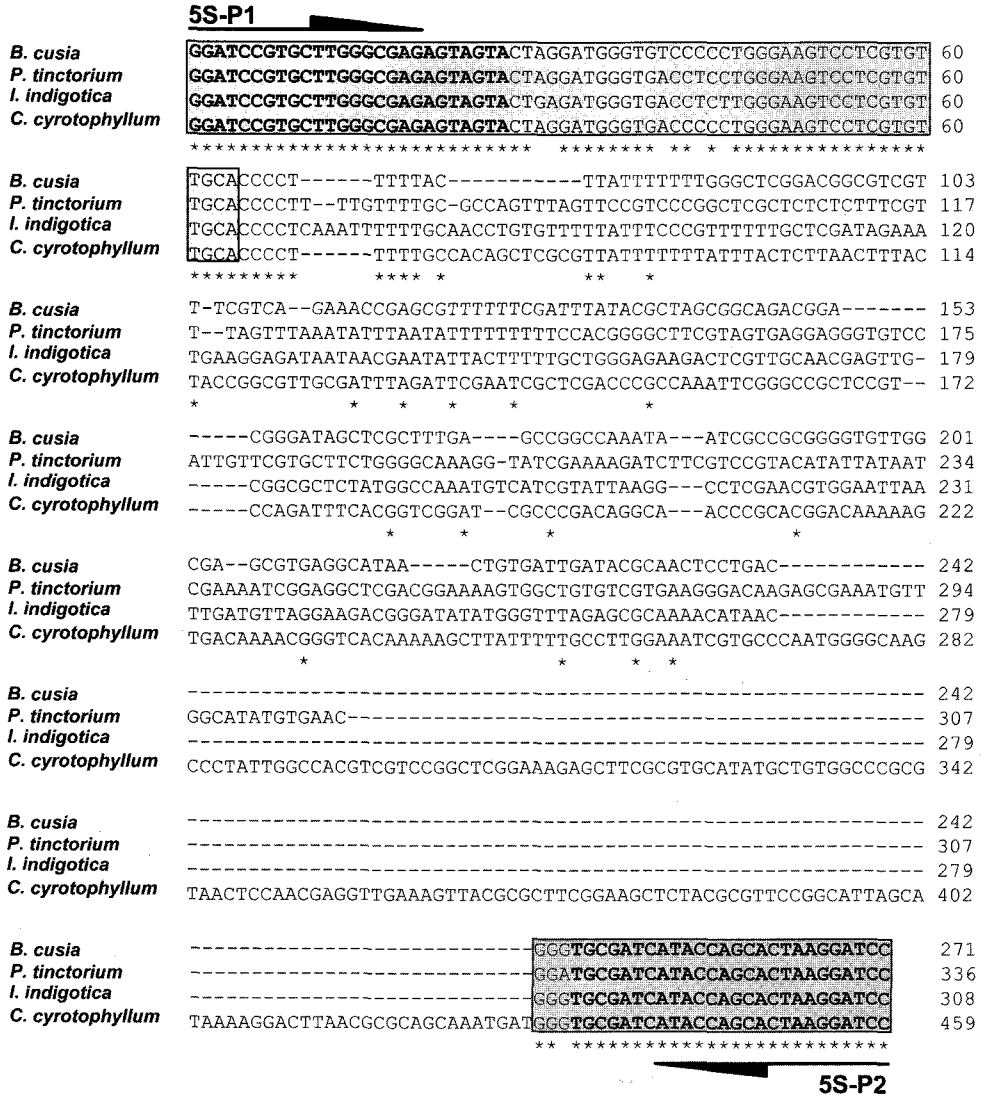

Fig. 2. Sequence analyses of the spacer regions of 5S-rRNA genes from *I. indigotica* (GenBank accession number AY078239), *P. tinctorium* (AY078240), *B. cusia* (AY078241) and *C. cyrtophyllum* (D63490). Alignment of the DNA sequences of 5S-rRNA spacer domains is shown. The coding regions are boxed. Primers (5S-P1 and 5S-P2) used for amplification were indicated by arrow. Identical sequences are indicated by (*). Gaps (-) are introduced for the best alignment.

C. cyrtophyllum. In *I. indigotica*, *P. tinctorium* and *B. cusia*, PCR products having ~300 bp were detected in agarose electrophoresis, while a ~450 bp PCR product was revealed in *C. cyrtophyllum* (Fig. 1A). Both fresh leaf and dried herb showed identical PCR product. Over 10 individual plants from the same species were analyzed. The PCR products were subcloned and sequenced three times in both directions. Several individual clones of the same PCR product were sequenced to avoid any mutation introduced by *Taq* polymerase. The respective lengths of the 5S-rRNA spacer domains in different species are: *I. indigotica* 215 bp, *P. tinctorium* 243 bp, *B. cusia* 178 bp and *C. cyrtophyllum* 366 bp. By DNA sequence search, the predicted restriction map of 5S-rRNA spacer domains after digestion of different restriction enzymes was revealed. The application of Bgl II, Dra I, Hind III, Msc I, Nhe I, Sac II and Ssp I restriction enzymes, therefore, provided a rapid method for identification of the four tested species (Fig. 1B).

A consensus sequence was obtained from the alignment of different individual clones of the same species. Identical sequences were obtained when the amplification was done with DNA isolated from either fresh leaf or dried herb of the same species (data not shown). Each species was found to have a unique DNA sequence in the 5S-rRNA spacer domain, which could be easily discriminated (Fig. 2). The spacer domains of these 4 species are phylogenetically distinct as determined by neighbor-joining analysis. The highest degree of identity, only about 28%, was found between *I. indigotica* and *P. tinctorium*. In contrast, the greatest diversity of the space domain sequence was found between *C. cyrtophyllum* and other species.

Additionally, RAPD analysis was carried out to determine the genetic variation among different members of Dyers woad leaf. Two 10-base oligonucleotide primers (A2 and Z8) were used in RAPD to screen the genetic distinctions of *I. indigotica*, *P. tinctorium*, *B. cusia* and *C. cyrtophyllum*. Primer A2 generated unique RAPD patterns for each species (Fig. 3A). These characteristics included: two clear bands at ~450 bp and ~550 bp for *B. cusia*, a single band at ~500 bp for *P. tinctorium*, three bands at ~300 bp, ~750 bp, ~1500 bp for *I. Indigotica* and two bands at ~950 bp, ~1250 bp for *C. cyrtophyllum*. On the hand, the primer Z8 also gave unique RAPD patterns for different species of Dyers woad leaf (Fig. 3B). These characteristics included: three bands at ~730 bp, ~1100 bp and ~1500 bp for *B. cusia*, a single band at ~400 bp for *P. tinctorium*, three bands at ~300 bp, ~500 bp, ~850 bp for *I. Indigotica* and three bands at ~450 bp, ~750

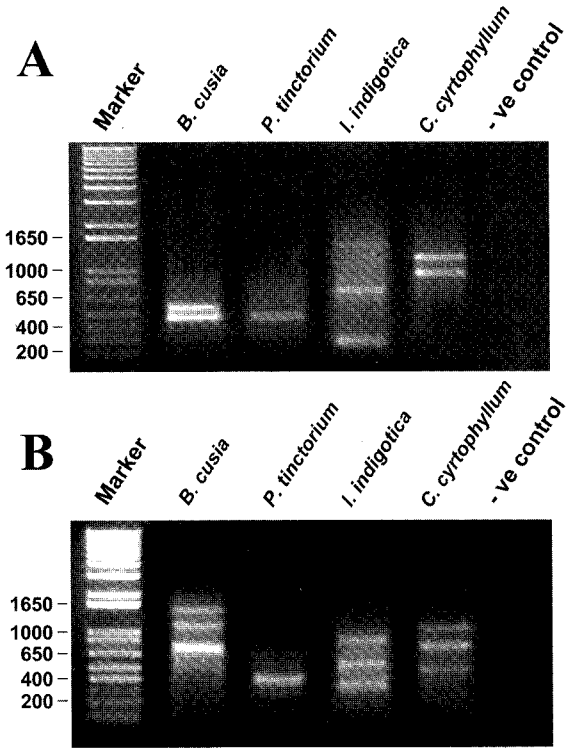

Fig. 3. Random amplification of polymorphic DNA (RAPD) of *I. indigotica*, *P. tinctorium*, *B. cusia* and *C. cyrtophyllum*. Two 10-base oligonucleotides A2 (**A**) and Z8 (**B**) were indicated. The PCR products were separated by a 1.2% agarose gel and visualized by ethidium bromide staining. The PCR products having the same size were obtained from five individual samples (N = 5). Same PCR products were obtained from either crude drugs or fresh materials (data not shown). The negative control is the one without genomic DNA. DNA markers in bp are indicated.

bp, ~1100 bp for *C. cyrtophyllum*. Although A2 and Z8 primers could able to distinguish different members of Dyers woad leaf as described here, other primers as described in Cui *et al.* (2003) did not able to show such a distinction (data not shown). The interpretation by using only RAPD analysis therefore could be misleading by using a pair of primers that is commonly flanking the same fragment between the genomic DNAs. Indeed, this is a common limitation of using RAPD instead of sequence analysis. Having RAPD and 5S-rRNA spacer sequence together, our results clearly indicate the genetic distinction of these species.

Although China Pharmacopoeia (2005) recorded officially that Dyers woad leaf should be derived from leaf of *I. Indigotica*, leaves from *P. tinctorium*, *B. cusia* and *C. cyrtophyllum* are commonly used as substitutes in different regions of China. *P. tinctorium*, a member of Polygonaceae, is also listed as another species of Dyers woad leaf but named as Liaodaqingye in Chinese Pharmacopoeia (Zheng, 2005). *B. cusia*, a member of Acanthaceae, is commonly used as Dyers woad leaf in Hong Kong, Guangdong, Guangxi and Fujian. *C. cyrtophyllum* is a member of Verbanaceae and being used in Jiangxi and Hunan as the substitute. This problem of different plant species being used as the same herb, therefore, jeopardizes the clinical efficacy and reliability of the herbal medicine. There are two possible reasons why these four unrelated species are being used: (i) the leaves from these four plants show a very close clinical efficacy throughout the history; and (ii) the blue pigment, usually used as indicator of Dyers woad leaf, is found in the four species, which subsequently leads to the inappropriate usage of the plant species. Besides the aforementioned four species, other plants also carry the name of Dyers woad leaf, which included the leaves from *Isatis tinctoria* L., *Idigofera suffruiticosa* Mill. and *Idigofera tinctoria* L.. Although these plants are more phylogenetically related to *I. Indigotica*, these species are rarely found on the herbal market as substitutes, and they are now being used mainly for the large scale purification of indigo.

Not only the morphological appearance of leaves from *I. indigotica*, *P. tinctorium*, *B. cusia* and *C. cyrtophyllum* show a close resemblance, the chemical composition of these leaves also shows some degree of similarity. Indirubin, indigo and tryptanthrin are considered to be the chemical and pharmacological markers of Dyers woad leaf. In aforementioned four species, they all contained indirubin, indigo and tryptanthrin; they are different only in their absolute amounts of such chemicals. However, individual species also processes distinct chemicals. *I. indigotica* contains 2,4 (1*H*, 3*H*)-quinazolinedione, qingdainone, isatin, indican, *n*-nonacosane (Gilbert *et al.*, 2000); *P. tinctorium* contains N-phenyl-2-naphthylamine, indican, kaempferol, gallic acid, caffeic acid (Kimoto *et al.*, 1999; Kataoka *et al.*, 2001); *B. cusia* contains isoindigo, 2,4 (1*H*, 3*H*)-quinazolinedione, 4 (3*H*)-quinazolinone, lupeol, betulin (Li *et al.*, 1993); and *C. cyrtophyllum* contains cyrtophyllin, 4 (3*H*)-quinazolinone (Xiao, 2002). Unfortunately, these chemicals could not serve as markers for authentication because none of them could chemically differentiate the identity of the herb. Besides, these chemicals might change according to the growth environment.

The biological functions of these chemical markers within Dyers woad leaf have been described. Indirubin showed anti-leukemic activity and inhibited leukemia L_{7212}, Lewis lung carcinoma and Walker carcinosarcoma 256 in rats (Zheng *et al.*, 1997) as well as its anti-leukemia activity in human (Gan *et al.*, 1985). Indigo can also be used to resist the fungal and viral infections (Xiao, 2002), and tryptanthrin has cytotoxic effects on malignant tumor cells (Kimoto *et al.*, 1999; Kimoto *et al.*, 2001). The content of indirubin and indigo in Dyers woad leaf is ranked as *P. tinctorium* > *I. indigotica* > *B. cusia* for indirubin (Deng, 1986) and *P. tinctorium* > *B. cusia* > *I. indigotica* in term of their indigo content (Lou and Qin, 1995). In the pharmacological actions of anti-bacterial, anti-pyretic and anti-viral agents, *I. indigotica* is more effective than *P.*

tinctorium, *B. cusia* and *C. cyrtophyllum*; however, only *P. tinctorium* showed activity resisting to influenza virus A (Ma and Xia, 2000). On the other hand, *P. tinctorium* has the protective effect on ferric nitrilotriacetate-induced renal damage and can improve hyperlipidemia induced by a high fat diet (Kimoto *et al.*, 1999; Inoue *et al.*, 2000).

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