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Assessing product adulteration in selected Malaysian herbal product using DNA barcoding

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Abstract

In the local Malaysian context, herbal plants such as *Eurycoma longifolia* (Tongkat Ali) and *Ficus deltoidea* (Mas Cotek) are known and widely used for its therapeutic properties. The raw material used as active ingredients is almost exclusively sourced from wild populations. Consequently, it is widely suspected that the commercial herbal products claiming to contain these species may be adulterated or contaminated. In this study, we have attempted to assess product authentication and the extent of adulteration using DNA barcoding. Amplification of these three markers (ITS2, rbcL and psbA-trnH) was successful in leaves and root (positive control). The barcode sequence were identified based on top matches in the GenBank and BOLD. However, in terms of the herbal products, only the ITS2 marker had been successfully amplified in all sample tested compare to rbcL and psbA-trnH marker. Out of the 10 herbal products examined, DNA was not amplified from 2 herbal products sample. The present study indicates that all the Ficus deltoidea herbal products sold in Malaysian market were 100% authentic based on ITS2 marker while in terms of Eurycoma longifolia herbal products, all were mislabelled. DNA barcodes such as that demonstrated in this study could be effectively used as a regulatory tool to control the adulteration of herbal products and contribute to restoring quality assurance and consumer confidence in natural health products

Keywords: ITS2, rbcL, psbA-trnH marker, authentication, DNA Barcode, *Eurycoma longifolia*, *Ficus deltoidea*

INTRODUCTION

There have been a general increased in the consumption of herbal product globally due to their claimed health benefits. This tremendous increase in the demand rate of these herbal products resulted in a massive development of economy in both developed and developing countries for the past decades. The World Health Organization (WHO) estimated that 5.6 billion people, approximately 65-85% of the world population currently use herbal products as remedies for primary care purposes (Azmin et al., 2016). Malaysia is not an exception in the use of all these forms of herbal products which are inform of capsule, tablet or tea. Malaysian National News Agency, BERNAMA report stated that the annual sales of traditional medicine from 2000-2005 increases tremendously from US$385 million (RM1 billion) to US$1.29 billion (RM45 billion) (Azmin et al., 2016) and this is expected to grow subsequently after each year.

However, along with the massive increase in the growth of herbal products in the market, the adulteration of the medicinal plant is also growing rapidly and these serve as the major challenge in the international trade (Seethapathy et al., 2014). Such kind of challenges includes product contamination, substitution, the used of unlabelled fillers and these types of adulterations is always common because the original morphological characteristics are

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absent (Wallace et al., 2012). Although some herbal medicines have promising potential and widely used, many of them remained untested and not monitored. This makes knowledge of their adverse effect and toxicity very limited and therefore compromised their safety and efficacy.

Authentication at the DNA level is more reliable when compared to the other macromolecules such as RNA and protein which are less stable and easily affected by external factors (Mishra et al., 2016). So, there is a potential need for the development of robust DNA based markers for plant identification and authentication at commercial level or DNA barcoding technique (Sucher and Carles, 2008). DNA barcoding is a useful methods which use short DNA sequence in order to identify and discriminate species as well as authenticating the herbal products (Jian et al., 2014). This type of technique is broadly assessable, accurate, stable and generally used to detects the level adulteration in different herbal product (Newmaster et al., 2013).

In Malaysia, *Eurycoma longifolia* known as Tongkat Ali and *Ficus deltoidea* known as Mas Cotek are widely used for its therapeutics properties. *Eurycoma longifolia* is a common tropical herbal medicinal plants which belongs to the family *Simaroubaceae* and it is distributed in several parts of southern Asia including Malaysia, Indonesia, Vietnam, and certain regions in Myanmar, Thailand and Cambodia (Bhat and Karim, 2010). *Eurycoma longifolia* is well known for its aphrodisiac properties which is mostly contributed as a result of it testosterone enhancing effects (Mohamed et al., 2015). Other pharmacological effects which this plant has been reported to exhibits includes anti-parasitic effects (Kavitha et al., 2012), anti-bacterial and anti-fungal (Farouk and Benafri, 2007) and anti-cancer effect (Kuo *et al.*, 2004).

Ficus deltoidea belongs to the family *Moraceae* which is known as Mistletoe in English. It is native and widely distributed throughout peninsular Malaysia, Thailand and Philippines (Bhore and Shah, 2009). Mas Cotek as commonly known in Malaysia is used for the treatment of several types of ailments such as diabetes, high blood pressure, heart problem, rheumatism, diarrheoa and pneumonia (Abdullah et al., 2009). Several studies have confirmed that this plant species has anti-hyperglycemic, anti-diabetic, antioxidant, anti-melanogenic, anti-photo aging, anti-inflammatory and anti-nociceptive properties (Bunawan et al., 2014).

In Malaysia, these plants species are commercialize, distributed and formulated as capsules, tea and tonic tea (Fatihah et al., 2012, Vejayan et al., 2013). Despite the fact that there is a high demand of herbal products of these plants in Malaysia, there are no studies of DNA barcoding used for authentification. Therefore the aim of our study was to identify the best DNA marker (*rbcL*, *psbA-trnH* or *ITS2*) and to assess its versatility in authenticate of herbal products from *Eurycoma longifolia* and *Ficus deltoidea*.

MATERIALS AND METHODS

Collection of Plant and Herbal Product

Ficus deltoidea plants were obtained from Pak Ali garden near Johor resort, Johor while for *Eurycoma longifolia* was obtained from Forest Research Institute Malaysia (FRIM), Selangor. Their different types of herbal products used in this study were purchased from several a local over the counter (OTC) at retail shop within Johor Bahru, Johor, Malaysia.

DNA Extraction and Amplification

Genomic DNA was extracted from the leaves of *Ficus deltoidea* and roots of *Eurycoma longifolia* using Cetyltrimethyl ammonium bromide (CTAB) method (Doyle and Doyle, 1987) with minor modifications. Approximately, 0.1g of the tissue were homogenised into fine powder using liquid nitrogen. Then powdered tissue was transferred into 2mL microcentrifuge tube and mixed with 700µL of pre warm extraction buffer at 65^{o}C (100mM Tris HCl, pH 8.0; 20mM Na_2 EDTA; 2% (w/v) CTAB; 1.4M NaCl) along with 2% (v/v) β-mercaptoethanol and 2% (w/v) of Polyvinylpyrrolidone (PVP). The mixture were thoroughly mixed and incubated at 65^{o}C for one hour with intermittent shaking after each 10 minutes.

After incubation, 700µL of chloroform: isoamyl alcohol (Ch: Iaa) (24:1 v/v) was added and mixed gently by inverting the tubes until two phase formed an emulsion and centrifuge at 12,000rpm for 10 minutes. The aqueous phase was carefully transferred into another microcentrifuge tube and the process was repeated with equal volume of Ch: Iaa and centrifuging at the same rate. Finally double volume of chilled isopropanol was added and the microcentrifuge were incubates for another one hour in -20°C and later centrifuge centrifugation at 12,000rpm for 10 min. Then the supernatant was discarded and the pellet was collected. The pellets was further washed twice with 70% (v/v) ethanol and centrifuged at 12,000rpm for 5min. After discarding the supernatant, the pellet was dried at 37°C and the pellet was re-suspended in 100µL of TE buffer (10mMTris–HCl, pH 8.0; 1mM Na_2 EDTA; 1.0M NaCl). The RNA was removed by incubating the re-suspended pellet in RNase at 37°C for 30 min.

The quality of the DNA extracted was analyzed using spectrophotometric nanodrop and quantitatively visualize on 1 % (w/v) agarose gel electrophoresis and photographed. The three universal primers used for the amplification of the barcode regions, namely *rbcL*-F (3'CTT GGC AGC ATT CCG AGT A 2'), *rbcL*-R (3'TCA CAA GCA GCA GCC AGT TC2') (Hamdan *et al.,* 2013), *ITS2* - F (3'GGG GCG GAT ATT GGC CTC CCG TGC 2'), ITS2-R (3'GAC GCT TCT CCA GAC TAC AAT2') (Chen *et al.*, 2010), *psbA3*_F (GTT 3' ATG CAT GAA CGT AAT GCT C 2'), *trnHf*_R (3'CGC GCA TGG TGG ATT CAC AAA TC 2') (Shaw *et al.*, 2005). The component of the PCR reaction were optimized and performed in 25µL reaction in a PCR machine (Eppendorf). The 25µl PCR reaction mixture consists of 1µl template DNA (20-100ng/µl), 5µl PCR buffer at 5X, 2µl of $MgCl_2$ (25mM), 0.4µl dNTPs mix (10mM) (Promega), 1µl of each forward and reverse primers (10mM), 0.125µl of Taq DNA polymerase at (5U/µl) (Promega), and the volume made up to 25µl with sterile distilled water.

The total genomic DNA from the leaves of *Ficus deltoidea* was amplified using thermocycler program for *rbcL, ITS2* and *psbA-trnH* regions set at 95°C for 2 min, followed by 35 cycles of 95°C for 1min, 45°C for 1min, and 72°C for 2 min with a final extension step at 72°C for 5 min. For the root of *Eurycoma longifolia,* for *rbcL* and *ITS2* set at 95°C for 2 min, followed by 35 cycles of 95°C for 1min, 49.4°C (at 55.9 for *psbA-trnH*) for 1min, and 72°C for 2 min with a final extension step at 72°C for 5 min. The PCR product was then purified and sequenced in First Base Sdn Bhd, Malaysia.

DNA Barcode Sequence Analysis

All amplified sequences results obtained were manually edited using BioEdit software and used as query sequence in Basic Local Alignment Search Tool (BLAST) algorithm (Altschul et al., 1997) for identification of the closest matched sequence in the GenBank nucleotide database. The generated sequences from the reference sample for *rbcL, psbA-trnH* and *ITS2* regions were subsequently used to identify the level of adulteration in the herbal products. All sequences were analysed using the BLAST algorithm search tool in the GenBank in order to assess the concordance or authenticity of label information. A top match (highest percentage similarities) of 97% was used to designate potential identification (Newmaster et al., 2013). Three definition terms according to Newmaster et al., (2013) such as authentic, contamination and substitution were used to assess the identity of the DNA barcodes found for the unknown species. When the DNA barcode for a species which is the main ingredient labelled on the herbal products (tested) is found, the products are said to be authentic. Thus, any sample that generates a barcode other than what is labelled on the tested herbal product are said to be substitution. Contamination is when the DNA barcodes is found for a species other than what is labelled in addition to the authentic barcode.

RESULTS AND DISCUSSION

The yield and quality of the genomic DNA extracted was assessed using spectrophotometry. The result obtained shows that genomic DNA of high quality was extracted from both plant species. The yield of the genomic DNA extracted from *Ficus deltoidea* leaves and *Eurycoma longifolia* roots was 412.1 and 350.4 ng/µl respectively.

However, in terms of purity, A_{260}/A_{280} absorbance ratio for *Ficus deltoidea* leaves and *Eurycoma longifolia* roots was 1.91 and 1.87 respectively. A_{260}/A_{280} absorbance ratio ranging between 1.8-2.0 indicates high level of purity (Moyo et al., 2008). The result indicates that the genomic DNA extracted from both plants was free from contamination as they are above the optimal range. The quality and integrity of the DNA was also high when analysed on 1% (w/v) agarose gel electrophoresis (Figure 1).

Out of the 10 herbal product samples of the *Ficus deltoidea* and *Eurycoma longifolia* herbal products examined, amplifiable DNA could be extracted from 8 samples (80%). Most herbal materials containing cells such as nucleic acid (DNA) are removed during extraction process and if they should remain in the botanical extract, they will occur in low quality and concentration as the original long region will be fragmented into short (Reynaud et al., 2015). Exposure to heat or drying process under sun has been reported to affect the quality of genomic DNA (Särkinen et al., 2012). Apart from different factors which interfere with the PCR reaction, amplification failure in some of the herbal products can be as a result of degradation at the primer site which is common with potentially degraded DNA (Wallace et al., 2012, Newmaster et al., 2013).

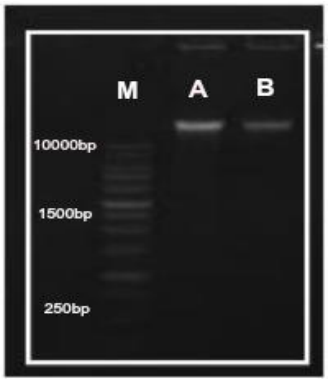

Figure 1. Total genomic DNA extracted from *Ficus deltoidea* leaf (A) and *Eurycoma longifolia* root (B) using CTAB method on 1% (w/v) agarose gel electrophoresis. Lane M: 1kb DNA ladder (Promega).

Based on Figure 2, the *rbcL, ITS2* and *psbA-trnH* regions were successfully amplified producing a single fragment from the genomic DNA in both plant (positive control). The expected size of specific amplified PCR fragment of *rbcL, ITS2* and *psbA-trnH* were approximately 1430, 160-320 and 330-630bp respectively. All the barcodes generated from these three regions were of high quality as the peaks from the chromatogram were less noisy and none overlapping.

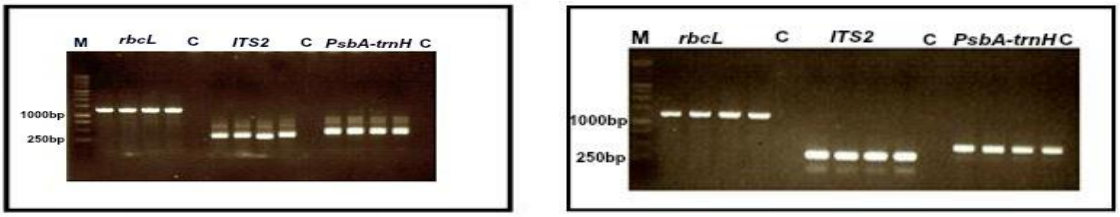

Figure 2. PCR products amplified from leaves of *Ficus deltoidea* (left) and root of *Eurycoma longifolia* (right) with *rbcL, ITS2* and *psbA-TrnH* primers respectively on 1% (w/v) agarose gel electrophoresis. Lane M: 1kb DNA ladder (Promega), C: Negative Control.

Even though the *rbcL* and *psbA-trnH* regions have been amplified from genomic DNA of *Ficus deltoidea* plant (Figure 2 left) and *Eurycoma longifolia* (Figure 2 right); however only ITS2 region of *Ficus deltoidea* and *Eurycoma longifolia* herbal products were successfully amplified. The *ITS2* region is a short region which is located within the nuclear *ITS2* region and is made up of about 160-320bp (Chen et al., 2010) Shorter target regions are easily to be

amplified than longer regions in degraded DNA sample especially when the amplicon size is greater than 200bp (Little and Jeanson, 2013) and this might be as a result of severe fragmentation of the DNA. The unsuccessful amplification of *rbcL* and *psbA-trnH* region could be as a result of fragmentation of the DNA which occurs during the processing of the plant herbal material. Large size region are always difficult to be amplified in fragmented or degraded DNA because the barcode region are larger than the average fragment size in DNA extract of the herbal product to be amplified (Little and Jeanson, 2013). These results are also in conformity to what was reported by Sarkinen et al., (2012). They found that there is a negative correlation between PCR success and amplicon size, which shows that amplification of shorter fragment from herbarium DNA are easier when compared to the long fragments. Amplifiable DNA could not be extracted from two types of the herbal products (20%) Which could be attributed due to the presence of inhibitors compounds which can interferes with the amplification process or the amplifiable DNA has been degraded during the processing progress.

As research on the authentication of herbal product in Malaysia using DNA barcoding is new, the information on the field is limited. Out of the total number of herbal products tested based on the top matched in the GenBank, 4 herbal products from *Ficus deltoidea* were found to be correctly labelled (authentic) as they contained the DNA barcode for the species which serve as the main ingredient labelled on the tested product. On the other side, the remaining 4 herbal product from *Eurycoma longifolia* were substituted with other species. According to NCBI engine search, DNA barcoding revealed that the herbal product were from *Holoptele integrifolia*, *Clerodendrum cyrtophyllum*, *Aradichrachta indica*, *Brucia javanica* and *Ficus stenophylla* and this is contrary to what was labelled on the tested product of *Eurycoma longifolia* (Table 1). The result from this finding is flabbergasting as this is coming at a time that the rate of consumption of herbal product in Malaysia is increasing tremendously together with an increase in the establishment of herbal product industries. The expected growth of the herbal product industries in Malaysia is between 15-20% per year (Azmin et al., 2016).

Table 1. DNA barcodes results for the tested individuals herbal product samples.

Sample Code (Product Type)	Sequences/Length of product	Market Label	Top Similarities To The Sequence from NCBI	Genbank Accession Number	Barcode ID
Sample 001 (Tea)	rbcL/NS	*Ficus deltoidea*	-	-	Ficus deltoidea
	ITS2/306	*Ficus deltoidea*	98%	HM159448.1	
	psbA-trnH/NS	*Ficus deltoidea*	-	-	
Sample 002 (Tea)	RbcL/NS	*Ficus deltoidea*	-	-	Ficus deltoidea
	ITS2/305	*Ficus deltoidea*	99%	HM159453.1	
	psbA-trnH/NS	*Ficus deltoidea*	-	-	
Sample 003 (Capsule)	rbcL/NS	*Ficus deltoidea*	-	-	Ficus deltoidea
	ITS2/307	*Ficus deltoidea*	99%	HM159449.1	
	psbA-trnH/NS	*Ficus deltoidea*	-	-	
Sample 004 (Capsule)	rbcL/NS	*Ficus deltoidea*	-	-	Ficus deltoidea
	ITS2/343	*Ficus deltoidea*	98%	HM159447.1	
	psbA-trnH/NS	*Ficus deltoidea*	-	-	
Sample 005 (Capsule)	rbcL/NS	*E. longifolia*	-	-	Holoptele Integrifolia + Clerodendrumc yrtophyllum
	ITS2/341	*E. longifolia*	97%	KC896699.1 +JX856576.1	
	psbA-trnH/NS	*E. longifolia*	-	-	
Sample 006 (Tea)	rbcL/NS	*E. longifolia*	-		Ficus stenophylla
	ITS2/344	*E. longifolia*	97%	EU091640.1	
	psbA-trnH/NS	*E. longifolia*	-	-	

Sample 007 (capsule)	rbcL/NS	*E. longifolia*	-	-	Aradichrachta indica +Brucia javanica
	ITS2/347	*E. longifolia*	86%	JX856538.1	
	psbA-trnH/NS	*E. longifolia*	-	-	
Sample 008 (capsule)	rbcL/NS	*E. longifolia*	-	-	Ficus carica
	ITS2/341	*E. longifolia*	97%	KF454312.1	
	psbA-trnH/NS	*E. longifolia*	-	-	

≠NS= No Sequence

CONCLUSIONS

The following conclusions can be drawn from the study:

- The study shows that all the four *Ficus deltoidea* herbal product based on top matched in the Genbank are authentic as they have more than 97% similarities. For the *Eurycoma longifolia* herbal product, they are substituted as the barcode for the main ingredient on the label of the herbal product is absent.
- This study strongly suggests that some Malaysian herbal products are adulterated and therefore there is a need for them to be properly authenticated before used. DNA barcoding and other molecular diagnostic tools can be effectively used in identification of medicinal plants.
- Our study suggests that inadvertent or intentional adulteration/ substitution is rampant in the herbal industry.

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