DNA BARCODING AND CHEMICAL ANALYSIS FOR EVALUATION OF AUTHENTICITY OF SELECTED HERBAL MEDICINAL PRODUCTS IN MALAYSIA

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This Thesis is dedicated to my beloved late MUM; May the Almighty Allah forgive her shortcomings and makes Aljannatul Firdaus her final abode.

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ABSTRACT

The increasingly demand of *Ficus deltoidea* (Mas Cotek) and *Eurycoma longifolia* (Tongkat Ali) medicinal plants due to antidiabetic and aphrosidiac therapeutic properties respectively have resulted in an increase in demand for their herbal medicinal products (HMPs) in Malaysia. The safety and efficacy of such HMPs which are in the form of tea, capsules or tablets relies on the authenticity of the plant raw material used as most of them are exclusively sourced from wild population. Consequently, commercial HMPs claiming to be authentic may be adulterated or contaminated with other plants species. Current identification methods such as organoleptic, microscopic and macroscopic examinations cannot identify plant species in a processed herbal product form due to lacking of indicative morphological features and plants taxonomy. Evaluation of DNA analysis such as the direct DNA sequence analysis (BLASTn and Neighbour-Joining) of the chloroplastic (*rbc*L and *psb*A-*trn*H) and nuclear (ITS2) regions for rapid detection of *F. deltoidea* and *E. longifolia* was evaluated. A standard reference materials (SRM) from all barcode regions were successfully cloned into p-Easy-T3. The sequence analysis of these 3 barcode regions showed that same species or closely related species shared high percentage of DNA identity. The phylogenetic analysis showed that *rbc*L had high discriminatory power in *F. deltoidea* var. kunstleri and ITS2 for *E. longifolia.* Identification of *F. deltoidea* HMPs using DNA barcoding showed that 67% were authentic, 11% were substituted while the identity of 22% could not be conclusively determined. For *E.longifolia* HMPs, 36% of the tested HMPs were authentic, 27% were substituted and the identity of 37% could not be conclusively determined. Further analysis using High Performance Liquid Chromatography (HPLC) revealed that 100% of the tested *F. deltoidea* HMPs were authentic as all samples contained the expected vitexin marker. This include 14% of tested HMPs identified as substitutions using DNA barcoding. Eurycomanone was detected in 43% of *E. longifolia* HMPs but at lower concentration than root extract In contrast, 14% of the tested HMPs which were authentic using DNA barcoding were found not to contain the expected marker. The ITS2 proved to be the ideal marker for the authentication of both plants HMPs. Using DNA barcoding, the overall study showed that there was fraudulence activities occurred in the HMPs tested. Even though HPLC of herbal compounds require different protocol and different standards for each biomarker, DNA sequence methods were the same for all HMPs. Hence, DNA barcoding should be utilised in authenticating other HMPs available in the Malaysia market.

ABSTRAK

Tumbuhan herba Mas Cotek (*Ficus deltoidea*) dan Tongkat Ali (*Eurycoma longifolia*) merupahan tumbuhan perubatan popular yang masing-masing mempunyai terapeutik berkaitan diabetik dan aprodisiak. Ini menyebabkan permintaan yang tinggi terhadap produk herba di Malaysia. Walau bagaimanapun, tahap keselamatan dan keberkesanan produk herba dalam bentuk teh, kapsul atau tablet bergantung kepada pencirian tepat bahan mentah tumbuhan tepat dari kawasan hutan. Akibatnya, keaslian produk herba komersial diragui kerana mungkin telah dicemari oleh bendasing. Kaedah pencirian terkini seperti organoleptik, mikroskopik dan pemeriksaan makroskopik tidak boleh mengenalpasti jenis spesis di dalam produk dalam bentuk serbuk atau yang telah diproses kerana tiada maklumat ciri morfologi dan taksonomi tumbuhan. Penentuan dan pengenalpastian *F. deltoidea* dan *E. longifolia* dengan cepat melalui analisis DNA adalah satu strategi berdasarkan analisis BLASTn dan Penyambungan Jiran (NJ) dalam kawasan barkod kloroplastik (*rbc*L and *psb*A-*trn*H) dan nuklear (ITS2). Bahan rujukan piawai (SRM) telah berjaya dihasilkan dengan mengklonkan ke dalam p-Easy-T3 untuk semua kawasan barkod yang dikaji. Analisis jujukan DNA untuk 3 kawasan barkod menunjukkan peratusan identiti yang tinggi daripada spesis yang sama atau spesis yang terdekat. Analisis filogenetik menggunakan Penyambungan Jiran (NJ) menunjukkan *rbc*L mempunyai kuasa diskriminasi yang lebih tinggi dalam *F. deltoidea* var. Kunstleri manakala ITS2 untuk *E. longifolia*. Ujian pengenalpastian terhadap 9 sempel produk herba *F. deltoidea* menggunakan barkod DNA menunjukkan 67% adalah asli, 11% telah dicampur sementara identiti untuk 22% tidak dapat ditentukan secara menyeluruh. Untuk produk 11 berdasarkan herba *E.longifolia*, 36% daripada produk herba yang diuji adalah asli, 27% telah dicampur dan identiti untuk 37% tidak dapat ditentukan secara menyeluruh. Analisis Kromatografi Cecair Prestasi Tinggi (HPLC) menunjukhan bahawa semua (100%) produk herba *F. deltoidea* yang diuji adalah asli kerana mengandungi penanda viteksin yang ditetapkan. Ini termasuk 14% produk herba yang dikenalpasti sebagai penggantian menggunakan kaedah barkod DNA. Eurikomanon telah dikesan dalam 43% produk herba *E. longifolia* tetapi pada kepekatan yang lebih rendah daripada ekstrak akar. Walau bagaimanapun, 14% produk herba yang diuji dikenalpasti sebagai asli menggunakan barkod DNA tetapi didapati tidak mengandungi penanda yang dijangka. Barkod ITS2 terbukti penanda yang ideal untuk pengesahan produk herba daripada *F.deltoida* dan *E.longifolia* ini. Keseluruhan kajian menunjukkan terdapat ketidakpatuhan terhadap penggunaan bahan mentah tumbuhan yang betul dalam produk herba ini. Walaupun sebatian bioaktif herba untuk HPLC memerlukan protokol dan piawai molekular yang berbeza untuk setiap tumbuhan, kaedah jujukan DNA adalah sama untuk semua produk herba. Kesimpulannya, produk herba yang lain di Malaysia juga boleh dikenalpasti menggunakan kaedah barkod DNA.

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LIST OF ABBREVIATIONS

ANOVA	-	Analysis of Variance
BHMA	-	British Herbal Medicine Association
BLAST	-	Basic Local Alignment Search Tool
BOLD	-	Barcode of Life Data System
CBOL	-	Consortium for the Barcode of Life
CTAB	-	Cetryltrimethylammonium Bromide
DAD	-	Diode Array Detector
DNA	-	Deoxyribonucleic Acid
EDTA	-	Ethylenediaminetetraacetic Acid
ELSD	-	Evaporative Light Scattering Detector
FDA	-	Federal Drug Administration
FDB	-	Full Length DNA Barcodes
FRIM	-	Forest Research Institute Malaysia
GC	-	Gas Chromatography
GBIF	-	Global Biodiversity Information Facility
HMP	-	Herbal Medicinal Product
HPLC	-	High Performance Liquid Chromatography
HPLC-ELSD	-	Liquid Chromatography- Evaporative Light Scattering Detector
HPLC-PDA	-	Liquid Chromatography- Photo-Diode Array
HPLC-UV	-	Liquid Chromatography- Ultraviolet-Visible
ITS2	-	Internal Transcribed Spacer
LSC	-	Large Single Copy
LB	-	Luria Bertani
LOD	-	Limit of Detection

LOQ	-	Limit of Quantification
*Mat*K	-	Maturase K
MDB	-	Mini DNA Barcodes
MCP	-	Mas Cotek Product
MMDBD	-	Medicinal Material DNA Barcodes Database
MSA	-	Multiple Sequence Alignment
NCBI	-	National Centre For Bioinformatics Information
NJ	-	Neighbour Joining
NMR	-	Nuclear Magnetic Resonance
PCR	-	Polymerase Chain Reaction
PDA	-	Photodiode-Array
RE	-	Restriction Enzyme
*rbc*L	-	Ribulose bisphosphate carboxylase
SD	-	Standard Deviation
SDS	-	Sodium dodecyl sulfate,
SSC	-	Small Single Copy
SRM	-	Standard Reference Material
RI	-	Refractive Index
RSD	-	Relative Standard Deviation
TAC	-	Tongkat Ali Product
TAE	-	Tris Acetic EDTA
TE	-	Tris-EDTA,
TLC	-	Thin Layer Chromatography
WHO	-	World Health Organization

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CHAPTER 1

INTRODUCTION

1.1 Background of the Study

In the last two decades, there was a tremendous increase in the global use herbal medicinal products (HMP) due to their claimed health benefits, easily availability, perceived effectiveness and safety (Posadzki *et al.*, 2013; Shaw *et al.*, 2012). This tremendous increase in the demand of these HMP resulted in a massive increased in their trade. The HMP generally referred to any medicinal product exclusively contained different part of medicinal plants which includes roots, leaves, bark, stem or herbal preparation (extract) (Agbabiaka *et al.*, 2016).

It is estimated that 5.6 billion people, approximately 80% of world population rely on HMPs for their primary health care (Azmin *et al.*, 2016; Khan *et al.*, 2009). More than 90% of Africans as well as Asia with 70% and 40% of Indian and Chinese population, respectively, continue to rely on HMP for general health care (Avigan *et al.*, 2016). Despite the great advances achieved in the field of modern medicine, plants still play a significant role in health care. At least, 25% of drugs in modern pharmacopoeia are directly or indirectly derived from plants (Nyirimigabo *et al.*, 2015; Palhares *et al.*, 2015; Thatoi and Patra, 2011) and more than 60% of anti tumour drugs are generally derived from natural product (Brower, 2008).

The international trade of herbal medicinal products is becoming a lucrative business due to their high demand, the global market is estimated at US$83 billion (Palhares *et al.*, 2015) and is projected to reach US$115 billion by the year 2020 (Ahmad *et al.*, 2015). Malaysia is not an exception to the increase in demand of HMPs. The Malaysian market for HMPs is project to reach RM32 billion in 2020 with 8-15% annual growth rate (Zakaria, 2015). This is due to the fact Malaysia is blessed with diverse biodiversity of medicinal and flowering plants. Out of the approximately total number of 14,500 diverse flowering plant present in Malaysia, 2000 species have been reported to have medicinal properties and large numbers of them have been scientifically proven (Jaganath and Ng, 2000).

The presence of this diverse biodiversity of medicinal plants has made the government of Malaysia to believe that medicinal plants have the potential to propel and substained the national bioeconomic sector. In fact medicinal plants have been made into the first Entry Point Project (EPP1) for Malaysia Agriculture National Key Economic Area (NKEA). The NKEA agriculture EPP1 has many objectives which includes i) to achieve an increase of RM2.2 billion GNI by 2020, ii) to produce safe, high quality and efficacious high-end herbal products, iii) to strengthen the supplies across the value chain, and iv) to enhance research and development R&D in herbs (Fariazah, 2015). The projects also focused on the commercialisation of 11 medicinal herbs so as to improve their productivity. Among the recognised medicinal plants focused on this project, *Ficus deltoidea* and *Eurycoma longifolia* are among them and these have been widely used in the treatment of various type illness.

The *F. deltoidea* which is known as Mas Cotek is one of the potential medicinal plant in Malaysia that is used for the treatment of various ailments (Misbah *et al.*, 2013). Several important bioactive compounds such as vitexin, isovitexin (Choo *et al.*, 2012), flavonoid and proanthocyanidins (Omar *et al.*, 2011) have been reported to be present in *F. deltoidea* based on experimental evidence. Apart from using this plants to tradionally treat ailments such as headache, toothache etc, the most pronounce properties of *F. deltoidea* based on ethno botanical approach is it claim to treat diabetes (Adam *et al.*, 2012).

Other properties of *F. deltoidea* plants with scientific evidence includes anti-inflammatory (Adam *et al.*, 2012), anti-bacterial (Uyub *et al.*, 2010), anti-cancer (Akhir *et al.*, 2011) and anti-diabetic property (Adam *et al.*, 2011). The well known anti-diabetic property can result in the increase in patronage of *F. deltoidea* HMP in Malaysia. The prevalence rate of diabetes mellitus in Malaysia keeps rising among adults of 30 years and above by more than 2 folds over the last 2 decades (Minhat and Hamedon, 2014).

In terms of *E. longifolia* locally known as Tongkat Ali is one of the most popular medicinal plants in Malaysia that is traditionally used for the treatment of various ailments such as high blood pressure, persistence fever and glandular swelling (Bhat and Karim, 2010). The roots of this plant are the most used part for the treatment of such ailments. Several studies with proven scientific evidence have reported different pharmacological properties of this plant, including aphrodisiac effects effects (Ismail *et al.*, 2012; Zanoli *et al.*, 2009), anti-diabetic, anti cancer (Tee and Azimahtol, 2005; Tee *et al.*, 2007), anti inflammatory (Varghese *et al.*, 2013), anti-malarial (Yusuf *et al.*, 2013). Both *E. longifolia* and *F. deltoidea* HMP products exist either in the form of capsule, tea, drinks or tablets.

However, the increased in popularity and use of HMP is becoming an issue of concern as they are not rigorously regulated by Drug Control Authority (DCA) in Malaysia (Aziz and Tey, 2009). The wide usage of the HMP in Malaysia with a population reaching 28 million (Silvanathan and Low, 2015) is becoming relatively high (Hasan *et al.*, 2009). This is mostly patronized among middle-age individuals so as to improve their health being and sexual libido (Hassali *et al.*, 2012). It was also reported that about 69.4% of the Malaysian population use HMP in their whole life time (Mitha *et al.*, 2013). As a result of these tremendous increase in demand of the HMP together with the presence of diverse medicinal and flowering plant species in Malaysia the market value is projected to reach RM32 billion in 2020 with annual growth of 8 to 15 (Zakaria, 2015).

Despites the wide usage of HMPs globally, their efficacy and safety depends on the authenticity of the raw material as most of them suffered from various types of adulteration such as substitution, contamination or the use of fillers (Techen *et al.*, 2014). Adulteration of HMP can be detrimental to the health condition of the consumers. According to World Health Organization (WHO), substitution or adulteration of HMP of any kind is not only fraud but a threat to the health of consumers as this might result in adverse effects and eventually death (Newmaster *et al.*, 2013). Despite all the advocacy by the WHO regarding the importance of maintaining authentic raw material for the production of HMP, several studies (Han *et al.*, 2016; Little and Jeanson, 2013; Parvathy *et al.*, 2015; Vassou *et al.*, 2015; Wallace *et al.*, 2012) have shown that majority of the HMP present in the market are adulterated.

The basic plant diagnostic morphological features in which Linnaean taxonomic system is based cannot identify related medicinal species in cut, processed or modified products form such as powder, tablets and capsule (Newmaster *et al.*, 2013) as this may be difficult to differentiates. However, this prompted to the introduction of a novel technique which utilises short DNA sequence from a standard genome for species identification. This technique is termed DNA barcoding and it was first coined by Hebert *et al.*, (2003) in which it is cost effective, reliable and can identify species even in processed product form. This is due to the fact that this technique utilizes short standardized genes sequences which are easily retrievable.

Contrary to the other types of identification methods, DNA barcoding is more reliable and consistent in the authentication as DNA is stable and is not influenced by age of the of the sample, environmental factors, physiological condition, growth condition, harvesting period, cultivation area, plant part either leaf, stem or root, storage sample condition and it is found in all tissue samples (Techen *et al.*, 2014). Therefore, combining the DNA barcoding noble technique together with high performance liquid chromatography (HPLC) which is one of the most popular, versatile, widely accepted chromatographic fingerprint technique used for the analysis of HMP (Weon *et al.*, 2012) will help to validate the authenticity of *E.*

longifolia and *F. deltoidea* HMP sold in the Malaysian market. Detailed knowledge about the main composition of the HMPs will help to ascertain their efficacy and safety as these are very important towards quality control of product in the market.

1.2 Problem Statement

Currently, there is a complete absence or extreme scarcity in understanding the extent in which Malaysian HMP have been substituted or contaminated even though it is believed to have a wide range of remedies against different type of infection. However, even though the efficacy and safety of these HMPs solely relied on the correct usage of the authentic plant material and to date, there is no standard protocol for the authentication of Malaysian HMP. Therefore, the use of DNA barcoding can serve as a quick and highly reliable tool for species identification but due to the lack of standard reference material (SRM) and poor DNA yield from plant material for PCR amplification of the DNA barcode region, the application is less successful. Therefore, the use of DNA barcoding for the authentication of *F. deltoidea* and *E. longifolia* HMP in conjunction to HPLC chromatographic fingerprint analysis could serve be an added advantage to check the quality these HMPs sold in the market as this is very important towards proper efficacy and safety. Thus, this will help to increase the consumer confidence in the consumption of the HMP in Malaysia.

1.3 Objectives

DNA barcoding was used to analysed the ingredient composition of *F. deltoidea* and *E. longifolia* HMPs based on *rbc*L and ITS2 barcode region. Currently, no universal method for extraction of high quality DNA from HMPs, a preliminary study was conducted in which 4 different extraction were tested to ascertain which of the extraction method will yield DNA of high quality. HPLC analysis was further

used to determine the presence and concentration of bioactive compound responsible for their theurapetic properties. The specific objectives of this study are

1. To establish a Standard Reference DNA barcode dataset for *F. deltoidea* and *E. longifolia* plants using chloroplast (*rbc*L and *pbs*A-*trn*H*)* and nuclear (ITS2) markers.
2. To confirm the authenticity of selected Malaysian HMP via DNA barcoding.
3. To validate the authenticity of the selected Malaysian HMP using HPLC chromatographic fingerprints.

1.4 Scope of Study

The reliability of any DNA barcoding study for detecting the level of adulteration depends on the presence of reliable reference database and this is lacking in most of the cases more especially for Malaysian in medicinal plants. Therefore, in this study, genomic DNA extracted from the fresh leaves and roots of *F. deltoidea* and *E. longifolia* plants were amplified using two chloroplast (*rbc*L amd *psb*A-*trn*H) and one nuclear (ITS2) barcode regions and subsequently cloned into pEasy-T3 cloning vector. The sequences' generated from these barcodes were used to establish a standard reference material (SRM) and then deposited into the NCBI Genbank. The *F. deltoidea* and *E. longifolia* HMP were subsequently authenticated using the BLASTn and neighbour joining (NJ) tree analysis using the *rbc*L and ITS2 barcodes regions. In addition, the HPLC chromatographic fingerprint analysis was further performed on the HMP in conjunction with DNA barcoding to qualitatively and quantitatively detect certain active compounds so as to arrive species identity and admixtures.

1.5 Significances and Original Contributions of This Study

The success in establishing a standard reference material (SRM) DNA barcode dataset for *F. deltoidea* and *E. longifolia* will assists in overcoming the issues of insufficiency of authentic reference data set for quality control of their HMP's which are sold in the market. This is due to the fact that proper authentication of HMPs using DNA barcoding technique solely depends on the presence of reliable reference data set. This will in turn assist to facilitate accurate species resolution when testing HMPs. However, the use of DNA barcoding in conjunction with HPLC analysis in the authentication of HMP's will help to provide detailed information about their safety and efficacy as these are very important towards quality control and assurance. Therefore, these could make herbal industries to produce high quality HMPs which are safe and efficacious as this is one of the primary objectives of EPP1 project, thus gaining the consumers' confidence in their consumption.

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