PROFILING OF ADULTERANT IN TONGKAT ALI HERBAL PRODUCT USING DNA BARCODING IN COMBINATION WITH HIGH RESOLUTION MELTING ANALYSIS

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DEDICATION

-My success is only by Allah-

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ABSTRACT

Eurycoma longfiolia or known as Tongkat Ali is popular in Malaysia for its aphrodisiac and therapeutic properties. However, the increasing demand of this herbal remedy make it prone to adulteration due to the limited availability of Tongkat Ali plant resources. Hence, to fulfil the market demands, unscrupulous manufacturers may intentionally add Tongkat Ali herbal product with cheaper plant species as substitute to increase their profit. Misidentification of plant species during collection or false mixing with other plant species during processing stage also contribute to unintentional adulteration of herbal products. Therefore, this study was conducted to assess the authenticity of *E. longifolia* herbal products by using DNA Barcode combined with a new sensitive method of High Resolution Melting Analysis (Bar-HRM). In order to obtain high quality genomic DNA, extraction was done using modified Cetyl Trimethyl Ammonium Bromide (CTAB) method and Nucleospin Plant II kit. The rbcL and ITS2 was chosen as the plant DNA Barcode region in PCR amplification for *E. longifolia* root and four selected herbal products samples (P1, P2, P3, P4). For sensitivity evaluation of HRM analysis to detect traces of targeted DNA in admixture sample, *E. longifolia* DNA was mixed with *Camellia sinensis* DNA in increasing percentages of 0%, 1%, 5%, 10%, 30%, 50%, 75% and 100%. DNA melting profiles recorded from the different percentages of admixture was then used as a standard to detect traces of *E. longifolia* DNA in three admixture herbal tea products containing *E. longifolia* and *C. sinensis* on the packaging label (P5, P6, P7). Results showed that genomic DNA obtained using Nucleospin Plant II kit method recorded better DNA quality as compared to modified CTAB method. From HRM analysis result, P1 and P4 herbal products were authentic while P2 and P3 herbal products were not genuine based on the DNA melting profiles of rbcL. On the other hand, ITS2 DNA melting profile successfully detected three herbal products (P1, P2 and P4) that were genuine containing *E. longifolia*. However, *E. longifolia* was undetectable in P3 herbal product. The results of bioinformatics analysis including BLASTn, Multiple Sequence Alignment and Phylogenetic tree also supported the HRM analysis data for both genes. The result suggested that ITS2 primer used in this study was more specific and suitable to detect *E. longifolia* in herbal products up to species level, while rbcL managed to identify only up to genus level. From the HRM sensitivity results, rbcL can only detect *E. longifolia* DNA at 5% level while ITS2 managed to detect at lower than 1%. This proved that ITS2 had more DNA sequence variations and discrimination power than rbcL. In conclusion, Bar-HRM analysis is a reliable, fast and sensitive method to detect the true targeted plant species in herbal products and can be used to monitor food product authenticity issue in the future.

ABSTRAK

Eurycoma longifolia atau dikenali sebagai Tongkat Ali adalah terkenal di Malaysia kerana sifat aprodisiak dan terapeutiknya. Walau bagaimanapun, dengan permintaan yang semakin meningkat menjadikannya terdedah kepada pencemaran mutu disebabkan kekurangan sumber pokok Tongkat Ali. Oleh itu, bagi memenuhi permintaan pasaran, pengeluar yang tidak bertanggungjawab mungkin dengan sengaja menggantikan atau menambah produk herba Tongkat Ali dengan spesis pokok yang lebih murah bagi meningkatkan keuntungan. Pengecaman spesis pokok yang salah semasa pengumpulan atau pencemaran spesis semasa pemprosesan yang tidak disengajakan juga boleh menyumbang kepada pencemaran mutu dalam produk herba. Oleh itu, kajian ini dijalankan untuk menilai keaslian produk herba yang dilabel mengandungi *E. longifolia* dengan menggunakan gabungan Barkod DNA dan kaedah terbaru dan sensitif, Analisis Penguraian Resolusi Tinggi (Bar-HRM). Bagi mendapatkan kualiti DNA genomik yang tinggi, proses pengesktrakan menggunakan kaedah pengubahsuaian Setil Trimetil Ammonium Bromida (CTAB) dan kit Nucleospin Plant II telah digunakan. Barkod DNA, rbcL dan ITS2 telah dipilih sebagai kawasan DNA barkod tumbuhan dalam mengamplifikasi sampel akar *E. longifolia* dan empat produk herba terpilih (P1, P2, P3, P4). Bagi mengkaji tahap sensitiviti analisis HRM ke atas DNA *E. longifolia* di dalam campuran produk herba, DNA *E. longifolia* telah dicampurkan dengan DNA *Camellia sinensis* pada kadar 0%, 1%, 5%, 10%, 30%, 50%, 75% dan 100%. Profil suhu penguraian DNA yang direkodkan daripada peratusan sampel campuran tersebut digunakan sebagai rujukan untuk mengesan DNA *E. longifolia* di dalam tiga produk teh herba campuran yang telah tertulis mengandungi *E. longifolia* dan *C. sinensis* pada label pembungkusan (P5, P6, P7). Kajian mendapati DNA genomik yang diekstrak menggunakan kit Nucleospin Plant II merekodkan bacaan kualiti yang lebih baik berbanding kaedah pengubahsuaian CTAB. Daripada keputusan analisis HRM, dua produk herba P1 dan P2 adalah asli manakala produk herba P2 dan P4 adalah tidak asli berdasarkan penguraian suhu DNA oleh rbcL. Sebaliknya, melalui analisa penguraian suhu DNA, ITS2 berjaya mengesan tiga produk herba (P1, P2 and P4) adalah asli mengandungi *E. longifolia*. Walau bagaimanapun, *E. longifolia* tidak dapat dikesan di dalam produk herba P3. Analisis bioinformatik termasuk BLASTn, Penjajaran Turutan Sejajar dan Hubungan Jujukan Akar juga menyokong data analisis HRM untuk kedua-dua primer. Keputusan mencadangkan barkod DNA, ITS2 yang digunakan di dalam kajian ini lebih spesifik sehingga tahap spesis manakala barkod DNA, rbcL hanya berjaya mengenalpasti sehingga tahap genus. Daripada keputusan kajian sensitiviti HRM, rbcL hanya mengesan DNA *E. longifolia* sehingga tahap 5% manakala ITS2 berjaya mengesan di bawah 1%. Ini membuktikan bahawa ITS2 mempunyai lebih variasi urutan DNA dan kekuatan diskriminasi yang lebih tinggi daripada rbcL. Sebagai kesimpulan, analisis Bar-HRM adalah kaedah yang dipercayai, cepat dan sensitif untuk mengesan spesis yang tepat dalam produk herba dan boleh digunakan untuk mengawal isu keaslian produk makanan di masa akan datang.

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LIST OF ABBREVIATIONS

rbcL	-	Ribulose Bisphosphate Carboxylase Large Chain
ITS2	-	Internal Transcribed Spacer 2
CTAB	-	Cetyl Trimethyl Ammonium Bromide
IAA	-	Isoamyl Alcohol
HRM	-	High Resolution Melting
BLASTn	-	Basic Local Alignment Search Tool Nucleotide
NJ	-	Neighbour Joining

LIST OF SYMBOLS

mg	-	miligram
kg	-	kilogram
pg	-	picogram
mL	-	mililitre
µL	-	microlitre
µM	-	micromolar
ng	-	nanogram
w/v	-	weight/volume
v/v	-	volume/volume
ng/µL	-	nanogram/microlitre
±	-	plus minus
rpm	-	revolutions per minute
°C	-	degree celcius
Ct	-	threshold cycle
%	-	percentage
>	-	more than

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CHAPTER 1

INTRODUCTION

1.1 Background of Study

Nowadays, processed herbal medicinal products industry had increasing tremendously due to the high market demand. According to the Global Industry Analyst (2017), herbal product industry is projected to reach incremental opportunity of more than US$ 140 billion between 2017 to 2027. Herbal medicines are preferable by consumers because it comes from natural resources and is known to have many benefits in improving human's health. However, with the increasing demand of herbal products nowadays, there are some problems regarding the quality and purity of those herbal remedies marketed worldwide. The issues of misidentification of herbal plants, intentional or unintentional adulteration of herbal product content and contamination during processing stage has raised concern in food safety and quality. According to the Food and Agriculture Organization of the United Nations, food safety and quality can be controlled by the mandatory act of national or local authorities to ensure all food are safe during production, handling, processing, storage and distribution, thus suitable for consumer's consumption and health. Therefore, a better method for detection of adulterants in food products is needed.

Eurycoma longifolia, commonly known by the locals as Tongkat Ali, is considered the most popular traditional herbal plant in Malaysia. It is widely acclaimed for its energy boosting and aphrodisiac properties. Malaysian government had announced the NKEA EPP1 project that emphasizes on the enhancement of herbal value-added products from eleven medicinal plants including Tongkat Ali with

expected gross national income (GNI) amounting RM 2.2 million by 2020. According to Malaysia Herbal Cooperation, the value of herbal industry will be escalating from 17 million in 2013 to 29 million by 2020 (Ahmad *et al.,* 2015). Herbal product registration had also gone up by 20% with National Pharmaceutical Control Bureau (NPCB) indicating there was an increase in demand for herbal remedies in Malaysia.

In Malaysia, Tongkat Ali herbal products are commonly sold as tea bag, capsules or as an additive in tea, coffee or beverages. However, these products are prone to adulteration due to deliberate addition or substitution with low quality plant substitute. In Malaysia, *E. longifolia* shares the same common name with other species; *Polyalthia bullata* (Tongkat Ali Hitam), *Jackia ornata* (Tongkat Ali Merah) and *Eurycoma apiculata* (hilly area Tongkat Ali species). Moreover, Tongkat Ali also has been known by the locals by several other names such as Pasak Bumi, Payung Ali, Penawar Pahit, and Tongkat Baginda. These common names may unintentionally result in confusion among plant collectors or manufacturers. Hence, a proper identification of *E. longifolia* species needs to be established in order to avoid fraudulence in herbal products industry.

In the scope of biological science, continuing research to identify plant species has been studied from time to time in order to meet the demand and challenges in plant identification including from processed products containing degraded DNA samples. In 2003, DNA Barcode was introduced as one of the successful method to identify species based on the short DNA markers. Chen *et al.,* (2010), had highlighted a successful history of DNA Barcoding in identifying medicinal plants. However, limitations of this method were identified such as not suitable for samples containing severely degraded DNA, difficulty to obtain high quality genomic DNA due to the high amount of pharmaceutical excipients in processed herbal products, lacking of some DNA references in Genbank database and difficulty in identifying a plant species in admixture sample (Raclariu *et al.,* 2018; Abu Bakar *et al.,* 2018; Parveen *et al*., 2016). This has urge the development of a new technique for better species identification mainly in herbal products. Hence, this study advocates the use of a novel

method known as High Resolution Melting (HRM) analysis that will help to compliment the result of DNA barcoding.

HRM is a sensitive analysis method that focus on the melting behaviour of double stranded DNA to single stranded DNA by capturing signals from saturated fluorescence dye. The idea of combining these two methods of DNA Barcoding and HRM creates a new method known as Bar-HRM. It is an interesting subject to be studied especially in the identification of herbal products. For the past several years, the application of HRM analysis has been established in high throughput genotyping, gene mapping and efficiently applied in food authentication and products testing (Simko, 2006). Hence, the present study was carried out to explore and investigate the use of Bar-HRM to detect adulterants in Tongkat Ali herbal products, thus identifying the non-authentic one.

1.2 Problem Statements

Herbal medicinal products industry is expanding each year with the increasing demand from the consumers. Hence, to fulfil the market demands, irresponsible manufacturers may deliberately substitute or add cheaper plant species to gain profits. Unintentional adulteration also can be contributed from misidentification of plant species during collection or false mixing with other plant species during processing stage. Since *E. longifolia* species or known as Tongkat Ali is valuable for its various therapeutic and aphrodisiac properties, the exploitation and non-selective harvesting of Tongkat Ali plant had raised concern in medicinal plant collections (Nordin, 2014). Substitution of *E. longifolia* with other species may be contributed from the low availability due to the slow growth rate and lack of robust conservation program. Besides, poor labelling of the ingredients and lack of information written on the products also can create confusion to consumers. These problems may jeopardize its nutritional values and risk consumer's health. Hence, due to the adulteration issues, any measures that may aide for better authentication of *E. longifolia* species in herbal products would be beneficial.

In Malaysia, *E. longifolia* herbal products are sold in various forms including capsules, tea bag and beverages/drinks. Using molecular biological technique, it is possible to detect traces of *E. longifolia* DNA in processed herbal product. However, by using only DNA Barcoding approach, it is sometimes hard to identify complex plant species using certain molecular marker. Besides, the harsh physical or chemical treatments in food processed products will lead to DNA degradation, reducing the effectiveness in molecular biology detection. The difficulty to identify single species in mixed samples also is challenging by using this method alone. These highlighted problems need to be tackled in order to reduce challenges in food safety and monitoring. Hence, the combined molecular biology techniques, DNA Barcoding and HRM analysis (Bar-HRM) is an interesting subject to be studied due to its high sensitivity, simplicity and reliability to detect traces of targeted DNA especially in admixture herbal products.

1.3 Research Objectives

i. To determine the best extraction method for genomic DNA from fresh plant and commercial herbal product.
ii. To analyse the melting profile using High Resolution Melting analysis and bioinformatics data of the chloroplastic region (rbcL) and nuclear ribosomal (ITS2) from *E. longifolia* root and four selected commercial herbal products.
iii. To analyse the melting profile of *E. longifolia* DNA in three selected admixture commercial herbal tea products containing (*E. longifolia* and *Camellia sinensis*) by using High Resolution Melting analysis.

1.4 Scope of Study

The extraction of high quality genomic DNA would be the first crucial step before conducting any molecular studies. One of the critical factor that needs to be ensured for both successful real time PCR amplification (Cankar *et al*., 2006; Murray *et al.,* 2009) and HRM analysis (Jin *et al.,* 2015; Sun *et al.,* 2016) is the quality of DNA and carryover of impurities from extraction process. The challenge that needs to be tackled in extraction of high purity DNA is to eliminate excipients and other PCR inhibitors from processed food and degraded products. Determination of suitable DNA extraction method was employed in the present study for fresh plant and herbal product samples. In this study, two genomic DNA extraction methods; conventional modified CTAB method and commercial Nucleospin Plant II kit were compared and the quality of DNA obtained were determined.

Since the herbal product comes in the form of powder or dried herbs, it may contain degraded DNA and it is difficult to identify the constituent species. Hence, by using real time PCR, data from High Resolution Melting analysis generated was used to compare and profile the adulterants present in the samples. Few parameters such as the type of extraction method, amount of DNA template and primer concentrations were optimized to study the effect on HRM analysis. In HRM analysis, positive control *E. longifolia* root was used as the plant reference to distinguish the melting behaviour between four commercial herbal products tested. Derivative plots from melt curve generated was further analysed for subtle differences in terms of both melting temperature and shape of melting pattern displayed in the form of normalized and difference plot graph. The combined technique of DNA Barcode with HRM (Bar-HRM) was applied using two potential plant DNA markers rbcL and ITS2. The amplified products from real time PCR were then sequenced. Bioinformatics analysis was carried out by analysing the sequencing results. Multiple sequence alignment and phylogenetic trees were constructed between *E. longifolia* reference sample, selected herbal products and sequences retrieved from Genbank.

In Malaysia, commonly a small amount of *E. longifolia* was added in tea bag product to enhance the health benefits. Therefore, the versatility and sensitivity of HRM to detect the presence of traces *E. longifolia* DNA in admixture herbal tea samples was also conducted in this study. Different percentages of *E. longifolia* (Tongkat Ali) was mixed with *Camellia sinensis* (tea) and analysed by using HRM analysis. The melting profiles of these different percentages of admixtures was then used as reference to authenticate selected three admixture commercial herbal tea products.

1.5 Significance of Study

The issue of fraudulent in herbal products had tremendously jeopardized the consumer's perception. Hence, a valid and reliable method to identify genuine from adulterated products needs to be established with more accurate, faster and simple authentication process. The vast development in technology has urged the findings of a reliable method at molecular levels which are DNA Barcode and High Resolution Melting analysis (Bar-HRM) for herbal products authentication. This method may serve as a basis to detect adulterants in herbal products which is commonly hard to recognize in their processed form. The advancement of High Resolution Melting sensitivity also serves as a reliable method to detect adulterants at a very low concentration in admixture herbal products. Implementing this Bar-HRM method could be beneficial for regulatory agencies in developing new policy related to food safety in the future.

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