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Research Article

Comparative Evaluation of Different DNA Extraction Methods from *E. Longifolia* Herbal Medicinal Product

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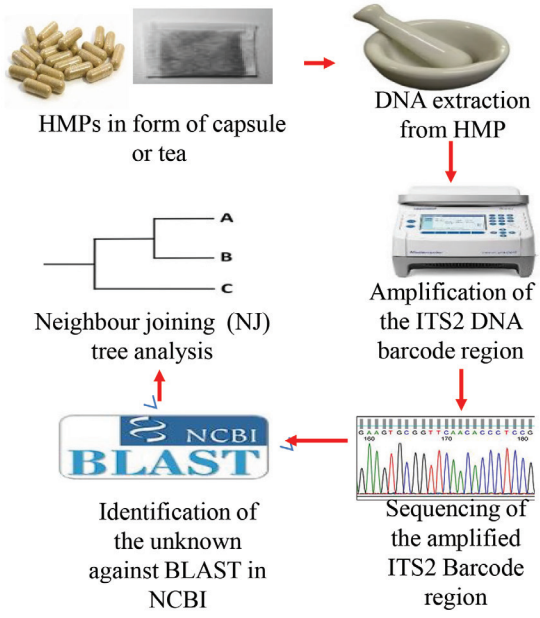
ABSTRACT

The aphrodisiac property of *Eurycoma longifolia* has led to an increase in the demand for its Herbal Medicinal Products (HMPs). However, the efficiency of such HMPs depends on the usage of their genuine raw materials. The conventional methods cannot identify species in processed form. The authentication of HMPs can be achieved effectively using DNA barcoding as the method species-specific. However, the use of this method solely relied on the extraction of high-quality DNA from the HMPs. Therefore, it is necessary to establish a satisfactory method for extracting high-quality DNA from the HMPs. Here, four DNA extraction methods were compared to evaluate the best protocol in yield, purity, polymerase chain reaction (PCR) amplification, sequencing, and species identification. The spectrophotometer analysis showed that the Nucleospin Plant II extraction kit has the best purity as this can be severely affected by the presence of various contaminants in the HMPs. Our findings reveal that DNA purity was more important as a predictor for PCR amplification than yield. Therefore, the present study results demonstrate that the Nucleospin Plant II extraction kit is the best because it produces the purest, amplifiable, and sequenceable DNA for identification and authentication of *E. Longifolia* HMPs.

GRAPHICAL ABSTRACT



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1. INTRODUCTION

Eurycoma longifolia is an essential evergreen medicinal plant belonging to the family Simaroubaceae [1,2]. The bitter characteristics of these plants result from the abundant amount of quassinoid, which usually contributes to most of their pharmaceutical properties [3]. The name of "Tongkat Ali" is due to its appearance as a walking stick with the presence of its long twisted roots. As a medicinal plant, *E. longifolia* has been used in Malaysia for its anti-malarial property, anti-cancer property, and toxicity effect [1,4]. It is still more widely used due to its aphrodisiac property, mostly contributed by its root part [5]. Presently, over 200 commercialize Herbal Medicinal Products (HMPs) from this plant, especially the roots, are available in the market. The HMPs from this plant species are usually processed into modified forms such as tea, capsule, coffee, powder, and carbonated drinks. These are generally recommended to improve health conditions and libido.

At the moment, the consumption of HMPs is increasing significantly as a result of the general perception that they are safe and can be used for the treatment of various health-related problems. This increase in demand has increased its trade both locally and internationally. A large number of people, estimated to be around 5.6 billion, solely depend on HMPs when they have a health problem [6,7]. Simultaneously, the massive increase in the demand for HMPs has resulted in some unethical malpractice such as fraudulent replacement of authentic material with inferior ones and mislabelling and misidentification of the raw plant. All these types of unethical activities practiced by the traders of the medicinal plants and practitioners are not suitable for the health of the consumers [8]. Other types of adulteration of the HMPs, such as contamination, could also occur at the processing point. Thus, there is a need to use proper identification and authentication methods for HMPs as their safety and efficacy solely rely on the appropriate use of authentic raw materials.

DNA barcoding is a reliable tool used to achieve quick and accurate species identification [9]. The technique is species-specific, cost-effective, and not restricted to any species' morphological characteristics [10,11]. However, the extraction of high-quality DNA from HMPs is the prerequisite for implementing this novel technique. This is because successful Polymerase Chain Reaction (PCR) amplification solely relied on the extraction of high quality of good yield and purity. Still, DNA extraction from medicinal plants is always difficult due to high secondary metabolites such as phenolic compounds, polysaccharides, and other proteins that generally interfere with DNA quality to be extracted [12,13]. The situation becomes more problematic if the material under investigation is HMPs. This is because the DNA in HMPs are easily destroyed, fragmented, or degraded after undergoing a series of processing procedures such as stewing and drying [14]. Degradation or fragmentation of DNA at the primer binding site may also fail amplification success [8,15].

Here, to rank the performance of different DNA extraction methods for HMPs, we compare four different extraction methods from HMPs to ascertain the one that will yield high-quality DNA in yield and purity. The various methods compared in the present study include the conventional method such as the Cetyltrimethyl Ammonium Bromide (CTAB) methods and commercial DNA extraction kit. The performance of each extraction method was tested against four *E. longifolia* HMPs. This plant is popular in Malaysia due to its aphrodisiac property [16,17]. The quality of the DNA extracted was further assessed by PCR amplification of the ITS2 DNA barcoding region. This step is crucial in this study because only high-quality DNA extracted can be successfully amplified using PCR. In other words, the efficiency of PCR amplification solely relied on high-quality DNA, which will help distinguish between the excellent and poor DNA extracted. Therefore, this study aims to assess various DNA extraction methods' efficiency to ascertain the most appropriate to yield high-quality DNA from *E. longifolia* HMPs. Three commercial DNA extraction kits, Nucleospin, DNeasy and wizard, and conventional method (CTAB) DNA extraction method, were tested. Each method's efficiency was compared in terms of DNA quality, the possibility of ITS2 barcode region amplification through PCR, and finally, sequencing of the PCR product.

2. MATERIALS AND METHODS

2.1. Material Used

Four samples of *E. longifolia* HMPs were acquired from some designated retail stores within Johor Bahru, Malaysia. As mentioned, this study aimed to evaluate the DNA extraction methods' efficiency and not the herbal product company; therefore, the company name was not provided.

2.2. DNA Extraction and Measurement

DNA extraction from *E. longifolia* HMPs was carried out using four different protocols; Conventional CTAB methods with minor modification. The other three commercial protocols used include Wizard resin (Promega, Madison, WI, USA) kit, Nucleospin plant II kit (Macherey-Nagel, Germany), and DNeasy (Qiagen, Germany) kit. Before the extraction process, pestle and mortar were used to manually homogenized 0.1 g of HMP in liquid nitrogen. This is necessary because the capsule contains additional materials such as glidant, lubricant, stabilizers, and fillers material, which can interfere with the DNA extraction process [15]. The sample's incubation time in the water bath in all the methods was increased, making the DNA extraction method more effective. To test the extraction method's reproducibility and reliability, DNA extracted from the HMPs was repeated three times (triplicates).

The extraction methods for the commercial kit mentioned were carried out following the instruction of the manufacturer. For the CTAB method, DNA was extracted in accordance to Doyle [18] with minor modifications. 10% Polyvinylpyrrolidone powder was added to fresh leaves or herbal capsule samples and quickly homogenized in liquid nitrogen. The grinded material were transferred into 2 mL microcentrifuge tube and mixed with 700 µL of pre-warm extraction buffer at 65°C (100 mM Tris HCl, pH 8.0; 20 mM Na_2 ethylenediaminetetra-acetic acid (EDTA); 2% CTAB Hexadecetyltrimethylammonium bromide (Sigma-Aldrich, Germany); 1.4 M NaCl along with 2% β-mercaptoethanol. The mixture was thoroughly mixed and incubated at 65°C for a longer 120 min with intermittent shaking every 10 min. After incubation, 700 µL of chloroform:isoamyl alcohol (Ch:Iaa) was added to the mixture to promote partitioning of phases into the aqueous and organic phases. The mixture was further

centrifuged at 17,709*g* for 10 min. Ch:Iaa's equal volume was added to the aqueous phase and centrifuged at the same rate. The pellets which contain the DNA were collected after incubation for 1 h in chilled isopropanol. 70% Ethanol was used to wash the pellet twice and centrifuged at 17,709*g* for 5 min. Finally, the pellets containing the DNA were re-suspended in 100 µL TE buffer after discarding the supernatant. The RNA was removed by incubating the re-suspended pellet in RNase at 37°C for 30 min.

A Nanodrop spectrophotometer (ND1000, Thermo Scientific, USA) was used to analyze the yield and purity of the genomic DNA extracted. The UV absorbance at 260 nm was used to determine the concentration, and DNA purity was analyzed using absorbance ratio A_{260}/A_{280} measurement. The quality (integrity) of the genomic DNA isolated was further analyzed using 1% agarose gel electrophoresis before visualization.

2.3. PCR Amplification and Sequencing

The extracted genomic DNA obtained from the *E. longifolia* HMPs were subjected to PCR amplification with reference ITS2 region to check for amplification success. The 25 µL PCR reaction mixture consists of approximately 30 ng genomic DNA as a template, 5X standard buffer, 25 mM of $MgCl_2$ (Promega), 10 mM of deoxynucleotide triphosphate (dNTPs) (Promega), 10 mM of each primer and 5U of Taq DNA polymerase (Promega). The sequence for the universal ITS2 forward primers used was ITS2 - F (GGGGCGGATATTGGCCTCCCGTGC), and the reverse primer was ITS2-R (GAC GCT TCT CCA GAC TAC AAT) [19]. The addition of sterile water to the PCR mixture was used as the negative control.

The reaction PCR reaction was performed in a thermal cycler (Eppendorf) using 95°C for 2 min, 35 cycles at 95°C for 1 min, 45°C for 1 min and 72°C for 2 min and final extension cycle at 72°C for 5 min. The amplified product was run on an agarose gel to ascertain the size of the amplified product. The PCR product was then purified and sequenced by First Base Sdn Bhd, Malaysia.

The sequences of the *E. longifolia* HMPs that were successfully amplified were manually edited using Bioedit software. The sequences were then blasted against the nucleotide database of NCBI using the Basic Local Alignment Search Tool (BLAST) program. Sequences with the best match in the BLAST program were downloaded in FASTA format and included in the analysis. A Neighbor-Joining (NJ) tree was constructed using MEGA 6 to test for a phylogenic relationship. However, using a tree-based method is not to draw a phylogenetic conclusion but rather to identify an unknown species based on the clustering compared to reference species.

3. RESULTS AND DISCUSSION

3.1. Evaluation of DNA Concentration, Purity, and Integrity of Four DNA Extraction Methods on the HMPs

Extraction of high-quality DNA from HMPs can always be a challenge because they usually undergo a series of heavy processing activities that may result in the DNA's degradation or fragmentation. This is in addition to the presence of secondary compounds (alkaloids, carbohydrates, and pigment), which interfere with high-quality DNA extraction [14,20].

The NanoDrop spectrophotometric analysis result to verify the quality and the yield of the extracted DNA shows a remarkable difference between the four methods. The DNA extracted using the CTAB method contributed the highest yield of DNA than any other method, as tabulated in Table 1.

The application of different separation principles in the extraction methods may result in that fact. The CTAB method uses the salting-out precipitation method, while all the three commercial kits utilize the silica binding methods. This result was also in accordance with reports by Cheng et al. [21] stating that the modified CTAB method proves to be acceptable methods in achieving the highest yield of Liuwei Dihuang Wan traditional Chinese medicine DNA with the complexity of ingredient compared to the commercial kit.

In the present study, compared with the other three protocols, the best extraction method in terms of purity was achieved using the Nucleospin Plant II extraction kit. With this method, the DNA purity ($A_{260/280}$) was between the optimal ranges of 1.76–1.81 except in TAP-3 (Table 1). The result showed that the DNA extracted using the Nucleospin Plant II extraction kit is free from contaminants. This finding was also in conformity with the discovery of Särkinen et al. [22], which shows that the degraded form of DNA extracted from herbarium gave higher purity when extracted with Nucleospin Plant II extraction kit.

The tested HMPs using gel electrophoresis analysis showed that the genomic DNA extracted were degraded as indicated with a light smearing (only observed using the CTAB). This could be due to a series of processing activities that they undergo (Figure 1). The findings indicate that there is some level of DNA fragmentation or degradation due to the exposure of the HMPs to high temperatures during the processing activities. The light intensity of the smear observed in the CTAB could result from the higher yield observed in the method than any kits. This finding is in agreement with that

Table 1 | Mean yield (ng/µL) ±SD and purity ($A_{260/280}$) ±SD and PCR amplification success of the ITS2 barcode region for the four different DNA extraction methods

Samples	Protocol	Yield (ng/µL)	Purity ($A_{260/280}$)
TAP-1 (Tea)	Modified CTAB	**462.5 ± 247.80**	**1.54 ± 0.13**
	Nucleospin	**41.2 ± 7.48**	**1.79 ± 0.25**
	DNeasy	**27.8 ± 2.46**	**1.62 ± 0.29**
	Wizard	93.4 ± 29.45	1.08 ± 0.08
TAP-2 (Capsule)	Modified CTAB	66.2 ± 35.7	1.12 ± 0.23
	Nucleospin	18.13 ± 3.45	1.13 ± 0.13
	DNeasy	13.7 ± 5.57	1.04 ± 0.04
	Wizard	28.2 ± 9.5	0.84 ± 0.07
TAP-3 (Capsule)	Modified CTAB	229.7 ± 47.2	1.35 ± 0.06
	Nucleospin	**43.3 ± 7.32**	**1.76 ± 0.17**
	DNeasy	27.6 ± 5.62	1.28 ± 0.21
	Wizard	63.4 ± 13.7	1.15 ± 0.14
TAP-4 (Tea)	Modified CTAB	**295.2 ± 56.3**	**1.75 ± 0.02**
	Nucleospin	**48.2 ± 7.78**	**1.81 ± 0.11**
	DNeasy	28.6 ± 0.98	1.41 ± 0.11
	Wizard	49.43 ± 6.02	1.14 ± 0.07

Bold = PCR amplification successful.

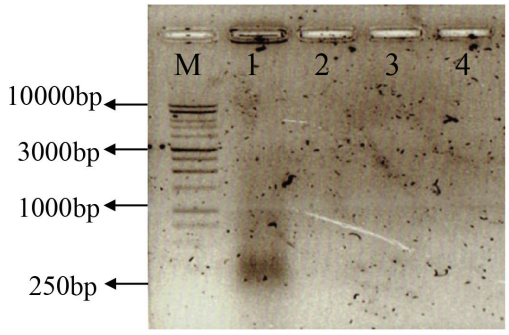

Figure 1 | Extracted genomic DNA from *Eurycoma longifolia* HMPs. Lane 1: CTAB method, Lane 2: Wizard resin method (Promega), Lane 3: Nucleospin Plant II kit, Lane 4: DNeasy plant extraction kit, Lane M: 1 kb DNA ladder.

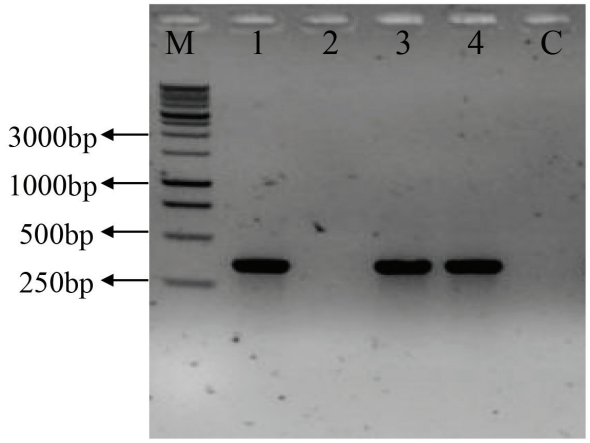

Figure 2 | Amplified ITS2 barcode region of *Eurycoma longifolia* HMPs. Lane 1: CTAB method, Lane 2: Wizard resin method, Lane 3: Nucleospin Plant II kit, Lane 4: DNeasy plant extraction kit, Lane C: Negative control and Lane M: 1 kb DNA ladder.

reported by Hale et al. [23]. They stated that the CTAB extraction method often produces a much higher DNA than any extraction kit. Degradation and fragmentation of DNA in HMPs, which occurs during processing activities, always serve as a challenge in any high-quality DNA extraction.

3.2. PCR Amplification, Sequencing, and Species Identification

Selecting a good DNA extraction technique is a critical step for authentication of HMPs as the extracted DNA's yield and quality directly bear on PCR results. To evaluate the efficiency of the DNA extraction methods used, PCR was performed using the ITS2 DNA barcode region. The ITS2 barcode region was chosen in the present study due to its small size, ease to be amplified (universality), high-quality sequence, and high discriminatory power [24–27].

The DNA extracted from each method was subjected to PCR amplification. Out of the four *E. longifolia* HMPs examines, amplifiable DNA could not be extracted in only one of the tested HMP samples (TAP-2) after repeated attempts. This could be due to the fragmentation of the DNA that can occur at some stage of the manufacturing process, which can affect other downstream processes. Secondary metabolites such as polysaccharides, phenolic compounds, glycoprotein, etc., can result in difficulty extracting high-quality DNA and amplification. Secondary metabolites are natural inhibitors that can hinder DNA extraction and other downstream processes [28,29]. Other factors such as fragmentation of the DNA at the primer annealing site may also result in fail PCR amplification of any DNA barcode region [8,11].

Out of the total number of extracted DNA from the present study, the Nucleospin kit has the highest amplification success, three out of the six *E. longifolia* HMPs amplified. This was followed by CTAB methods and the DNeasy kit (Qiagen), having two and one amplification success. On the other hand, none of the HMPs was amplified using the Wizard resin, as shown in Figure 2.

Even though the yield of the DNA extracted using the CTAB method was higher than any tested method, it has less amplification success when compared with the Nucleospin Plant II kit. A possible explanation of this might be the high purity of DNA extracted using the Nucleospin Plant II kit. This finding was in agreement with that described by Llongueras et al. [30]. They found that the Nucleospin kit produces the best DNA extracted from 13 anti-diabetic herbal supplements for PCR amplification. This result suggests that there was a strong link that exists between DNA purity and PCR success. This indicates that any extraction method that will maximize the high purity of HMPs rather than yield will be of enormous importance for successful PCR amplification of DNA. A suitable and efficient extraction protocol should not only give high yield but high purity [31]. The efficiency of PCR amplification and selected DNA extraction methods suggests that DNA purity was more important as a predictor for PCR amplification than yield.

To further evaluate the amplified ITS2 barcode region of *E. longifolia* HMPs, the PCR products were purified and subjected to sequencing to determine the actual biological ingredient in the HMPs samples. The result based on the sequencing revealed that all the three *E. longifolia* HMPs whose DNA was extracted using the Nucleospin Plant II extraction kit were successfully identified to species level. The BLASTn analysis shows that only two (TAP-1 and TAP-3) matched with *E. longifolia* sequences found in the GenBank database. The sequences generated from TAP-1 and TAP-3 have a high similarity of 99%. On the other hand, the sequence generated by TAP-2 HMPs revealed that the sample has a sequence similarity of 97% with *Ficus deltoidea*. This result showed a possible substitution in the HMP. Newmaster et al. [8] described a possibility of generating new DNA barcodes other than what was written in the label of the HMPs in a situation where substitution has occurred. These findings were further confirmed using the NJ analysis. The NJ analysis showed that TAP-1 and TAP-3 shared the same clade with other *E. longifolia* sequences retrieved from the GenBank. This finding indicates that TAP-1 and TAP-3 were truly authentic as they are found on the same clade with *E. longifolia* sequences in addition to having high sequence similarity. However, one of the HMPs tested samples (TAP-2) was located outside the *E. Longifolia* clade, implying that the HMPs contained certain ingredients contrary to the packaging label (Figure 3). The present study's findings showcase some unethical activities in the HMPs sold in the market places. However, these results need to be confirmed by comparing with a developed standard reference database similar to what

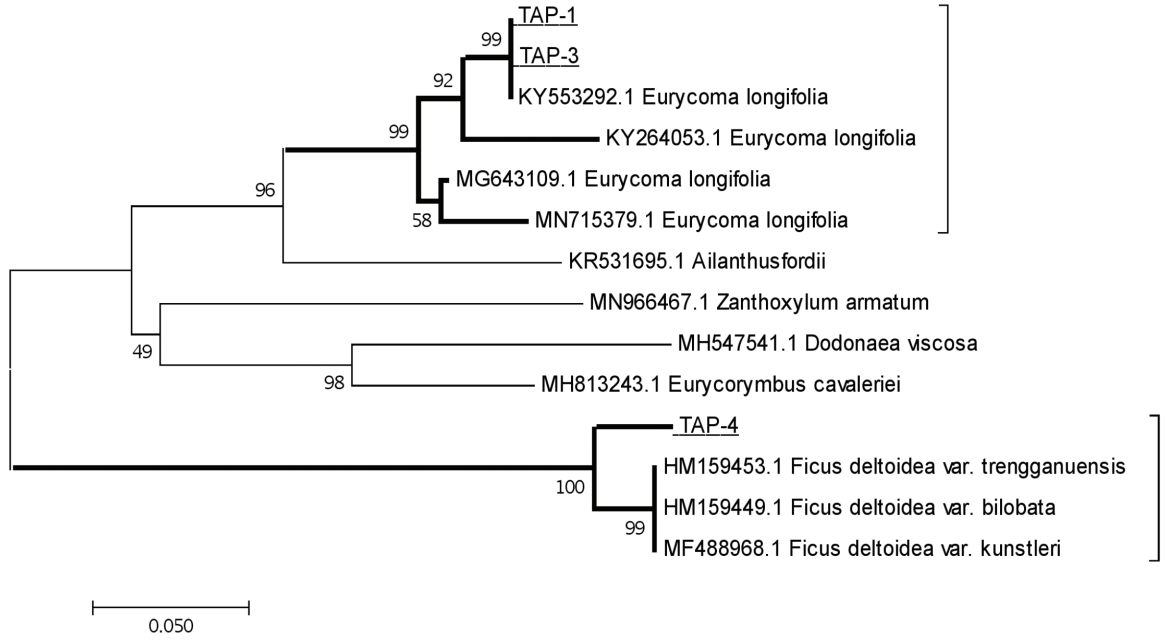

Figure 3 | Neighbour joining tree constructed using ITS2 barcode region showing *Eurycoma longifolia* HMPs and some Genbank retrieved sequences. Underlined TAP represent the HMPs.

was created in other parts of the world such as China [9], North America [8], and India [32].

4. CONCLUSION

We tested four different types of DNA extraction methods from *E. longifolia* HMPs. Our findings show that the Nucleospin plant II kit performed best in terms of purity and amplification success, thereby providing sequenceable DNA for molecular identification studies. This is followed by CTAB methods, which used to have a high yield. However, we concluded that yield is not a good predictor for DNA quality as some might have high yield but with impurities that can affect PCR analysis. This finding indicates that purity is more important as a predictor for amplification success than yield. Therefore, the present study results demonstrate that the Nucleospin Plant II extraction kit is the best because it produces the purest, amplifiable, and sequenceable DNA for identification and authentication of *E. Longifolia* HMPs. Thus, any DNA extraction method that will maximize high purity should be used to isolate high-quality DNA from HMPs.

CONFLICTS OF INTEREST

The authors declare they have no conflicts of interest.

AUTHORS' CONTRIBUTION

AW contributed in conception, design and supervision of the experiment. BMA contributed in development of methodology, conducted the experiment and provided the data and writing. FMA contributed in writing and review of the manuscript. MA analysed the data. All authors read and approved the final manuscript.

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