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EVALUATION OF DNA QUALITY FROM MODIFIED DNA EXTRACTION AND AMPLIFICATION OF ITS2 FROM *Eurycoma longifolia* CAPSULE HERBAL PRODUCTS

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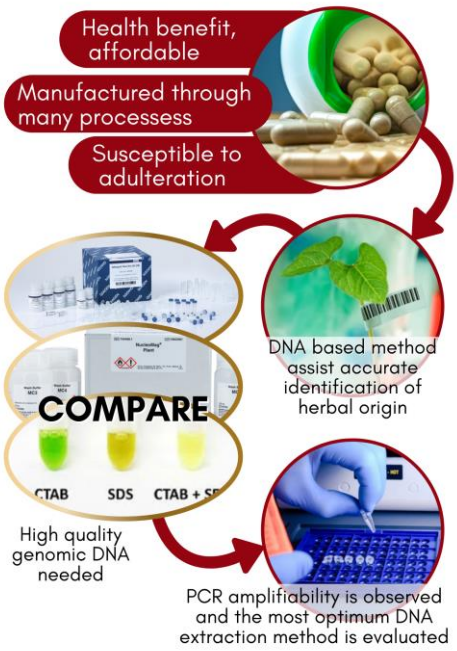
Graphical abstract



Abstract

Demand for herbal products has increased because of their purported health benefits and economic value. However, they are susceptible to adulteration, making accurate identification of herbal origin essential, especially for quality control. In recent decades, DNA-based methods have played a crucial role in the development of authentication tools that required good quality of DNA. The manufacturing process of herbal products involved heating, grinding or other mechanical procedures, and addition of excipients/additives caused DNA to degrade which in turn influenced DNA quality. In this study, nine different conventional methods with some modifications were evaluated to determine the best technique producing good quality DNA from capsule herbal products. Assessment was conducted using spectrophotometric measurements and agarose gel electrophoresis. To determine the quality of gDNA, amplification of ITS2 amplicon was performed by PCR. The DNA extraction finding showed that DNA quality from each method resulted in a different DNA purity and yield, hence the ITS2 amplification. Each of the modified DNA extraction methods performed has its own strengths and limitations when it comes to obtaining high quality gDNA. In addition, the study demonstrated the success of ITS2 amplification with the modified DNA extraction methods used.

Keywords: DNA extraction, herbal products, ITS2, modified conventional methods, gDNA

Abstrak

Permintaan untuk produk perubatan herba telah meningkat disebabkan oleh faedah kesihatan dan nilai ekonominya. Walau bagaimanapun, pemalsuan kandungan boleh berlaku, maka pengenalpastian sumber herba yang tepat adalah sangat penting, terutamanya untuk kawalan kualiti. Dalam dekad ini, kaedah berasaskan DNA telah memainkan peranan penting dalam pembangunan alat pengesah sumber herba yang memerlukan kualiti DNA yang baik. Pembuatan produk herba melibatkan pelbagai proses seperti pemanasan, pengisaran, mekanikal serta penambahan bahan-bahan asing menyebabkan degradasi DNA sekaligus menghalang kejayaan pengekstrakan DNA berkualiti tinggi. Dalam kajian ini, sembilan kaedah pengekstrakan DNA konvensional yang diubahsuai telah dibandingkan untuk menentukan teknik terbaik untuk produk herba daripada kapsul. Penilan kualiti DNA dilakukan melalui bacaan spektrofotometri dan elektroforesis gel agaros. Untuk menentukan kualiti DNA, amplifikasi ITS2 dijalankan melalui PCR. Kajian menunjukkan kaedah pengekstrakan yang digunakan menghasilkan kualiti dan kuantiti DNA berbeza. Setiap kaedah pengekstrakan DNA yang digunakan

mempunyai kekuatan dan kelemahan tersendiri. Seterusnya keberhasilan fragmen ITS2 yang diamplifikasi dari PCR telah ditunjukkan dengan jayanya.

Kata kunci: Pengekstrakan DNA, Produk perubatan herba, ITS2, kaedah konvensional, DNA genomic

1.0 INTRODUCTION

Herbal products have been portrayed in different formulation forms such as capsules, dietary supplement, beverages (tea/ herbal drink), powder and dried materials. Currently, consumption of these products is increasing significantly as they are generally considered safe and can be used to treat various health problems. This increasing demand has increased trade both locally and internationally with an estimated 5.6 billion, relying exclusively on herbal products for treatment [2]. At the same time, the massive increase in demand for herbal products has led to some unethical practices, such as fraudulently substituting authentic raw material with inferior one from plant collection to processing. All these types of unethical activities were not suitable due to hidden risk of toxicity for the health [19]. Therefore, it is necessary to use appropriate identification and authentication methods for herbal products, as their safety and efficacy depend solely on the appropriate use of authentic raw botanical materials.

DNA barcoding has been used in the last decade as modern and reliable tools to authenticate and identify the species at molecular level. Among the DNA barcode used were ITS2 region that situated between 5.8S and 28rRNA in ITS region. Previous study had shown that ITS2 has ability to discriminate approximately 13307 genus and 5240 species accessed from GenBank and no argument on considering ITS2 as universal barcode for wide taxa range of medicinal plant [13]. In addition, Pang *et al*., reported that ITS2 was able to distinguish 100% correctly species from adulterants, however, it was not easy to discriminate herbal products, because of degraded DNA and substituted by other species [11, 20]. Therefore, high quality of DNA extracted was a crucial step in any DNA molecular techniques. This DNA barcoding approach served as a new detection tool for identifying adulterated herbal medicinal products [4, 16, 18, 25]. However, the DNA extraction from complex herbal products is challenging due to mixture of several of plants species that containing different polyphenols, alkaloids, and flavonoids [15] or mixed with other substance (talc/pharmaceutical excipients in their formulation, pigment, stabilizer, impurities, admixtures). Other than that, exposure of raw materials to high temperature, grinding, exposure to strong pH changes through manufacturing causing further DNA degradation and low DNA recovery [11, 10, 17, 19]. These factors contribute to low DNA yield and DNA integrity while presence of PCR inhibitory contaminants requires more efforts in optimization or testing different extraction protocols. There were several reports regarding the study of DNA quality and quantity from processed herbal products [10, 22, 17, 26].

However, this research was only limited to certain botanical origins (raw materials/ extract/ powder), leaving a gap of knowledge on whether modified conventional methods could retrieve sufficient DNA from capsule herbal products compared to commercial kits.

There were various commercial column-based DNA extraction kits available in the market has been tested on herbal products with various degree of successfulness [6, 7, 20, 26]. Apart from commercial kits, most widely used extraction methods were classical versions of cetyltrimethylammonium bromide (CTAB) but depend on species and differ in steps. The main cause of differences in CTAB protocols was due to cell walls composition and intracellular components of tested plants. Despite the limitation, several extractions modified methods from CTAB-based, SDS or TCM etc. have been established for medicinal plant species and herbal products [8, 21]. Until now, there was no DNA extraction protocol that could eliminate the PCR inhibitory completely with most of the protocols needing to be improvised accordingly due to the complexity of the herbal products ingredients.

Production of high-quality DNA is characterized predominantly by high molecular weight fragments with an $A_{260}/_{280}$ ratio between 1.8 to 2.0 and lacking contaminating substances such as polysaccharides and other secondary metabolites [24]. In a previous study, the commercial kit, NucleoSpin Plant II kit, was the best method for DNA extraction from *Eurycoma longifolia* products [1]. An economical DNA extraction method for dried herbal powder has been developed to retrieve DNA with successful PCR amplification [3]. Their finding suggested that DNA quality did contribute to successful amplification rather than DNA yield similar in our finding. Hence, it is possible to produce good DNA purity in conventional DNA extraction methods that can be improved by modifying the steps.

Therefore, the present study was conducted to evaluate the variability of DNA quality and yields when using nine modified CTAB-based methods in extracting DNA from capsules of *E. longifolia* herbal products. Further PCR amplification of ITS2 was performed to evaluate the quality of DNA obtained. Even though the conventional methods seem to be laborious compared to commercial kits, the findings in the present study would be useful to the laboratory which has limited financial resources.

2.0 METHODOLOGY

Herbal Products

Capsule herbal products used in this study were from *E. longifolia* designated as TACP-1, TACP-2 and TACP-3 which stated as a mixture of different plant species known as polyherbal.

DNA Extraction

In the present study, nine types of modified conventional DNA extraction methods [9] and two commercial DNA extraction kits were employed; NucleoSpin® Plant Mini Kit II (Macherey-Nagel) and DNeasy Plant Mini Kit (Qiagen). For commercial kits, the DNA extraction was carried out as per manufacturer's instruction. A total of 100 mg of capsule herbal products were grounded in pestle and mortar separately using liquid nitrogen until fine powder was produced. After that, the sample was transferred into a sterile microcentrifuge tube before the extraction began. Meanwhile for conventional methods, the extraction was carried out following the modified method as listed in Table 1.

Table 1 Summary of modified DNA extraction methods used in the present study

Protocol	Reference	Variation
Modified CTAB I	Doyle & Doyle (1987) [9]	4 hours lysis stage. Overnight isopropanol precipitation. Phenol-Chloroform extraction.
Modified CTAB II	Doyle & Doyle (1987) [9]	4 hours lysis stage. Overnight isopropanol precipitation. Chloroform extraction only.
Modified CTAB III	Doyle & Doyle (1987) [9]	2 hours lysis stage. 2 hours isopropanol precipitation. Phenol- Chloroform extraction.
Modified CTAB IV	Doyle & Doyle (1987) [9]	2 hours lysis stage. 2 hours isopropanol precipitation. Chloroform extraction only.
Modified CTAB V	Harini *et al.*, 2008 [12]	Two times Phenol-Chloroform extraction. Precipitation using salt (NaCl). Increased salt concentration from 4M to 5M of NaCl, with proportional increase in CTAB concentration (3%).
Modified CTAB VI	Saghai-Maroof *et al.*, 1984 [23]	Precipitation of DNA using sodium acetate & absolute ethanol. Re-extracting with phenol and reprecipitation with sodium acetate and ethanol.
Herbal Supplements Dietary (HSD)	Little (2014) [14]	Lower concentration of salt. Without using CTAB & PVP for lysis. Addition of sucrose, sodium acetate & potassium acetate in lysis buffer & precipitation buffer, respectively.
Traditional Chinese Medicine (TSM)	Cheng *et al.*, 2014 [6]	No liquid nitrogen used.
Modified SDS method	Doyle & Doyle (1987) [9]	Addition of 10% SDS solution and 3% of PVP.
Nucleospin Plant II Kit	Macherey-Nagel	Silica-based resin. Procedures followed according to manual.
DNeasy Plant Mini Kit	Qiagen	Silica-based resin. Procedures followed according to manual.

Quantitative and Qualitative Spectrometric Analysis of DNA

The quantitative readings of DNA purity were performed by absorbance ratio (A260/280) and concentration (ng/µL) by using NanoDrop® 1000 Spectrophotometer (Thermo, USA). The quality of gDNA extracted (5µL sample) was electrophoresed on 1% (w/v) agarose gel and viewed under Alphalmager® gel documentation system (BioRad).

Agarose Gel Electrophoresis

The quality (integrity) of genomic DNA extracted was run through 1 % (w/v) agarose gel electrophoresis pre-stained with Ethidium Bromide. A total of 5 µL of extracted gDNA was mixed with 2 µL of loading dye (Promega®), and the mixture was loaded in the gel carefully. For a genomic DNA marker, 6 µL of 1 kB or 100 bp ladder (Promega®) was loaded into the well. The gel electrophoresis was running and set at 74 Volt, 420 mA current in 40 minutes' duration. The

agarose gel was taken off from the electrophoresis set and viewed.

PCR Amplification

The 20µL components of the PCR mixture reaction (Promega) containing 5X GoTaq Buffer, 0.5µL of *ITS*2F 5'- GGG GCG GAT ATT GGC CTC CCC TTG C -3' and *ITS*R 5'- GAC GCT TCT CCA GAC TAC AAT -3' [(Chen *et al.*, 2010), 0.4µL of dNTPs (10mmol/L), 0.125 µL of Go Taq Polymerase (50 mmol/L), 2µL of Magnesium Chloride (10mM/L), 300ng of gDNA, 1µL of Bovine Serum Albumin (BSA) and 13.475µL sterile ionized water. The PCR reaction program was conducted for 30 cycles, with initial denaturation at 94℃ for 2 minutes, denaturation at 94℃ for 1 minute, annealing at 56.9℃, extension and final extension at 72℃ for 2 minutes and 5 minutes respectively. The PCR product was then electrophoresis on agarose gel and later viewed under Gel Documentation System (BioRad).

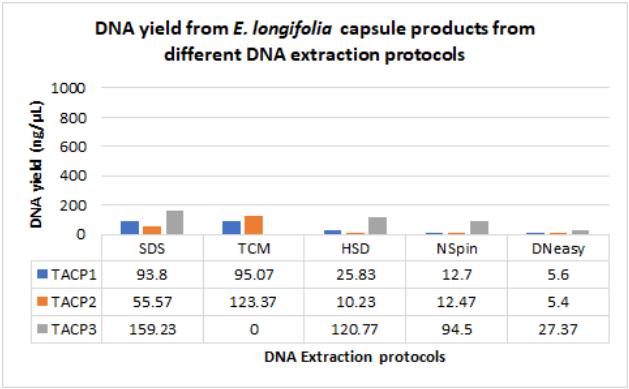
3.0 RESULTS AND DISCUSSION

Analysis of DNA Yield and Purity from Herbal Products

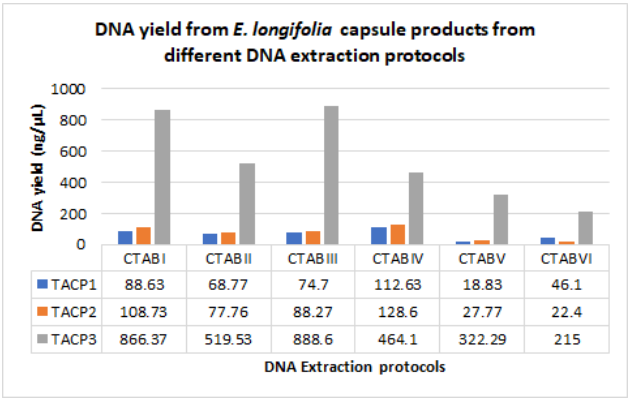
The results showed that the genomic DNA yield (Figure 1A and B) and purity readings (Figure 2A and B) for *E. longifolia* herbal products tested were varied with different extraction protocols used. Meanwhile for TACP-1 and TACP-2, the highest DNA yield was recorded at 112.63ng/µl and 128.6ng/µL respectively when extracted with modified CTAB IV. The present study proposed the purity reading of DNA for these high yielded modified extraction methods was between 1.4-1.66 which was considered acceptable for herbal products. Since genomic DNA is extracted from post-processed products, the quality of DNA may exhibit low quantitative performance due to intricate processing procedure and presence of excipients [7].

In the present study, extraction methods of modified CTAB I, II, II and IV were differentiated based on duration of DNA lysis and the use of phenol solution. From the results, increasing lysis duration in modified CTAB I and II lead to a better separation of DNA from impurities, resulting in higher DNA yield as compared to modified CTAB III and IV. In terms of phenol extraction (4 hours' lysis stage), modified CTAB I produced higher yield of DNA than non-phenol extraction from modified CTAB II with minor difference in DNA purity ratio between them. However, vice versa for TACP-1 and TACP-2 when extracted with phenol extraction (2 hours' lysis stage), the result showed that modified CTAB III scored lower DNA yield than non-phenol extraction (modified CTAB IV), with also a slight difference of DNA purity between them.

The use of double extractions of chloroform: isoamyl alcohol/ phenol: chloroform: isoamyl alcohol in modified CTAB V and modified CTAB VI produced good purity DNA at 1.41-1.87 absorbance ratios. In the CTAB V method, increased CTAB solution to 3% with 5M of sodium chloride in the lysis buffer resulted in good DNA purity (1.75) for both TACP-1 and TACP-2 but not for TACP-3 (1.4). Both modified CTAB V and VI gave slightly higher DNA yield for TACP-3 but not DNA purity. Sodium acetate was a common salt that could effectively help ethanol to precipitate nucleic acid by increasing the disaggregation of DNA. From the results, precipitation and re-precipitation of DNA using sodium acetate was not giving a high impact on DNA yield in TACP-1 and TCP-2. The function of SDS was to enhance cell disruption and retrieve more DNA. From the modified SDS method result, DNA purity was better in TACP-1 compared to TACP-2 and TACP-3 but highest DNA yield for TACP-3. The findings revealed that suitability of SDS based methods varied according to the individual type of herbal products presumably due to different types of impurities.



(A)



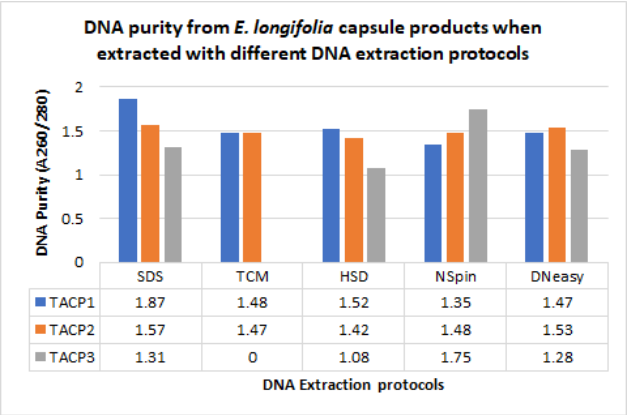
(B)

Figure 1 (A) and (B) The DNA yield of *E. longifolia* products using nine modified conventional methods and two commercial kits

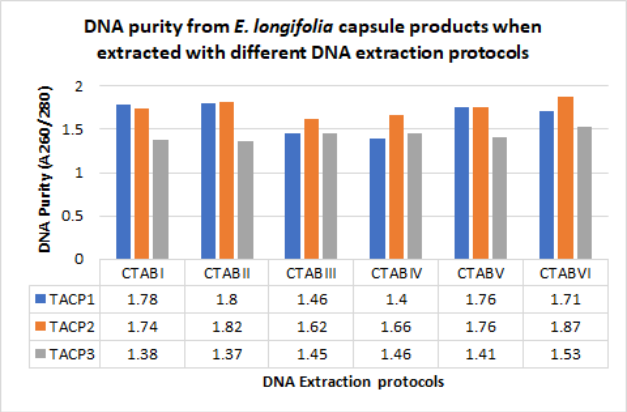
The procedure from TCM methods resulted in lower DNA purity (1.4) in both TACP-1 and TACP-2 but did not impact the DNA yield (95ng/µL and 123ng/µL respectively). No gDNA retrieved from TACP-3 from all tested conventional methods due to viscous and sticky pellet formation. However only commercial kits (NucleoSpin Plant II) did manage to recover gDNA from TACP3 products with purity of 1.75 and DNA

yield at 94ng/µL. This is because spin column traps short DNA and degraded DNA on the resin membrane with chaotropic salt wash away the PCR inhibitors. In all modified DNA extraction protocols, TCM methods were unable to recover the gDNA from all tested herbal products and failed to amplify the ITS2 (DNA purity of 1.4).

From the result, higher DNA yield was recorded for TACP-1 and TACP-2 with moderate DNA purity ratio (1.42-1.52), while for TACP-3 showed higher DNA yield but low in purity. A range of DNA yields between 464-88ng/µL in TACP-3 when extracted from modified CTAB I to IV. The range of purity were from 1.08 to 1.75. In the present study, the absence of CTAB and Polyvinylpyyolidone (PVP) and low salt concentration from extraction components was unable to remove phenols and impurities extensively in the Herbal Supplements Dietary (HSD) method that gave the lowest DNA purity (1.08) in TACP-3. The overall results showed that certain modified CTAB methods were able to extract DNA with various degrees of DNA yield and purity. The components of Tris-base, EDTA, sodium chloride, PVP and other components were sufficiently used to remove secondary metabolites and synthetic coloring, expedient, and concentrated dyes in these herbal products.



(A)



(B)

Figure 2 (A) and (B) The DNA purity of *E. longifolia* products using nine modified conventional methods and two commercial kits

Amplification of *ITS2* Barcode from Herbal Products

From Figure 3, successful amplification of ITS2 has been recorded with a difference success percentage between herbal products tested. Out of nine modified protocols, the percentage of successful amplification was 66% both for TACP-1 and TCAP-2 while 11% for TACP3. Compared with modified protocols, 100% success were observed when using commercial kits for TACP-1 and TACP-2. All amplified ITS2 fragments were at 370bp in size. The amplicon from TACP-3 showed a very faint band when using commercial kits (Figure 3 C: lane 10) but none from modified protocols. This indicates that modified steps and its components have not been efficiently worked for TACP-3, possibly due to unknown additional ingredients during formulation preparation.

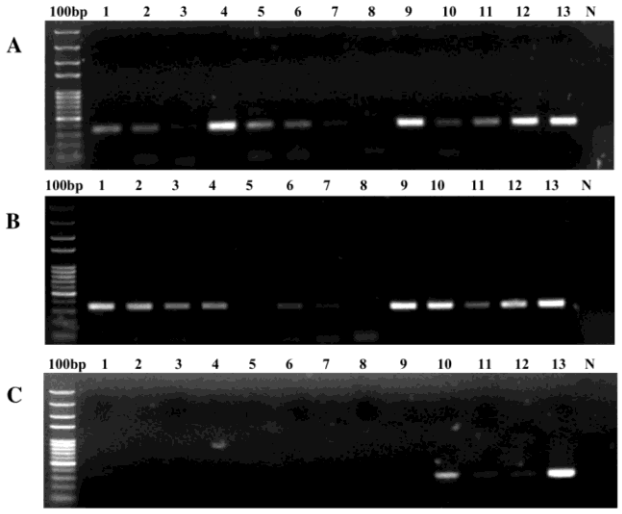


Figure 3 Amplification of *ITS2* from (A) TACP-1 DNA, (B) TACP-2 DNA and (C) TACP-3 DNA respectively. Lane 1: Modified CTAB I; Lane 2: Modified CTAB II; Lane 3: Modified CTAB III; Lane 4: Modified CTAB IV; Lane 5: Modified CTAB V; Lane 6: Modified CTAB VI; Lane 7: Modified SDS; Lane 8: TCM; Lane 9: HSD; Lane 10: NucleoSpin Plant II Kit; Lane 11: DNeasy Plant Mini Kit; Lane 12: *E.longifolia* roots; Lane 13: *E.longifolia* leaves; N: Negative control (Water). The DNA ladder was 100bp

Based on ITS2 fragment intensity, modified protocols from CTAB IV and HSD were best for TACP-1, HSD for TACP-2 while inconclusive for TACP-3. However, amplifiable ITS2 was recorded for TACP-3 from good purity of DNA. The PCR procedure showed no contamination from other DNA as indicated in Lane N without any band appearing. The present study indicates that failure of PCR amplification has resulted from low quality of DNA. The range of DNA purity for TACP-3 was 1-1.4 where unable to amplify the ITS2. However, the present study found that purity between (1.76 to 1.87) with appropriate DNA yield

resulted in PCR amplification. Based on observation, clearer and transparent aliquot of gDNA indicated purer DNA with less contamination compared to cloudy and pigmented aliquot that affected PCR amplification. In summary, the present study had shown that modified protocols from CTAB and HSD were able to retrieve the DNA and PCRed where these modifications can be used for other herbal products. The findings in the present study also showed that DNA yield and purity based on DNA extraction methods were different between the herbal products tested. The promising finding here would be beneficial to be used for DNA extraction from other herbal products such as tablet, beverages, and crude/dry roots in the same plant species or other plant species for molecular biology approach.

4.0 CONCLUSION

The present study revealed that each DNA extraction method has its own capabilities and limitations. These findings suggest that the quality of genomic DNA recovery was critical for successful PCR amplification.

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