

Genomic Extraction of Several Malaysian Herbal Plants

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Abstract

Herbal plants are found in abundant in Malaysian rain forests with various uses for its medicinal, pharmaceutical and nutritional value as well as the ordinary spice in our culinary. Among herbal plant studied include Java Tea, "Senduduk (purple & white)", "Daun Kaduk", Madagascar Periwinkle, Gardenia, Impatiens and Betel. As genomic DNA extraction method for each plant varies according to its properties and constituents, four extraction methods were chosen to determine the best method for each species. The methods selected were Doyle and Doyle Method (1989), Dellaporta *et al.* Method, Modified CTAB Method, and DNA and RNA Double Extraction Method. Modified CTAB Method was determined as the best extraction method for most of the plant studied except for one. Genomic DNA for Betel was best extracted using the Doyle and Doyle Method. The results obtained from the study varied for each plant and it proved that the constituents in each plant species contributed to the success of extracting genomic DNA of high quality and purity even from two closely related species such as "Daun Kaduk" (*Piper sarmentoum* Roxb.) and Betel (*Piper betel* Linn).

Keywords: Herbal plants, Genomic DNA, extraction, Doyle & Doyle, Dellaporta, Modified CTAB.

Introduction

Herbal and traditional medicines have been used for thousands of years to improve the health and well-being of civilization. They have been proven to have both medicinal and nutritive values. Herbs and medicinal plants are becoming popular these days as more and more people are inclined to use herbal remedies in their daily life. It is believed that as much as 80% of the world outside the industrialized countries especially the third world countries relies on herbs for health. As a matter of fact, various commercial pharmaceutical products originated from herbs.

Malaysia has abundance species of flowering and non-flowering plants of which a quarter are said to be of medicinal value. In the Malaysian rain forest, some 8,100 plant species are found and 10% are reported to have medicinal value [15]. However, only a few have been investigated fully for their potential. As the Malaysian flora diverse greatly, well diverse secondary metabolites can be anticipated from these medicinal plants. Biotechnology is expected to play a major role in the production of natural products through biosynthesis and bioengineering.

Genomic analysis is one of the elementary disciplines that pertains the manipulation of the genomes of an organism. A complete set of genes that control the overall organization of an organism is presented in its genome. In the molecular biology field, understanding an organism's genome, especially in plant, will considerably help impart essential genetic tools needed to maintain the plant development [1]. Plant exudes certain chemical compounds as secondary metabolites including the herbal plants and this can make the extraction of the plant's genome a hassle. The compounds such as alkaloids, tannins, essential oils and others while beneficial for treatment of different diseases [10, 14], can hinder the quality of DNA extracted.

As a science to identify the DNA sequences on various species, genomic analysis is the first essential step to study genes and its function in a particular species. In plant, various genes control the overall

development from the seed up until it bears the fruit. Technological advances in molecular biology and the application of these techniques to the analysis of plant gene structure and expression has increased the knowledge of cellular processes that control the plant gene production [2]. In order to further study the benefit and importance of herbal plant, genomic analysis is required for the identification, description and assessment of cellular functions on a molecular level of the gene in the plant genome.

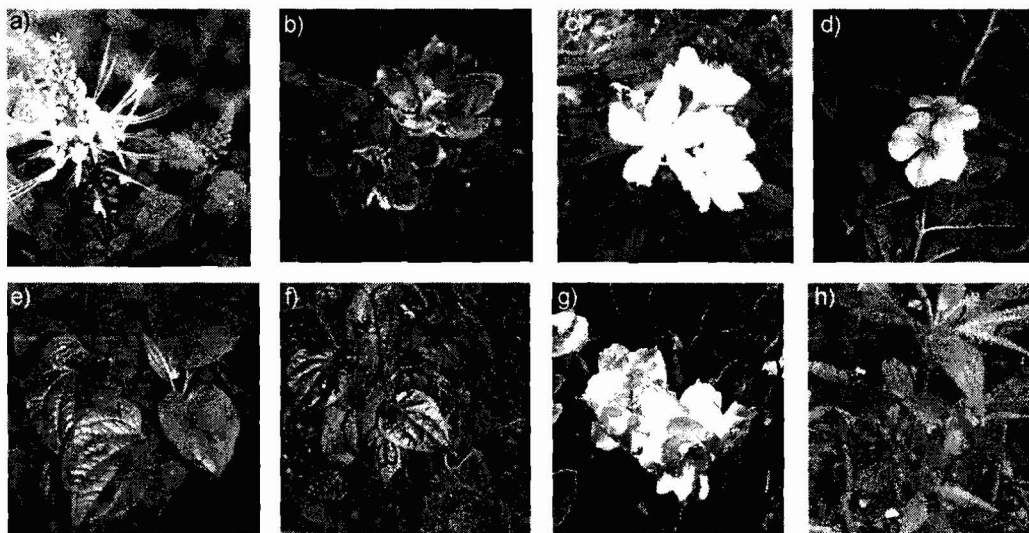


Figure 1: Local herbal plants. (a) Java Tea, (b) "Senduduk" (purple colored), (c) "Senduduk" (white colored), (d) Madagascar Periwinkle, (e) "Daun Kaduk", (f) Betel, (g) Gardenia and (h) Impatiens

Materials and Methods

Plant Material

Young leaves for each plant was used and ground to a fine powder.

Doyle & Doyle Method (1989)

The ground leaves were added with CTAB extraction buffer (1.4M NaCl, 1% PVP, 0.02M EDTA, 0.1M Tris-HCl, 2%CTAB, 0.2% mercaptoethanol), incubated at 65°C for 20 minutes before proceeded with phenol: chloroform purification. Following centrifugation, the aqueous phase was removed to a new tube and precipitated with isopropanol on ice for 10 minutes. The pellet collected was dissolved in TE buffer by gentle inversion.

Dellaporta et al. Method (1983)

A mixture of ground leaves with extraction buffer (100mM Tris-HCl, 50mM EDTA, 500mM NaCl, 10mM mercaptoethanol, 20% SDS) was incubated at 65°C for 10 minutes followed by the addition of 5M KOAc and incubation on ice for 20 minutes. After centrifugation, the aqueous phase was transferred to a new tube, added with isopropanol, incubated at -20°C for 30 minutes and spun. The pellet was dissolved in TE buffer while the aqueous phase was precipitated with 3M NaOAc and isopropanol. The pellet was then dissolved in TE buffer.

CTAB Modified Method

The hot CTAB buffer (without mercaptoethanol, heated at 65°C) was added to the ground leaves and incubated at 65°C for 5 minutes followed by chloroform: IAA purification. The top aqueous phase was transferred to a new tube, added with CTAB solution (5% CTAB, 0.7M NaCl) and the chloroform: IAA purification step was repeated until the aqueous phase became clear. The aqueous phase was then

transferred to a new tube; precipitated with 3M NaOAc and 100% cold ethanol for overnight and the pellet collected was resuspended in TE buffer.

DNA and RNA Double Extraction Method

Ground leaves with homogenization buffer (0.1M NaCl, 2% SDS, 50mM Tris-HCl, 10mM EDTA, 0.1mg/mL Proteinase K) was left at room temperature for 10 minutes and subjected to phenol: chloroform purification until the aqueous phase was clear. After final rinse with chloroform: IAA, 2M LiCl was added and the solution was incubated overnight at 4°C. Following incubation, the solution was centrifuged and the pellet containing the RNA was dissolved in TE buffer while the supernatant containing the DNA was subjected to overnight precipitation before the pellet was finally collected and dissolved in TE buffer.

DNA Purification

Genomic DNA was treated with equal volume of 2.5mg/mL RNase, incubated for 2 hours at 37°C, purified with phenol: chloroform, the aqueous phase precipitated before the pellet was collected and dissolved in TE buffer.

DNA Analysis via Electrophoresis

Genomic DNA from all methods was subjected to 1.2% agarose gel electrophoresis for verification.

Results and Discussion

Comparison of Methods Used for DNA Extraction

The isolation of pure and intact DNA of high molecular weight is essential in any molecular work. Molecular marker technology in plants especially for gene mapping and breeding in medicinal plants required such criteria [5, 18]. In order to determine the best extraction for all 8 herbal plants studied, four established methods were tested. Among the methods chosen for the study included the original version of one of the most established plant DNA extraction protocol by Doyle and Doyle in 1989 and Dellaporta et al. in 1983. As various plants with various medicinal properties may react differently with the extraction buffer in each procedure, the study could further enhance subsequent work with the best choice of method for obtaining pure and good quality DNA.

As shown in Figure 2 and Table 1, most of the plants were best isolated using the Modified CTAB Method. Only Betel (*Piper betel*) differs from the others as Doyle and Doyle Method suited the plant better. The result proved that even 2 plants with closely related family such as "Daun kaduk" (*Piper sarmentosum*) and Betel (*Piper betel*) differ in their properties, hence the isolation technique used. The other 2 plants that not only shared the same family and genus but look identical except for the color of the flower and bark; gave different result for each of the methods employed. While "Daun Kaduk" and Betel react differently to the methods used, the "Senduduk Putih" and "Senduduk" differ in terms of their genome sizes with the former having a much higher molecular weight than the latter.

Modified CTAB Method suited most of the plants studied but no single method can be used to obtain large quantities of pure DNA from all plant species as DNA isolation steps are empirical, mainly attributed to variability in plant tissue composition [7]. In addition, one method for extracting DNA may not be suitable and reproducible for all plant species [19].

In comparison, the procedures in the 4 methods did not differ much except for the components in the extraction buffer used as well as the incubation and precipitation time. Modified CTAB Method in truth is the modified version of Doyle and Doyle Method to simplify the process. The components in the extraction buffer remain the same except for the omission of β -mercaptoethanol in the modified version. Even though β -mercaptoethanol functions to inhibit oxidation by polyphenols, the modified method compensates that by applying an addition of CTAB and NaCl in a subsequent step to remove residual polysaccharide bound to the DNA [11, 13] and increase the yield of total cellular DNA [18]. CTAB a detergent, in conjunction with high salt concentration (NaCl) functions to solubilize the DNA complex [17]. Another difference between these two methods is the removal of contaminants or secondary metabolites. Doyle and Doyle Method employed phenol: chloroform followed by chloroform: isoamyl alcohol (IAA) [9] whereas the CTAB Modified Method escaped the phenol: chloroform step. While phenol: chloroform is more effective to remove protein, if phenol contamination

occurs, it might inhibits and reduces the efficiency of downstream reaction, sequencing and screening [6].

Dellaporta et al Method utilized the principle of salting out while the Double Extraction Method was able to extract both DNA and RNA. Both methods comprised of almost the same components with the previous two methods but at different concentration and the omission or addition of other components. In the salting out process, SDS together with high salt concentration precipitated the nucleic acid. However, the extraction buffer lacked PVP which functions to form complex hydrogen bonds with polyphenolic compounds. Thus the DNA obtained may still be contaminated heavily with these compounds as proved by some of the plants studied (Fig 2 (a), (b), (d), (f), (g)). Meanwhile, the Double Extraction Method applied Proteinase K that acted as an enzyme for protein degradation. Nevertheless, the enzyme gave varying yields of DNA as some may remain attached to protein and therefore lost in the phenol: chloroform extraction [8].

Table 1: Summary of the four extraction methods on Malaysian Herbal Plants

Plant	Doyle & Doyle Method, 1989	Dellaporta et al Method, 1983	CTAB Modified Method	DNA & RNA Double Extraction Method
Java Tea (<i>Orthosiphon aristatus</i>) Size/ Molecular Weight = 23 Kbp	Shearing of DNA with traces of protein / Polysaccharide (PS).	Good quality of DNA but with traces of protein / PS.	Good quality DNA.	Good quality DNA but slightly lower Molecular Weight (MW).
Madagascar Periwinkle (<i>Catharanthus roseus / Vinca rosea</i>) Size/ Molecular Weight = 23 Kbp	Shearing of DNA with traces of protein / Polysaccharide (PS).	Lightly lower MW DNA with traces of protein / Polysaccharide (PS).	Good quality DNA.	Good quality DNA but with traces of protein / PS.
Gardenia (<i>Gardenia jasminoides</i>) Size/ Molecular Weight = 23 Kbp	Bad quality DNA with shearing and contamination of protein / PS.	Low quantity DNA.	Good quality DNA.	Low quantity DNA with traces of protein / PS.
Impatiens (<i>Impatiens balsamina</i>) Size/ Molecular Weight = >23 Kbp	Bad quality DNA with shearing and contamination of protein / PS.	Bad quality DNA with shearing and contamination of protein / PS.	Good quality DNA with slight traces of protein / PS.	Bad quality DNA with shearing and contamination of protein / PS.
Senduduk Putih (<i>Melastoma decemfidum</i>) Size/ Molecular Weight = >23 Kbp	Good quality DNA with traces of protein / PS.	Inconsistent result as some produced sheared DNA.	Good quality DNA.	Good quality DNA but different MW and contamination of protein / PS
Senduduk (<i>Melastoma malabatrimum</i>) Size/ Molecular Weight = 10 Kbp	Low quantity DNA with protein/ PS contamination.	Low quantity DNA with traces of protein/ PS.	Good quality DNA.	No DNA obtained.
Daun Kaduk (<i>Piper sarmentosum</i>) Size/ Molecular Weight = 23 Kbp	Sheared DNA with protein/ PS contamination.	Sheared DNA with protein/ PS contamination.	Good quality DNA.	Low quantity DNA with protein/ PS contamination.
Betel (<i>Piper betel</i>) Size/ Molecular Weight = >23 Kbp	Good quality DNA.	Low quantity DNA with lower MW.	Sheared DNA with protein/ PS contamination.	Good quality DNA but contaminated with protein/ PS.

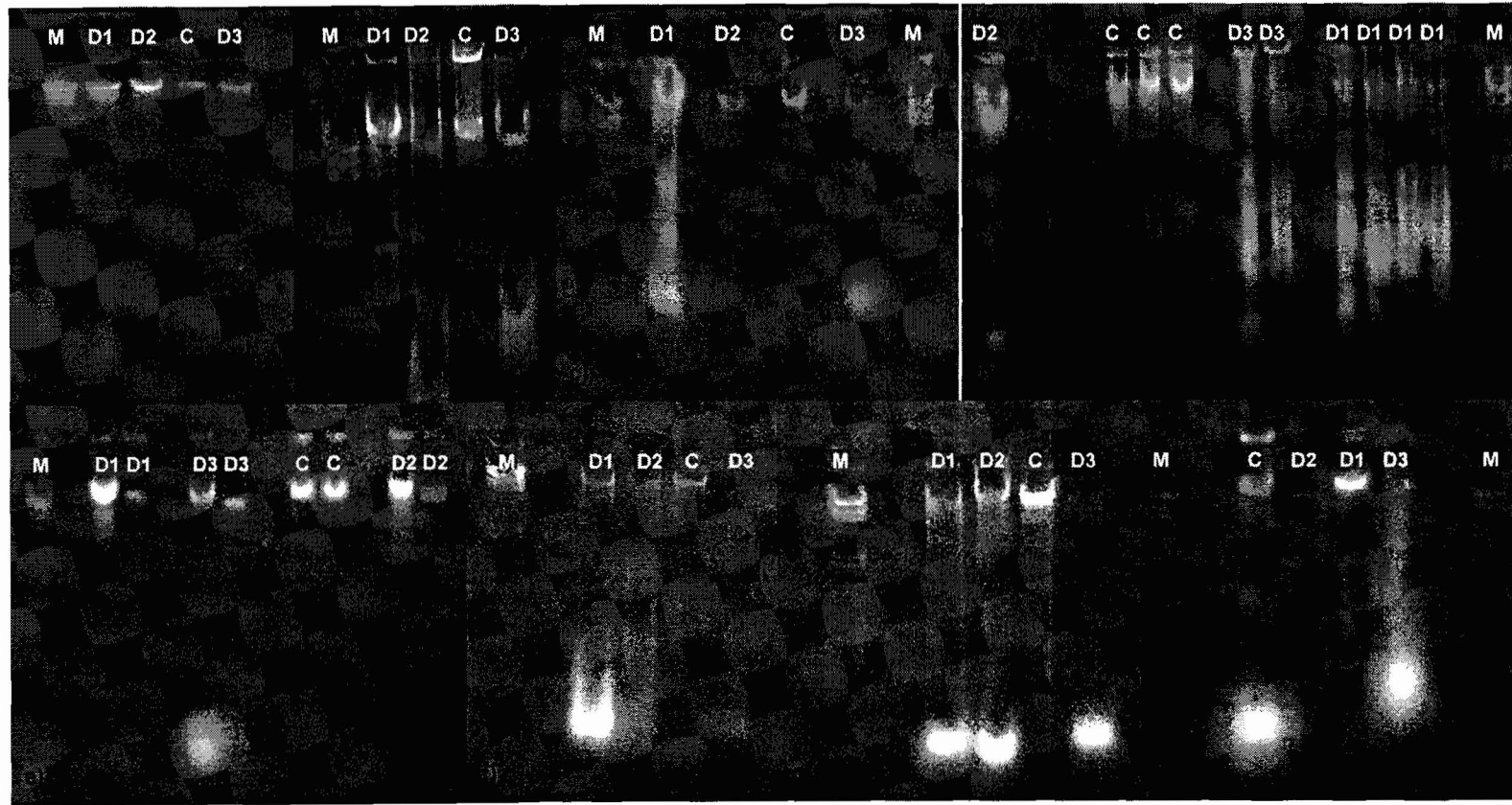


Figure 2: Genomic DNA extracted using the 4 methods from (a) Java Tea, (b) Madagascar Periwinkle, (c) Gardenia, (d) Impatiens, (e) "Senduduk" (white flowered), (f) "Senduduk" (purple flowered), (g) "Daun Kaduk" and (h) Betel. M= λ *Hind*III DNA marker, D1=Doyle & Doyle Method, D2=Dellaporta et al Method, C=Modified CTAB Method and D3=Double Extraction Method.

Other Contributing Factors to Pure and Intact Genomic DNA Isolation

Apart from the components in the extraction buffer and the steps involved in the isolation procedure, the source of material is equally important. In plant, various sources can be used from many of the plants' parts such as seed, leaf, embryo, petiole and flower. Among them, the most common source is the leaf as not all plants contain seed or flower [2]. Not just any kind of leaf can be used. Proper choice of leaf tissue is important in determining the quality of DNA extracted.

The age and the stage of leaf growth and development of the plant may play a role in the inconsistencies of extractions. Mature leaves have higher quantities of polyphenols, tannins and polysaccharides, causing contamination during the extraction [4, 16]. The concentration of the secondary metabolites and polysaccharides vary among tissue as well as with tissue age. Most of the plants used for this study were conducted using the young and partially expanded leaves as both are the best material for DNA extraction. The use of very young leaves however, has resulted in poor yields of DNA as reported by Lodhi et al. in 1994.

For the purpose of extracting DNA, the leaves should be thoroughly ground using liquid nitrogen. Freshly collected material is preferred. Another option would be to immerse the material briefly in liquid nitrogen until the leaves are frozen and kept in -20 to -80°C prior to use. Frozen material should be used within a month to preserve the DNA quality. By grinding the leaves, the homogenization of the plant material is made easier [3]. Grinding is also an important step as it releases the DNA from within the plant tissue. Certain study cautioned grinding the leaves to a very fine powder would result in shearing of DNA [7]. It was proven true for some plants conducted in this study, although the grinding technique plays a part as well. With the appropriate technique, the DNA should be intact as grinding to a fine powder gave greater yields [12].

Conclusion

From the study conducted, it was proven that the quality of DNA extracted from various plants vary depending upon the properties of the plant itself as well as the procedure used. Genomic DNA was successfully isolated from 8 Malaysian herbal plants. Among the 4 methods applied, 7 of the plants gave good quality of DNA using the Modified CTAB Method while Betel using the Doyle and Doyle Method. Different methods to obtain genomic DNA for plants that come from the same family and genus such as "Daun Kaduk" and Betel were used. In conclusion, no one method is suitable for all plant even from closely related family. In addition, the choice of material and grinding technique attribute to the success of acquiring high quality and pure DNA.

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