Journal of Medicinal Plants and By-products (2020) 1: 71-78

Original Article

A Simple, Quick, Efficient and Cost-effective Method for Isolating Genomic DNA from Medicinal Plant Lemon Balm

Sara Ghaffarian[1,2] and Seyyed Abolghasem Mohammadi[2,3*]

[1]Departement of Biology, Faculty of Natural Science, Azarbaijan Shahid Madani University, Tabriz, Iran
[2]Department of Plant Breeding and Biotechnology, Faculty of Agriculture, University of Tabriz, Tabriz, Iran
[3]Center of Excellence in Cereal Molecular Breeding, University of Tabriz, Tabriz, Iran

Article History: Received: 15 September 2019/Accepted in revised form: 07 December 2019

Abstract

Lemon balm (*Melissa officinalis* L.) is popular as an alternative to standard medicine for a variety of problems and its essential oil is widely used in pharmacology. The isolation of good quality DNA is the pre-requisite for molecular research. Due to high amounts of polysaccharides, polyphenols and various secondary metabolites, DNA extraction from lemon balm is problematic. In this study, three different CTAB based methods by some modification were compared for the isolation of high-quality genomic DNA from lemon balm. The DNA from the leaves of five lemon balm ecotypes was extracted using CTAB methods A (with PVP and phenol), B (PVP, Proteinase K and Na acetate) and C (PVP, Tri sodium citrate and NaCl). The quality of DNA samples was determined by physical appearance, agarose gel electrophoresis, spectrophometer, PCR amplification and restriction. Based on the results of various DNA quality analysis, the CTAB method C was found to be the most efficient one. Method C was found to be the most efficient DNA extraction method for lemon balm providing high DNA yields with better quality, in short time with less cost. It could affordable for DNA extraction from medicinal plants with similar secondary metabolites content.

Keywords: DNA extraction, *Melissa officinalis*, PCR amplification, Restriction digestion.

Introduction

Lemon balm (*Melissa officinalis* L.), a member of Lamiaceae, is a perennial plant originated from southern Europe, Asia and parts of North America. Lemon balm populations are distributed in all Mediterranean countries including regions of Turkey and northern Iran [1]. Currently lemon balm is widely used as sedative or calm, spasmolytic and antibacterial agent and sleep aid [2]. There are up to 70 different compounds in lemon balm oil. The known major components of lemon balm are reported to include geranial and neral [3]. It is essential oil is used in medicine as anti-tumor, anti-bacterial, antimicrobial, antispasmodic and antioxidant [4].

Based on the World Health Organization estimate, more than 80% of the world's population depends on herbal medicine for primary healthcare needs. Most plant materials used in herbal medicine and other related industries are taken from wild plant resources. The rapid growing demands for medicinal plants, compounded by habitat loss, are exerting pressure on many medicinal species [5]. Therefore, characterization of the medicinal plants for their conservation and utilization is the most important concern of biodiversity conservation worldwide [6,7]. By the advanced of molecular techniques, medicinal plants have been subjected to extensive genetic studies due to their worldwide medicinal importance and conservation objectives. DNA-based molecular marker technologies have been widely used in medicinal plants for analysis of genetic diversity and population differentiation [8-13] and construction of linkage map [14].

By the progress in the application of molecular techniques in medicinal plants studies, isolation of

*Corresponding author: Department of Plant Breeding and Biotechnology, Faculty of Agriculture, University of Tabriz, Tabriz 5166614766, Iran
Email Address: Mohammadi@tabrizu.ac.ir

impact, high-molecular mass genomic DNA becomes an important pre-requisite. In spite of published numerous protocols for DNA extraction from plants [15-20], the problem of DNA extraction from medicinal plants is still an important issue in the field of plant molecular biology. Plant species of the same or related genera can exhibit enormous variability in the complexity of pathways of unnecessary functions. Therefore, the biochemical composition in plant tissues of various species is vary considerably and this heterogeneity among species may not allow optimal DNA yield with a single protocol. Thus, even closely related species may require different DNA isolation protocols [21,22]. Therefore, it's necessary to change a protocol or combine more different protocols to obtain a high quantity DNA of the desired quality. A good extraction protocol should be simple, rapid and efficient, yielding appropriate levels of high quality DNA suitable for molecular analyses [23].In most cases, especially in population analysis, it is not cost effective to use extraction kits despite of their efficiency.

Several factors including degradation of DNA due to endonucleases, co isolation of highly viscous polysaccharides and inhibitor compounds like polyphenols and other secondary metabolites which directly or indirectly interfere with the enzymatic reactions restrict the isolation of high molecular weight DNA from medicinal plant species [24].In medicinal plants, presence of polysaccharides, polyphenols, essential oils and other secondary metabolites produces extra problems in the purification of DNA. The presence of polyphenols, can reduce the yield and purity of extracted DNA [25] and polyphenols binds to DNA, giving it a brown color and making problem in the most molecular analyses in their oxidize form [26]. Also, polysaccharides plenty presence in medicinal plant and interfere with activity of enzymes in restriction digestion and PCR amplification [27]. For example, acidic polysaccharides inhibit Hind III enzyme restriction, thereby precluding amplification [28, 29] by inhibiting *Taq* DNA polymerase activity [30].

There are several successful genomic DNA isolation protocols but none of these are universally applicable to all plants [31]. Moyo *et al*. [32] optimized CTAB-based protocol for extracting high quantity and quality of genomic DNA from various medicinal plant species and the isolated DNA was amenable to restriction endonuclease digestion and PCR amplification. Also Pateraki and Kanellis [33] described optimized protocols that yield high quality DNA and total RNA from *Cistus creticus*, with metabolites such as terpenoids, polyphenols, Xavonoids, glycosides, and resin.

The aim of this study was to develop a protocol for high quantity and quality DNA extraction from lemon balm leaves. Modifications were focused on minimizing phenolic compounds and polysaccharide co-isolation in DNA, maintaining the good quality of the DNA.

Material and Methods

Plant Materials

The plant materials included three lemon balm Iranian wild ecotypes and two varieties from Japan and Germany. Leaf samples were collected from 15 field grown plants for each genotype, transported to laboratory in liquid nitrogen and stored at -80ºC until use.

DNA Extraction

DNA was extracted from leaf tissue by means of the three modification of CTAB protocols.

Method A

Total genomic DNA from leaf tissue was extracted in CTAB isolation buffer as described by Saghai Maroof *et al*. [34] with modifications on concentration and amount of the components and using various chemicals for removing polyphenols, polysaccharides and etc. Also, salt solution applied for removing remaining contaminations.

Solutions:

- CTAB extraction buffer (20 mM sodium EDTA, 100 mMTris-HCl, 1.4 M NaCl, 2.0% (w/v), CTAB)
- TE buffer (pH:8) (10 mMTris-HCl and 1 mM EDTA)
- Salt solution 10X (pH: 8) (NaCl 2.5 M, EDTA 20mM and Tris-HCl 0.5 M)
- 10% Polyvinylpyrrolidone (PVP)
- 5% Sodium Dodecyl Sulfate (SDS)
- 5% N-lauryl sarcosin (sarcosyl)
- 0.2% β-mercaptoethanol
- Chloroform-isoamyl alcohol (24:1)
- Liquid nitrogen
- 8 mMNaOH
- Isopropanol
- 5M NaCl
- RNase A

Protocol:

1. Grind 0.1g of leaf tissue in liquid nitrogen using mortar and pestle, transfer to 4.0 mL Eppendorf tube, add 1000 µLwarm CTAB buffer, 200 µLPVP, 200 µL SDS, 200 µL sarcosyl and 7.5 µL β-mercaptoethanol and swirl.
2. Incubate at 65 ºC for 45-60 min and mix every 5 min during incubation.
3. Incubate at room temperature for 5 min and add 2 mL chloroform: isoamyl alcohol (24:1 v/v) and mix.
4. Centrifuge for 20 min at 4000 g at room temperature.
5. Transfer top aqueous phase to new Eppendorf tube, add 1000 µL of NaCl and mix.
6. Add 1000 µL of cold Isopropanol and leave it for 1-2 hour at -20ºC.
7. Centrifuge for 20 min at 4000 g.
8. Discard supernatant and dissolve pellet in 300µL NaOH.
9. Mix 1 volume of 10X salt solution and 9 volume of DNA for 4 hour.
10. Add 1/3 volume of absolute ethanol and incubate on ice for 15 min.
11. Centrifuge for 20 min at 4000 g (4 ºC).
12. Transfer top aqueous to new tube and add equal volume of isopropanol.
13. Incubated at -20 ºC for 30 min.
14. Centrifuge for 20 min at 4000g and discard top aqueous.
15. Dissolve pellet in 300 µL TE buffer and treat with RNase A at 37 ºC for 30 min.

Method B

This protocol was based on previous method with using phenol, proteinase K and Na-acetate for removing some of pollutions.

Solutions:

- CTAB extraction buffer
- TE buffer
- 10% Polyvinylpyrrolidone (PVP)
- 0.2% β-mercaptoethanol
- Chloroform-isoamyl alcohol (24:1)
- Liquid nitrogen
- Isopropanol
- Proteinase K
- Na acetate
- RNaseA

Protocol:

1. Grind 0.2 g leaf tissue in liquid nitrogen with mortar and pestle, transfer to 4.0 mL Eppendorf tube containing 1000 µL warm extraction buffer and swirl.
2. Add 200 µL PVP, 200 µL sarcosyl, 400 µL SDS and 7.5 µL β-mercaptoethanol and mix
3. Incubate at 65 ºC for 60 min and mix every 5 min during incubation.
4. Immediately add an equal volume of chloroform: isoamyl alcohol (24:1, v/v) and mix slowly. Centrifuge for 20 min at 4000 g and transfer supernatant to new tube.
5. Repeat chloroform: isoamyl alcohol stage to remove white aerosols completely.
6. Added 1/2 volume of NaCl and mix.
7. Centrifuge for 30 seconds at 4000 g and replace supernatant in new tubes.
8. Add an equal volume of isopropanol and incubate for 2-3 hour at -20 ºC.
9. Centrifuge at 4000 g for 20 min and air dry pellet at room temperature.
10. Dissolve Pellet in 300 µL TE buffer.
11. Add 3µL RNase per tube and incubate at 37 ºC for 60 min.
12. Add 3µL proteinase K per tube and incubate at 37 ºC for 60 min.
13. Add 150 µL phenol and 150 µL chloroform per tube.
14. Centrifuge for 15 min at 11500 g and transfer supernatant to new 1.5 mL tube.
15. Add 1/10 volume of absolute Na acetate and 2 volume of absolute ethanol and mix well.
16. Incubate over night at -80 ºC.
17. Centrifuge for 20 min at 11500 g.
18. Discard supernatant and wash with ethanol (70%).
19. Air dry pellet, dissolve in 100 µL TE buffer and treat with RNase A at 37 ºC for 30 min.

Method C

Solutions:

- CTAB extraction buffer
- TE buffer
- 10% Polyvinylpyrrolidone (PVP)
- 0.2% β-mercaptoethanol
- Chloroform-isoamyl alcohol (24:1)
- Tri sodium citrate
- Liquid nitrogen
- Isopropanol
- 5 M NaCl
- RNase A

Protocol:

1. Grind 0.2 g leaf tissue in liquid nitrogen using mortar and pestle and transfer to 2 mL tubes contain 800 µL warm extraction buffer.
2. Add 200 µL PVP and 60 µL β-mercaptoethanol per tube and incubate at 65ºC for 30-60 min.

3. Immediately add an equal volume of chloroform: isoamyl alcohol (24:1 v/v) and mix complete slowly.
4. Centrifuge for 20 min at 11500 g and transfer supernatant to new tube.
5. Add 1/3 volume NaCl and mix.
6. Add 0.15 g tri sodium citrate and dissolve.
7. Centrifuge for 30 second at 13000 rpm.
8. Transfer supernatant to new tube.
9. Add 700 µL isopropanol and incubate for 20 min at -20 ºC.
10. Centrifuge for 20 min at 13000 rpm.
11. Discard supernatant, air dry pellet, dissolve in 100 µL TE buffer and treat with RNase A at 37 ºC for 30 min.

Determination of DNA quality and quantity

The quantity of DNA extracts was estimated *via* spectrophotometry (Bio Photometer, Eppendorf AG, Germany) at 260 nm assuming that an absorbance of 1.0 U corresponds to a DNA concentration of 50 µg/mL [34]. In addition, DNA purities were evaluated via the absorbance rations $A_{260/280}$.Agarose gel (0.8%) electrophoresis was also performed to determine DNA quality.

PCR Amplification

For PCR analysis, DNA samples were diluted to a working concentration of 20 ng/µL. Inter retrotransposon regions were amplified in a DNA Thermal Cycler (AB, USA) using two RTN primers Nikita (5'CGCATTTGTTCAAGCCTAAACC3') and Sukkula (5'GATAGGGTCGCATCTTGGGCGTGAC3') barley's retrotransposon families based on IRAP technique. The PCR carried out in a final volume of 10 µL containing approximately 40 ng of genomic DNA, 1 U of *Taq* DNA polymerase (Cinagen, Iran), 2 mM of dNTP, 1.5 mM MgCl2 and 12 pmol of each primer. PCR thermal cycling conditions were initial denaturation at 95 °C for 4 min followed by 40 cycles of 95 °C for 1 min, 55 °C for 2 min, and 72 °C for 2 min and a final extension at 72 °C for 9 min. The amplified products were resolved using 4% polyacrylamide gel on GelScan 3000 (Corbett Robotics, Australia).

Digestion Quality of DNA

To further check of DNA quality, DNA samples were digested using *Hin*d III and *Eco* R I. The digestion reactions were carried out final volume of 15 µl containing 5 µL of DNA, 5 µL of ddH2o, 2 U of restriction enzyme (Fermentase, USA) and 3 U of restriction buffer. The reaction was incubated for 24 hours at 37 ºC. The digested DNA was resolved on 1% agarose gels.

Results

Studying physical appearance showed that using either of procedure A and B yields yellowish DNA with dark brown contaminations. In return using our protocol C colorless DNA was obtained from all samples. In this case, all samples of procedure A were deep dark brown, samples belonging to procedure B had amounts of yellowish contaminations and pellets obtained from procedure C were completely transparent.

In molecular biology extracted DNA must be completely intact without sharing for most goals in molecular biology. As evidenced by agarose gel electrophoresis, all methods yielded relatively high molecular weight DNA and obtained DNA was unshared, in all three methods. RNA pollutions were removed by RNase treatment. A representative 0.8% agarose gel containing DNAs extracted using procedure C after RNasetreatment is shown in the Fig. 1.

The ratio of absorbance at 260 nm and 280 nm is used to assess the purity of DNA and RNA. A ratio of ~1.8 is generally accepted as pure DNA. If the ratio is appreciably lower in either case, it may indicate the presence of protein, phenol or other contaminants that absorb at or near 280 nm. This ratio is used as a secondary measurement of nucleic acid purity [36]. Also, the ratio of absorbance at 260 nm is used to assess DNA quantity. Spectral absorbance ratio of 260/280 nm and 260 nm are shown in Table 1. The spectral absorbance of method A was not detectable because of viscous contaminations. The isolated DNA using procedure C had normal spectra in which the A260/A280 ratios were 1.8 in average. We used 0.2 g leaf tissue for extraction in method C. In comparison to procedure B that used 1 g leaf tissue, procedure C had higher quantity efficiency too.

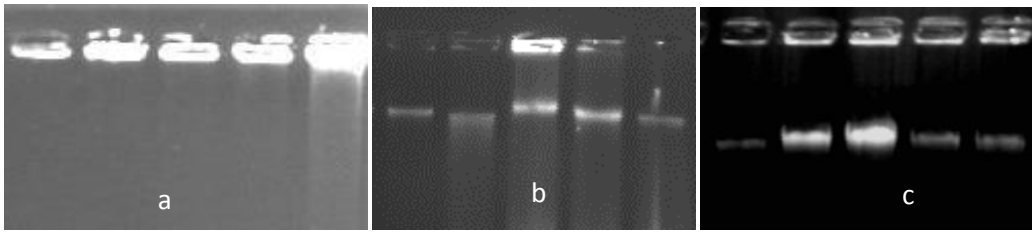

Fig. 1 Agarose gel (1%) electrophoresis of total uncut genomic DNA isolated as described in method C. Six to eight µL of DNA with 6 µL loading dye were loaded in each lane. Lane L, 100-3000 bp molecular weight marker (Fermentase, # SM0321).

Table 1 DNA yield (260nm) and purity (260/280nm) range obtained for all sample extracts using methods 2 and 3.

Method		B	C	
Absorbance	260	260/280	260	260/280
1	616	1.51	640	1.80
2	775	0.84	458	1.69
3	975	1.20	621	1.86
4	199	1.42	991	1.84
5	541	2.05	1019	1.83

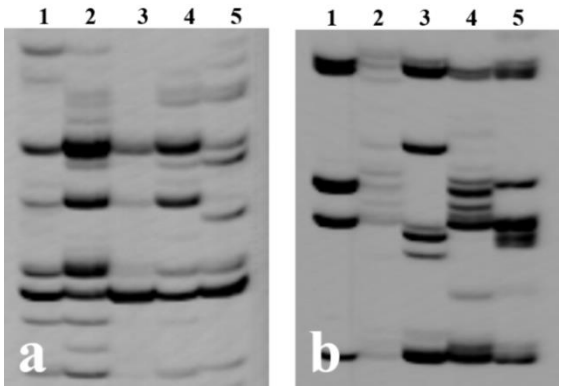

Fig. 2 Polyachrilamide gel electrophoresis (4%) of method C PCR products amplified with different primers, Sukkula (a) and 3´LTR (b).

Apart from importance of DNA quality and quantity, a protocol is reliable when extracted DNA is useful in enzymatic reactions such as restriction digestion and amplification which confirms contaminations elimination. So, the suitability of extracted DNA for downstream molecular processes was further verified by PCR amplification and DNA digestion. Polymerase chain reaction was used to amplify genomic fragments from lemon balm genotypes using primers designed based on barley Sukkula and Nikkita LTR retrotransposons. Amplification with two pair primers and digestion with two endonuclease enzymes were completely successful and DNA samples extracted using methods C were amplified fragments with high quality and quantity but DNAs yielded using procedure A and B failed to amplify retrotransposons (Fig. 2).

All genomic DNAs extracted using method C completely restricted with both Hind III and Eco RI enzymes. Digested genomic DNA isolated as described in materials and methods and fractioning of digestion products are showed in Fig. 3.

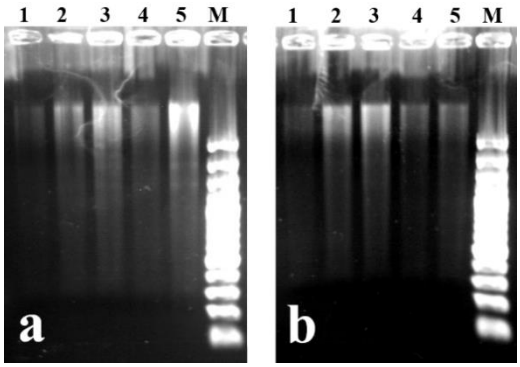

Fig. 3 Agarose gel electrophoresis (1%) of method C restriction digestion products digested with different enzymes, Hind III (a) and Eco RI (b). Lane L, 100-3000 bp molecular weight marker (Fermentase, # SM0321).

Discussion

The CTAB-based method as commonly used DNA extraction procedure has been modified several times to reduce contaminants such as polyphenols and polysaccharides that are present in the plant tissues [15-18]. Although all currently published methods of DNA extraction may have their effectiveness in isolating DNA that is suitable for PCR amplification or restriction digestion, in medicinal plants presence of polysaccharides, polyphenolic compounds, tannins, alkaloids and phenols limit the use of available methods [32].

Published methods of DNA isolation including those of Saghai Maroof *et al*. [34], Lodhi *et al*. [35] and their modification in a few ways and using Fermentase PCR cloning kit for purifying extracted DNAs by these methods proved unsuccessful and unreliable for lemon balm [36]. As the obtained DNA was dirty yellow, with high viscosity and unsuitable for PCR amplification. This may be due to high endogenous levels of polysaccharides, phenolic and other organic constituents which interfere with DNA isolation and purification, since there are various secondary metabolites especially large amounts of polyphenol compounds in *M. officinalis*. The none of isolated DNA samples were subjected to even agarose gel electrophoresis.

Finally, we attempted to establish a reliable method for isolation of high quality DNA from *M. officinalis*. To this purpose, DNA purity analyzed by standards including physical appearance, viscosity, A_{260}/A_{280} ratio, digestibility with restriction enzymes and so suitability for DNA digestion based molecular techniques such as Restriction Fragment Length Polymorphism (RFLP), capability to amplify by *Taq* DNA polymerase for Polymerase Chain Reaction based molecular techniques such as SSR and so suitable for other DNA based molecular techniques.

As showed in results, procedure A was not suitable for removal of contaminations. DNA products of procedure B had lower contaminations which might because of: (1) using phenol in extraction process, remaining low phenol pollutions with DNA and therefore low oxidation, and (2) centrifuging for depositing and removing brown contaminations that appears after adding NaCl. But still there were amounts of contaminations. Whereas Saghai Maroof *et al*. [34] and Lodhi *et al*. [35] extraction products had very high amount of brown contaminations. In procedure C extracted DNA was completely transparent and had no chromatic contamination, in absence of phenol. Some of contaminations removed by short time centrifuging which removed dark brown contaminations and some others by tri sodium sulfate salt.

Successful extraction of genomic DNA that can be amplified by PCR and digested by restriction enzymes confirms DNA high purity and can lead to the establishment of DNA fingerprinting for the individual genotypes for various molecular approaches. As discussed before three important problematic pollutions are polyphenols, polysaccharides and proteins. Protocol C applies some general compounds for lysis and removing of them. These included a detergent such as cetyltrimethyl ammonium bromide (CTAB) which disrupts the membranes, B mercaptoethanol which helps in denaturing proteins and for removing the tanins and polyphenols, chloroform: isoamyl alcohol which the nucleic acid solution is extracted by successively washing with, Alcohol for nucleic acid precipitation, tri sodium citrate which aids in precipitation by neutralizing the negative charges on the DNA [37], NaCl which helps in removing polysaccharides [30] and PVP to purge polyphenols [38]. Finally, procedure C found advantageous that, it's simple and rapid, there is no need to expensive and rare compounds, yields high DNA concentration using low amounts of tissue, provides high quality DNA despite mini prep process and its utilizable for plants with high concentration of polyphenols and polysaccharides

Conclusion

In this study, three DNA extraction methods were compared to present DNA isolation protocol for lemon balm which produces high quality DNA that can be efficiently amplified using PCR and digested using endonucleases. The most problematic object faced in the isolation of high molecular weight DNA from medicinal plant species is co isolation of highly viscous polysaccharides and inhibitor compounds like polyphenols and other secondary metabolites which interfere with the enzymatic reactions. Among the DNA extraction methods analyzed in this study, the modified extraction method C was found to be the most efficient in isolating high DNA yield with better quality from *M. officinalis.*Th DNA extracted using this protocol can be used for all DNA amplification and DNA digestion based molecular techniques such as molecular marker studies, cloning, gene mapping, advanced sequencing technologies, marker assisted selection and etc. Also, this method can be used to extract DNA from other medicinal plant which has similar secondary metabolite content as lemon balm.

Acknowledgements

We acknowledge the lab support by Center of Excellence in Cereal Molecular Breeding, University of Tabriz, Tabriz, Iran.

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