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Optimized DNA extraction and purification method from *Alchemilla* species using polyethylene glycol

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Abstract

Pure DNA is essential in various techniques of molecular biology and its extraction from plants to produce large amounts of secondary metabolites is a difficult task. *Alchemilla* is known to synthesize a large number of secondary metabolites which reduce the quality of the extracted DNA. This study, aimed to set up a method for high-quality DNA isolation from *Alchemilla* leaf. For this purpose, three extraction methods were examined and a comparison concerning price, simplicity, and security was carried out. We also optimized a CTAB-based method using increasing the volume and concentration of CTAB buffer, lysis time, and cold incubation period, performing six times dilutions, and three times precipitations, adding polyethylene glycol, and removing toxic or expensive materials. The results showed that, 260/280 and 260/230 ratios of extracted DNA by the optimized method with the concentration of 595–387 ng/µL were 1.75–1.82 and 1.56–1.68, respectively. The quality of extracted DNA by this method was significantly higher ($p < 0.001$) than that of other ways, so that all samples were positive for DNA, as assessed by electrophoresis and PCR. The optimized method was simple, effective, reproducible, relatively non-toxic, and inexpensive. The results revealed that, this method was successful in producing large amounts of high-quality amplifiable DNA.

Keywords: Nucleic acid purity, phenolic compounds, polysaccharide compounds, secondary metabolites, spectrophotometry

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روش بهینه‌سازی شده جداسازی و تخلیص DNA از جنس *Alchemilla* با استفاده از پلی‌اتیلن گلیکول*

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خلاصه

استخراج DNA برای مطالعات مولکولی ضروری بوده و استخراج خالص آن از گیاهان با مقادیر زیاد متابولیت‌های ثانویه بسیار مشکل است. جنس *Alchemiila* L. متابولیت‌های ثانویه متنوعی تولید می‌کند که باعث کاهش کیفیت DNA استخراجی می‌شوند. این مطالعه، با هدف استخراج آسان و ارزان DNA با کمیت و کیفیت بالا از نمونه‌های برگ این جنس انجام شد و نهایتا، سه روش استخراج DNA با هم مقایسه گردیدند. در انتخاب روش بهینه، چند نکته اساسی شامل هزینه، سادگی و حذف مواد شیمیایی خطرناک مدنظر بود. این مطالعه، با ایجاد تغییرات قابل توجهی در روش CTAB از قبیل تغییر حجم و غلظت بافر استخراج، مدت زمان انکوباسیون‌ها، انجام شش مرحله شستشو و سه مرحله رسوب، افزودن پلی‌اتیلن گلیکول و همچنین حذف مواد پرهزینه و خطرناک مانند نیتروژن مایع و بتا مرکاپتواتانول، قادر به جداسازی ایمن و ارزان، مقدار زیادی DNA با کیفیت مطلوب شد. کمیت و کیفیت DNAهای استخراجی توسط روش نانومتری، الکتروفورز ژل آگارز و واکنش زنجیره‌ای پلیمراز مشخص گردید. طبق نتایج به دست آمده در این مطالعه، نسبت ۲۶۰/۲۸۰ DNA استخراجی به روش بهینه‌سازی شده، بین ۱/۷۵ تا ۱/۸۲و نسبت ۲۶۰/۲۳۰ بین ۱/۵۶ تا ۱/۶۸ با غلظت ۳۸۷ تا ۵۹۵ نانوگرم در میکرولیتر اندازه‌گیری شد که این اعداد به طور قابل توجهی ($p < 0.001$) از اعداد مربوط به DNA حاصل از سایر روش‌ها بیشتر بود. پروتکل بهینه‌سازی شده، ساده، مؤثر، قابل تکرار، نسبتا غیرسمی و ارزان بود. نتایج این تحقیق همچنین نشان داد پروتکل مذکور در تولید مقادیر کافی از DNA با کیفیت قابل قبول برای سنجش‌های ژنتیکی موفق بود.

واژه‌های کلیدی: اسپکتروفتومتری، ترکیبات پلی‌ساکارید، ترکیبات فنلی، خلوص اسید نوکلیک، متابولیت‌های ثانویه

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Introduction

Molecular methods used for genetic studies of the plant species rely on the extraction of pure, intact, and high-quality DNA. Molecular methods mostly complete the classic, ususlly morphology-based systematics. DNA research, allow establishing the taxonomic identity of samples and reassessing the obtained results (Gaudeul & Rouhan 2013). Partial or total nuclear DNA degradation (by endogenous), presence of polysaccharides and phenolic compounds are common problems that can occur during isolation and purification of DNA from plant tissues. These chemical components with a strong attraction to DNA are powerful oxidizing agents that covalently bind to nucleotides and inhibit the function of Taq polymerase, thus affecting the PCR efficiency (Weishing *et al*. 1995, Varma *et al*. 2007, Saboora *et al.* 2019). Oxidized forms of polyphenol react with the nucleic acid and lead to browning and reducing the retention time of the DNA sample (Katterman & Shattuck 1983). Therefore, in the extraction procedure, the target nucleic acid should be the most intact and without contaminants.

Mature tissues have high quantities of polyphenols, polysaccharides, and tannins (Dabo *et al*. 1993) so it is suggested that, plant samples should be fresh and young. DNA degradation starts immediately after the collection of samples. Therefore, if leaf buds or young leaves (which include many cells with high DNA content) dry with silica gel within ca. 12 h after collection, DNA will degrade less (Gaudeul & Rouhan 2013). But it may not always be possible to obtain DNA from fresh tissues. Hence, there was a need to optimize the genomic DNA extraction method so that, the dried plant is also useful. On the other hand, because of biochemical composition differences among different species introducing one isolation method that is optimal for all species is almost impossible (Weishing *et al*. 1995). Many commercially available kits are easy to use and give positive results, but they are not affordable (Ahmed *et al*. 2009). Several DNA isolation methods have been introduced but unfortunately, occasionally they are inefficient.

Like most members of the *Rosaceae* family, *Alchemilla* L. species contain a large number of secondary metabolites especially: flavonoids, flavonol, phenols, polyphenols, glycosides, terpene, tannins, hydrocarbons and resins (Felser & Schimmer 1999, Fraisse *et al*. 2000, Falchero *et al*. 2009, Trendafilova *et al.* 2012, Duckstein *et al*. 2013). During our phylogenetic analysis (summer 2017 & summer 2019), we noticed DNA extraction problems, vigorously prohibiting the pure DNA extraction from *Alchemilla* leaves. We encountered some problems during the isolation and purification of DNA from fresh and dried leaves of *Alchemilla* by conventional methods DNA extraction. The colors of obtained DNA pellets at the end of extractions were yellow, brown or even black. None of them were successful in electrophoresis and PCR amplification. Although we performed different PCR methods and used different primers, no DNA band was observed. Yellow and brownish colored DNA pellets indicate contamination by phenolic compounds (Weishing *et al*. 1995). Also, genomic DNA of leaves of *Alchemilla* was previously extracted by Gehrke *et al*. (2008 and 2016) using the Qiagen DNeasy Plant Mini Kit with modification. They reported that the presence of secondary compounds to make it difficult target for PCR amplification and sequencing. On the other hand, these kits each having their limitations and are very expensive.

The primary purpose of this work was to found a reproducible, affordable, relatively non-toxic, and efficient method for isolation of pure and high-yield DNA from the leaf of the genus *Alchemilla*. For this reason, several factors affecting DNA isolation examined as follows: changing buffer composition and concentration and incubation time, adding some components, repeating some steps that improved the DNA quality. In the present work, we compared the effectiveness of three different procedures used for the extraction of high-quality genomic DNA from *Alchemilla* leaf. The quantity of DNA obtained with the optimized method compared to the DNA extracted by the original CTAB's (Doyle & Doyle 1987), SDS's (Mafra *et al.* 2008) methods, and the quality also compared to a commercial kit protocol. Spectrophotometer, gel

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electrophoresis, and PCR measurements used as standards for assessing the quantity and quality of the obtained DNA.

Materials and Methods

Fresh and herbarium (up to 40 years old) samples were selected based on the following materials and methods:

- Plant material

Dry samples obtained from Tehran University Herbarium (TUH) and fresh samples from Guilan province (Iran) collected and stored in –70 ℃ until use (Table 1).

Table 1. The species used in the current analysis along with their related data

Taxon	Locality	Country	Date	Altitude (m)	Collector	Herbarium No.
Alchemilla melancholica Fröhner	Gilan prov.: Espili, Larikhani	Iran	1993	1530	Saeidi	TUH 18841
A. hessii Rothm	Mazandaran prov.: Kandovan, Ghahreman	Iran	1974	2200	Augustine/ Sheikholeslami,	TUH 19418
A. pectinoloba Fröhner.	Gilan prov.: Deylaman, Larikhani	Iran	1993	1530	Saeidi	TUH 18837
A. sericata Rchb. ex Buser.	Gilan prov.: along Asalem to Khalkhal road and Almas neck	Iran	14.05.2018	2400	Faghir/ Shokatyari	GUH 8348
A. fluminea Fröhner	Gilan prov.: along Asalem to Khalkhal road and Almas neck	Iran	14.05.2018	2200	Faghir/ Shokatyari	GUH 8350
A. farinosa Fröhner	Gilan prov.: along Asalem to Khalkh al road and Almas neck	Iran	14.05.2018	2400	Faghir/ Shokatyari	GUH 8349

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- Extraction methods

- DNA extracted using the following methods:

- Method A: Gene all plant mini kit

DNA isolated from fresh and dried leaves using Gene All plant mini Kit (Pishgam, Songpa-gu, Seoul, Korea), according to manufacturer recommendations. The resulting samples stored at –70 ˚C until use.

- Method B: modified SDS-based DNA extraction method

Ground 100 mg of fresh leaves (for dry leaves 50 mg) in ice-cold condition (with powdered dry ice) to fine powder in presence of 1000 µL preheated SDS extraction buffer (65 ˚C) (2% Sodium dodecyl sulfate (SDS) w/v, 150 mM NaCl, 100 mM Tris-HCl, 25 mM EDTA, pH 8.0) by using a pre-chilled mortar and pestle (at –20 ˚C). Then incubated at 65 ˚C for 60 min, with gentle shaking by hand every 5 min. Left samples at the room temperature (RT) and centrifuged at 12,000 rpm, the upper layer extracted twice with phenol/chloroform/isoamyl alcohol (25:24:1) (first extraction) and chloroform/isoamyl alcohol (24:1) (second extraction), respectively. Then 0.1 volume potassium acetate solution (3 M, pH 5.5) and double volume of ethanol solution (95%, –20 ˚C) added to the upper aqueous phase (first precipitation), shacked mild and centrifuged for 10 min at 15000 rpm. Discarded the supernatant and washed the pellet with ethanol solution (70%, –20 ˚C) twice and air-dried for 20 min, the dried pellet dissolved with 400 µL Tris-EDTA buffer (10 mM Tris, 1 mM EDTA) and incubated for 30 min at 37 ˚C. Third extraction with chloroform/isoamyl alcohol (24:1) performed. Two and a half volumes of ethanol were added to the upper layer (second precipitation). Then centrifuged for 10 min at 15000 rpm, discarded supernatant, DNA pellet washed with cold 70% ethanol, and the dried pellet dissolved in 50 µL of deionized water.

- Method C: Modified CTAB-based DNA extraction method

Ground 100 mg of fresh leaves (for dry leaves 50 mg) to a fine powder in the cold condition in presence of 800 µL preheated 2x CTAB extraction buffer (65 °C) (2% (w/v) CTAB, 100 mM Tris, pH 8.0, 20 mM EDTA, 1.4 M NaCl, and 2% PVP (polyvinylpyrrolidone) by using a pre-chilled mortar and pestle (at –20 °C) and transferred to a new 2 µL sterile tube containing 300 mg activated carbon (PAC) and vortex 40–60s until thoroughly mixed, then incubated with gentle agitation at 60 °C for 30 min. Left samples at room temperature (RT), added 600 µL of a mixture of chloroform and isoamyl alcohol (24:1) and mixed by inversion for 15 min, then centrifuged for 7 min at 13000 rpm. The supernatant transferred to a new 2 µL sterile reaction tube. Added 1 volume of ice-cold isopropanol with invert gently and stored at –70 °C for 1 h, then centrifuged for 20 min at 14000 rpm. Discarded supernatant and pellet washed with 1000 µL of cold 70% ethanol. After centrifugation at 8500 rpm for 5 min, the supernatant discarded, and the pellet dried at 37 °C for 30 min. The pellet dissolved in 50 µL of deionized water and stored at –70 °C.

- Method D: Modified CTAB-based DNA extraction method

Ground 100 mg of fresh leaves (for dry leaves 50 mg) in cold condition to fine powder, in presence 2000 µL of 3x CTAB extraction buffer (65 °C) 3% CTAB (1M Tris-HCl pH 8, 1.4 M NaCl, 0.5 M EDTA), by using a pre-chilled mortar and pestle (at –20 °C), incubated with gentle agitation at 60 °C for 90 min. Samples left at the room temperature (RT), then 300 µL of buffer phenol-chloroform-isoamyl alcohol (25:24:1) added to supernatant and vortex 30–40s, incubated in ice for 15 min, then centrifuged for 7 min at 14,000 rpm. Added 700 µL of buffer chloroform-isoamyl alcohol to the supernatant and shacked mild for 15 min (bench or wrist shaker), then centrifuged for 7 min at 13,000 rpm. This step repeated. Added half the volume 3 M sodium acetate (pH 5) and 1 volume of ice-cold isopropanol, inverted trice, and stored at −20 °C overnight. Then centrifuged for 20 min at 14000 rpm. Discarded supernatant and pellet washed with 1000 µL of cold 70% ethanol. After centrifugation at 8500 rpm for 5 min, the supernatant discarded and the pellet dried at 37 °C for 30 min. The pellet dissolved in 50 µL of deionized water and stored at –70 °C.

- Method E: Modified CTAB-based DNA extraction method

All extraction process performed like method D except that after the addition CTAB buffer, 500 µL of polyethylene glycol (PEG) 8000 [13% (w/v)] solutions added to each tube.

- Method F: Modified CTAB-based DNA extraction method

All extraction process performed like method D except that after the pellet dissolved in deionized water, added 500 µL of PEG 8000 [13% (w/v)] solutions and NaCl (1.6 M) solution then gently inverted 2 to 5 times and stored in −20 °C for 1 h. Then centrifuged for 10 min at 10000 rpm. The supernatant discarded, and the pellet dried at 37 °C for 30 min. 500 µL of deionized water and then 500 µL buffer chloroform-isoamyl alcohol added to each tube and shake mild for 15–20 min. Centrifuged for 5 min at 10000 g. 40 µL of 3 M sodium acetate and 2–2.5 volume of 95% alcohol added to the supernatant and inverted gently five times. If not appear deposition, tube stored in –70 °C for 1–3 h (the longer the chilled incubation, the more the precipitation) and centrifuged for 10 min at 10000 rpm. Discarded supernatant and pellet washed with 1000 µL of cold 70% ethanol. After centrifugation at 8500 rpm for 5 min, the supernatant discarded, and the pellet dried at 37 °C for 30 min. The dried pellet dissolved in 50 µL of deionized water and stored at –70 °C.

- DNA Quantity and quality measurements

DNA quality and quantity evaluated using three methods: NanoDrop spectrophotometer, PCR amplification, and electrophoresis on the agarose gel.

- Concentration and purity of DNA

DNA quality and quantity characterized using a spectrophotometer base on Stulnig & Amberger (1994).

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The extracted DNA by different methods assessed at 230, 260, 280, and 320 nm wavelengths to investigate their concentration, yield, and purity.

- DNA visualization on agarose gel

The presence and quality of extracted genomic DNA assessed by electrophoresis of an aliquot of 5 µL DNA in a 0.8% agarose gel stained with SYBR Safe, using 1xTAE (Tris-Acetate-EDTA) buffer, at 90 V for 1 h and photographed with a Bio-Rad UVI gel documentation system.

- DNA amplification by polymerase chain reaction

The final test overall quality and quantity of the template DNA was PCR amplification success. The nrDNA ITS region amplified using the primers 17SE (5'ACGAATTCATGGTCCGGTGAAGTGTTCG3') and 26SE 5'TAGAATTCCCCGGTTCGCTCGCCGTTAC3') (Sun *et al*. 1994). The *trn*H- *psb*A cp DNA region amplified using the primers *trn*H-F (5'CGCGCATGGTGGATTCACAATCC3') (Tate & Simpson 2003) and *psb*A R (5'GTTATGCATGAACGTAATGCTC3') (Sang *et al.* 1997). The PCR amplification carried out in a volume of 25 µL containing 10.5 µL of deionized water, 12.5 mL of the 2X Taq DNA polymerase Master Mix Red (Amplicon Cat. No. 180301, 150 µM Tris-HCl pH 8.5, 40 µM (NH4) 2SO4, 3.0 µM MgCl2, 0.4 µM dNTPs, 0.05 units µL-1 Amplicon Taq DNA polymerase, inert red dye, and a stabilizer), 0.5 µL of each primer (10 pmol/µL), and 1 µL of template DNA (20 ng/µL). PCR protocol outlined in table 2. PCR products assessed by electrophoresis of an aliquot of 2 µL DNA in 1% agarose gel stained with SYBR Safe, using TAE buffer, at 70 V for the 30s and photographed with a Bio-Rad UVI gel documentation system.

Table 2. PCR thermocycler profile for *trn*H- *psb*A (numbers in parentheses) and 17SE-26SE primers

Step	Temperature	Time	Cycling
Initial denaturation	95 ℃	5 min	1
Denaturation	95 ℃	30 (45) s	-
Annealing	55 ℃	30 (45) s	28
Extension	72 ℃	90 (45) s	-
Final extension	72 ℃	7 min	1
Hold	4–20 ℃	-	-

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- Statistical data analysis

Results expressed as mean ± S.E. Two-way ANOVA test performed to check the presence or absence of significant differences between DNA concentration and purity values followed by Tukey's HSD post hoc test. All statistical analyses were done using R Gui Ver. 3.5.

Results

- Concentration and purity of DNA

The concentration of gDNA obtained by the different methods ranged from 20.05-1218.31 ng/µL (Table 4). The concentration of gDNA showed very significant differences ($p<0.0001$) amongst the investigated methods, which were 51.75 ng/µL (method A), 423.42 ng/µL (method B), 438.25 ng/µL (method C), 1031 ng/µL (method D), 791.3 ng/µL (method E) and 423.42 ng/µL (method F) (Table 3). The concentrations of DNA obtained from fresh samples with A, B, C, and E methods were higher than the dried samples (Table 3).

The analysis of variance showed significant differences among the examined methods in the 260/280 ratio, which were 1.28 (method A), 1.34 (method B), 1.25 (method C), 1.38 (method D), 1.47 (method E), and 1.81 (method F) (Table 3). Also, the DNA absorbance 260/230 ratio had significant differences among the tested. DNA extracted with the procedure F showed a high rate (260/230 = 1.59). PEG with NaCl addition to the extraction buffer improved the absorbance ratios both at 260/280 from 1.28

to 1.81, and 260/230 from 0.81 to 1.59 (Table 3). Dried and fresh samples did not show significant difference in 260/280 and 260/230 ratios (Table 4). Highest DNA yield obtained with the method D (51.57 µg/100 mg) followed by other methods: E (39.56 µg/100 mg), F (21.17 µg/100 mg), C (21.9 µg/100 mg), B (21.17 µg/100 mg) and A (2.58 µg/100 mg) (Table 3). Fresh and dry samples indicated a significant difference in DNA yield with A, B, C, and E methods (Table 4).

Table 3. Differences between groups in concentration and spectrophotometer absorbance ratios for DNA purity, depending on the method used for DNA extraction

Method	Concentration ± SD ng/µL	Abs. ± SD 260/280	Abs. ± SD 260/230	Yield ± SD µg/100 mg
A	51.75 ± 16.66 [c]	1.28 ± 0.40 [cd]	0.81 ± 0.10 [c]	2.58 ± 0.83[c]
B	423.42 ± 92.6 [b]	1.81 ± 0.05 [bc]	1.59 ± 0.06 [c]	21.17 ± 4.36 [b]
C	438.25 ± 71.08 [b]	1.25 ± 0.05 [d]	0.77 ± 0.03 [bc]	21.9 ± 3.55 [b]
D	1031 ± 67.17 [a]	1.38 ± .015 [bc]	0.83 ± .019 [bc]	51.57 ± 3.36 [a]
E	791.3 ± 2.033 [a]	1.47 ± 0.31 [b]	0.95 ± 0.07 [b]	39.56 ± 1.01 [a]
F	423.42 ± 92.6 [b]	1.81 ± 0.05 [a]	1.59 ± 0.06 [a]	21.17 ± 4.36 [b]

Means within columns that have the same letters are statistically similar (Tukey's multiple range test, $P < 0.001$). Different lowercase letters indicate significant differences. The values reported are means ± standard deviation.

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Table 4. Differences within group in concentration and spectrophotometer absorbance ratios

Method	Concentration ± SD ng/µL	Abs ± SD 260/280	Abs ± SD 260/230	Yield ± SD µg/100 mg
A (fresh)	66 ± 19.14 [a]	1.28 ± 0.47	0.7 ± 0.16	3.29 ± 0.95 [a]
A (dry)	20 ± 3.23[b]	1.31 ± 0.59	0.69 ± 0.094	1 ± 0.16 [b]
B (fresh)	537 ± 82.9 [a]	1.38 ± 0.05	0.74 ± 0.08	21 ± 2.75 [a]
B (dry)	305 ± 37.11 [b]	1.3 ± 0.04	0.68 ± 0.03	26.83 ± 4.14 [b]
C (fresh)	577 ± 38.7 [a]	1.17 ± 0.07	0.74 ± 0.03	28.8 ± 1.94 [a]
C (dry)	360 ± 79.53 [b]	1.24 ± 0.06	0.77 ± 0.04	18 ± 3.97 [b]
D (fresh)	1218.31±60.99	1.36 ± 0.01	0.76 ± 0.02	60.9 ± 3
D (dry)	1005 ± 78.57	1.39 ± 0.017	0.83 ± 0.02	50.27 ± 3.9
E (fresh)	1080 ± 97.8 [a]	1.44 ± 0.035	0.94 ± 0.03	53.99 ± 4.8 [a]
E (dry)	768 ± 16.7 [b]	1.48 ± 0.03	0.95 ± 0.1	38.42 ± 0.83 [b]
F (fresh)	595 ± 84.5	1.75 ± 0.016	1.56 ± 0.22	29.75 ± 1
F (dry)	387 ± 10.2	1.81 ± 0.38	1.68 ± 0.06	17 ± 5.8

Means within columns that have the same letters are statistically similar ($P < 0.01$). Different lowercase letters indicate significant differences. The values reported are means ± standard deviation.

- Agarose gel electrophoresis

No genomic DNA band was visible from the extracts using a commercial kit (Fig. 1, A). DNA extracted through methods B, C, D, and E exhibited smear and impurities (Fig. 1, B-D). For procedure F good and clear DNA bands were visible on the agarose gel (Fig. 1, E).

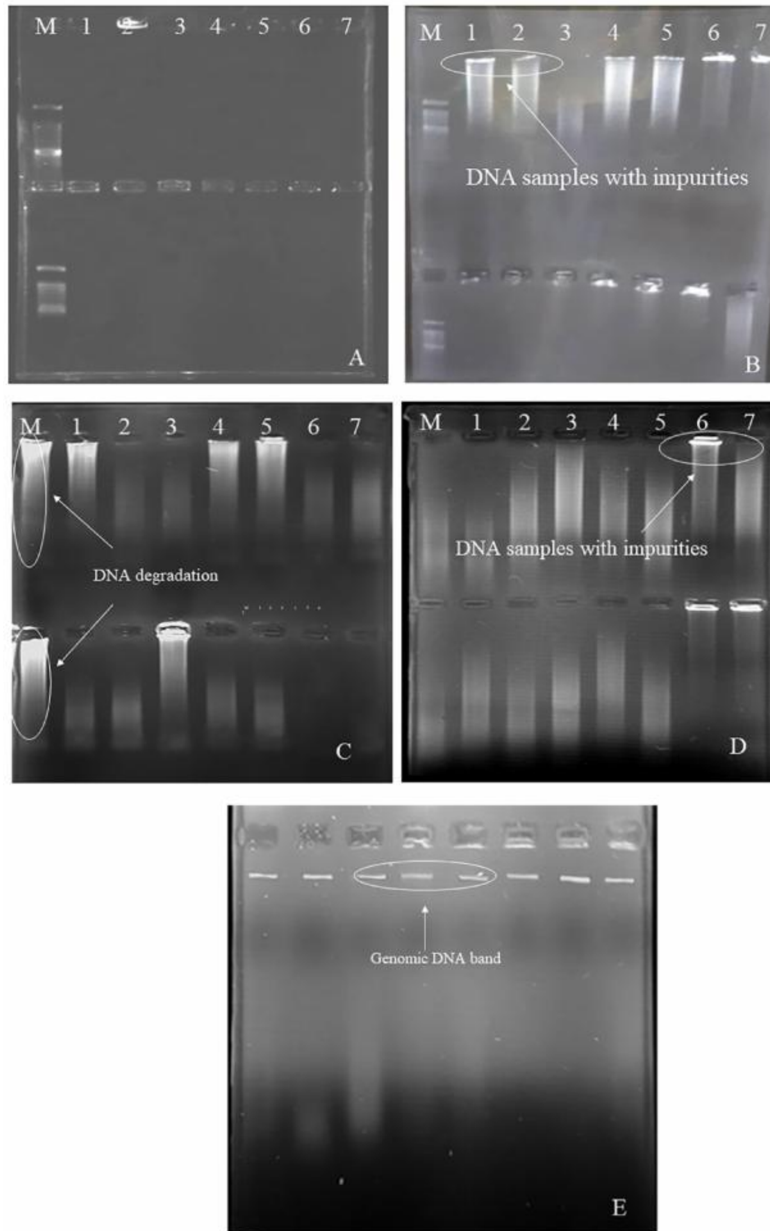

Fig. 1. Electrophoresis results on 0.8% agarose gel with DNA extracted from six species *Alchemilla* by different extraction DNA: A. Method A, B. Method B, C. Method C (upper row) and D (lower row), D. Method E, E. Method F., M. DNA size marker.

- PCR amplification

All extract methods failed to amplify PCR products in all samples with two primers, except methods F (Fig. 2, A-E). Clear and strong amplified band obtained from all samples prepared with method F for both of the primers (Fig. 2, F-G).

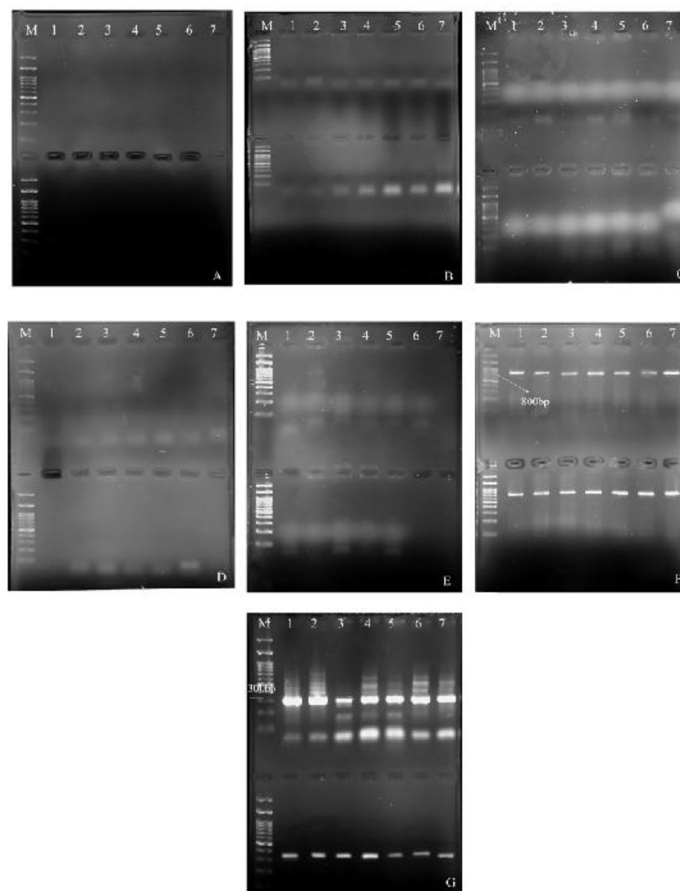

Fig. 2. Resolution of nrDNA and cpDNA segments amplified by 17SE-26SE and *trn*H- *psb*A primers respectively in PCR: A. Method A [*trn*H- *psb*A (upper row)], 17SE-26SE (lower row), B. Method B [*trn*H- *psb*A (upper row)], 17SE-26SE (lower row), C. Method C [*trn*H- *psb*A (upper row)], 17SE-26SE (lower row), D. Method D [*trn*H- *psb*A (upper row)], 17SE-26SE (lower row), E. Method E [*trn*H- *psb*A (upper row)], 17SE-26SE (lower row), F. Method F (17SE-26SE), G. Method F (*trn*H- *psb*A)., M. DNA size marker.

Discussion

The process of extraction of DNA is one of the most common techniques in molecular biology. Different nucleic acid extraction methods have been published to date, although each has its limitations. A suitable choice of leaf tissue is important to obtain high-quality DNA. Mature leaves are not a good choice for DNA extraction due to their high concentration of secondary metabolites (Dabo *et al*. 1993), as a result, the mature leaf is not the right choice. This problem is quite widespread in the genus of *Alchemilla*. Yellow and brownish colored DNA pellets indicate contamination by phenolic compounds (Weishing *et al*. 1995) where the biggest challenge we faced during DNA extraction from fresh and dried *Alchemilla* leaf.

Method A (GeneAll plant mini kit) was a commercial extraction method, but it did not indicate hopeful results during spectrophotometric assay, agarose electrophoresis, and PCR for *Alchemilla* species. Therefore, the current method is not useful for the isolation of DNA from leaves of *Alchemilla*. However, in some experiments, the DNA extraction Kit with some

modification was the best method for extraction of pure DNA from old dried leaves (Riahi *et al*. 2019).

Method B performed based on the SDS extraction method. SDS is an anionic surfactant that disrupts the cell membranes and denatures proteins (Natarajan *et al*. 2016). Relatively high yield of DNA obtained from both fresh and dry samples but in very poor purity. Brownish pellets obtained by this method and absence of band after agarose gel electrophoresis and PCR further substantiated the poor quality of extracted DNA. The method failed to obtain contamination-free DNA from *ALchemilla* leaf.

Method C carried out using the original CTAB method, with few modifications including: 1. Exclusion of -mercaptoethanol, which is a biological antioxidant which can inhibit oxidation of polyphenols (Kawata *et al*. 2003, Varma *et al*. 2007) but it cause central nervous system, respiratory and eyes damages, therefore we did not use it in this work (Anuradha *et al*. 2013), 2. Addition of PVP 2% (w/v), and 3. Addition of PAC. Polyvinylpyrrolidone is a water-soluble polymer. PVP or PAC/combination of both helps to remove polyphenols. PAC binds to polyphenols and staves off irreversible interaction of polyphenols with DNA, on the other hand, PVP has a synergistic effect in binding polyphenols on PAC additionally, PVP reduces the oxidation of polyphenols (John 1992, Bi *et al*. 1996). Brownish DNA pellets with very poor quality, presence of smear and contamination in the agarose gel, and lack of DNA band in PCR suggested that the current method is not an efficient method for DNA extraction from *Alchemilla.*

In method D, we used method C with some modifications as follows: 1. Addition of the different volume (four times, 2 mL of buffer per 50 mg of the leaf) and concentration (3 x) of CTAB buffer, 2. Elongation of lysis time for cells, 3. Addition of 300 µL of phenol/chloroform/ isoamyl alcohol buffer, 4. Addition of sodium acetate 3 M (pH5) together with isopropanol, and 5. An increase of cold incubation period. By using this method, a high yield of poor-quality DNA obtained. A high concentration of lysis buffer promotes interruption of the cell and nuclear membranes to expose the genetic components (Amani *et al.* 2011). These results are in agreement with that of Aboul-Maaty & Oraby (2019) which showed a higher CTAB concentration used to obtain a higher yield of nucleic acids. Moyo *et al*. (2008) reported that, the optimization of the correct balance between tissue amount and extraction buffer volume is key for a successful DNA extraction. Some cells require longer lysis time. Lade *et al*. (2014) used this parameter to increase the concentration of DNA.

Isopropanol and 3M sodium acetate led to precipitate nucleic acids out of supernatant and form a white precipitate. Simultaneously with this process, salts and other solutes, such as remaining phenol and chloroform, stay in the supernatant (Box *et al*. 2011, Greco *et al*. 2014, Lade *et al*. 2014). The modifications described above provided high yields of genomic DNA, as confirmed by spectrophotometric assay. In the current method and agreement with other research, increases in DNA yield observed when sodium acetate together with isopropanol used (Greco *et al*. 2014).

Brownish pellets and 260/280 and 260/230 values obtained by method C, indicated contamination by phenolic compounds, polysaccharides, protein, and carbohydrate. Therefore, phenol-chloroform-isoamyl alcohol mix used to remove these contaminations. It can also be first time checked with phenol/chloroform, and then only with adding chloroform. Chloroform mixed with phenol can better remove proteins than chloroform alone. Proteins, lipids, and carbohydrates, partition into the organic phase or remain in the interphase, while nucleic acids are soluble in the aqueous phase (Chomczynski & Sacchi 2006).

The NanoDrop results showed impurities in DNA samples. Our findings displayed that, mixing phenol-chloroform, modifying lysis time, changing the volume and concentration of CTAB buffer, using sodium acetate (3M) together with isopropanol, and increasing cold incubation period led to higher DNA concentration but did not show meaningful changes in the purity of DNA.

Method E described in method D except for using PEG 13% (w/v) solution. Brownish pellet, A260/A280,

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and A260/230 values, the result of electrophoresis and PCR amplification obtained by using the method D indicated the likely presence of contamination in the extracted DNA. PEG is a polyether compound with many uses, from industrial manufacturing to medicine. Precipitation of DNA with PEG inhibits the presence of plant metabolites (glycosides, polyphenols etc.), which would prevent Taq DNA polymerase activity (Del Castillo Agudo *et al*. 1995), so in this method, PEG 13% (w/v) solution added to lyse buffer before incubation. Quantity and quality derived from these samples didn't significantly differ from method D. Based on the result of NanoDrop, method E gave slightly but not significantly lower DNA yield and higher 260/280 and 260/230 ratios than that method D. Also, weak smear and impurity seen after electrophoresis suggested that the quantity of DNA derived from this method is still not good enough for PCR.

Method F described in method D except for repeating precipitation and dilution of DNA steps. The second precipitation of DNA carried out in the presence of 13% w/v PEG 8000 and 1.6M NaCl solutions. In the presence of salts, PEG leads to an increase in DNA concentration. This process is called polymer and salt-induced (psi) condensation or C condensation. PEG functions to provide a hydrophobic environment, while salt cations can neutralize the negative charge of phosphate backbone (Bloomfield 1996, Cheng *et al.* 2015). Residual inhibitors removed by PEG precipitation, and the white pellet dissolved more easily in deionized water. Remain likely contaminants eliminated by repeating chloroform: isoamyl alcohol treatment. Ethanol together with sodium acetate buffer utilized for the third precipitation of DNA fragments. In solution, sodium acetate breaks up into Na+ and [CH3COO]−. The positively charged sodium ions neutralize the negative charge on the PO3− groups on the nucleic acids, making the molecule far less hydrophilic, and therefore much less soluble in water. Ethanol, on the other hand, has a much lower dielectric constant, making it much easier for Na+ to interact with the PO3−, shield its charge, and make the nucleic acid less hydrophilic, causing it to drop out of solution (Lade *et al*. 2014). This method yielded sufficient amount of *high* quality genomic DNA from both the fresh and dry leaves of *Alchemilla.* The amount of obtained DNA by method F was less than that extracted by methods D and E but it is sufficient for amplification.

The modifications described above increased the absorbance ratios both at 260/280 from 1.47 to 1.81 and 260/230 from 0.95 to 1.59. This further supported by a completely transparent and colorless DNA pellet obtained by this method and thick and white bands of DNA saw after PCR amplification for both the primers. These results were similar to other studies (Cheng *et al.* 2015, Youssef *et al*. 2015).

We concluded that, this method results in significantly higher purity of DNA than other methods, indicating the repeating of precipitation and dilution steps is likely to have improved the ability of the DNA extraction and reducing the level of impurity and thus resulted in clear PCR bands. On the other hand, we did not use expensive liquid nitrogen and environmentally hazardous substance such as -mercaptoethanol. The use of pre-chilled mortar and pestle and powdered dry ice effectively replaced the use of costly liquid nitrogen. The current method is simple, effective, reproducible, affordable, and relatively non-toxic. Although the total time of the extraction for this method has increased, we believe that, obtaining amplifiable DNA from specimens of plants containing a large number of secondary metabolites is more important than increasing the extraction time. Even the isolation DNA from a fresh sample of *Alchemilla* by previous methods failed. As a result, the presented method is efficient enough to amplify the PCR reaction.

Conclusion

The described DNA extraction method in present study resulted in the production of enough amount of

high-quality DNA from fresh and herbarium specimens (older than 40 years) of *Alchemilla.* The method optimized step by step to produce a sufficient yield of high-quality amplifiable DNA. These changes include: the use of 3% CTAB buffer (1M Tris-HCl pH 8, 1.4 M NaCl, 0.5M EDTA), performing six times dilutions by phenol/chloroform/isoamyl alcohol (one time), chloroform/isoamyl alcohol (three times), alcholol (two times), performing three times precipitations, first with isopropanol, second with PEG and third with ethanol, and increasing the lysis time and cold incubation period. The method eliminates the need to use costly liquid nitrogen and toxic compounds such as -mercaptoethanol to obtain high-quality genomic DNA. This simple method is reproducible, affordable, effective and relatively non-toxic. Consequently, we suggest the optimized method presented here for DNA extraction from plant species with high contents of secondary metabolites, even in low-technology laboratories

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