

Comparison of Five DNA Extraction Methods in *Peganum harmala* L., *Tamarix ramosissima* Ledeb., and *Potentilla reptans* L

Zahra Salehi

Damghan University

Arezou Rezaei (✉ arezaei@du.ac.ir)

Damghan University

Atefe Amirahmadi

Damghan University

Parisa Farrokh

Damghan University

Javad Ghasemian

Damghan University

Research Article

Keywords: *Peganum harmala* L., *Tamarix ramosissima* Ledeb., *Potentilla reptans* L., DNA extraction, CTAB, PCR

Posted Date: July 22nd, 2022

DOI: <https://doi.org/10.21203/rs.3.rs-1859265/v1>

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Abstract

Extracting high-yield, high-quality DNA from plant samples is challenging due to the presence of the cell wall, pigments, and some secondary metabolites. The main CTAB method, two of its modified protocols (beta-mercaptoethanol or ammonium acetate were eliminated), the modified Murray and Thompson method, and the Gene All kit were statistically compared based on the quantity and quality of the total DNA (tDNA) extracted from fresh and dried leaves of three medicinal herbs *P. harmala*, *T. ramosissima*, and *P. reptans*. The suitability of the tDNAs for molecular studies was evaluated by polymerase chain reaction (PCR) of the fragments of the internal transcribed spacer (ITS) in nuclear DNA and the *trnL*-F region in chloroplast DNA. Some significant differences were found between the tDNAs extracted by five extraction methods. With the exception of *P. harmala*, where the PCR of both the ITS fragments and the *trnL*-F region worked successfully in all DNA samples, but only the ITS fragments, not the chloroplast *trnL*-F region, were amplified in the DNA samples of *T. ramosissima* and *P. reptans*. The chloroplast *trnL*-F region was amplified only in DNA samples extracted from fresh and dried leaves of the three studied herbs using the commercial kit. Gene All kit, the main CTAB method, and its modified protocols were the less time-consuming protocols that yielded DNA suitable for downstream PCR vis-a-vis the modified Murray and Thompson method.

Key Message

- The DNA extracted from *P. harmala*, *T. ramosissima*, and *P. reptans* was compared.
- Three CTAB methods, the modified Murray and Thompson method, and a kit were examined.
- PCR of the fragments of the nuclear ITS and chloroplast *trnL*-F regions was performed.
- Ammonium acetate and BME were not very important components in the main CTAB method.

1 Introduction

Medicinal herbs and their products are of health and economic value. (Ribeiro et al., 2007) Extraction of high-quality DNA is required to study the genetic, morphological, and ecological characteristics of medicinal herbs. (Ribeiro et al., 2007) The extraction of high-quality and high-yield DNA from plant samples is laborious due to the hard polysaccharide cell wall and secondary metabolites such as polysaccharides, polyphenols, alkaloids, and proteins (Varma et al., 2007; Mornkham et al., 2012; Friar, 2005; Pipan et al., 2018; Lucas et al., 2019; Abdel-Latif and Osman, 2017; Scobeyeva et al., 2018), which can precipitate with DNA during extraction and inhibit DNA digestion and PCR (Zhang and Stewart, 2000). Moreover, DNA extraction from medicinal herbs is often problematic in molecular studies as they are rich in polysaccharides or secondary metabolites such as polyphenols (Sahu et al., 2012; Križman et al., 2006).

In the current study, three medicinally important herbs, including *Peganum harmala* L. (Zygophyllaceae), *Tamarix ramosissima* Ledeb. (Tamaricaceae), and *Potentilla reptans* L. (Rosaceae) were investigated. *P. harmala*, known as esband in Persian, is a perennial herbaceous plant native to the arid regions of North Africa, the Mediterranean, the Middle East, Pakistan, and India (Herraiz et al., 2010). The herb is common in the provinces of Azerbaijan, Fars, Gorgan, Isfahan, Kerman, Khorasan, Khuzestan, Semnan, and Tehran in Iran (Varma et al., 2007; Akhane 2002). Since ancient times, *P. harmala* has been traditionally and commonly used for medicinal and psychoactive purposes (Herraiz et al., 2010). In Iran, *P. harmala* is traditionally used as an antiseptic by burning its seeds (Fathiazad et al., 2006). The herb is traditionally used in various countries to treat asthma, colic, fever, jaundice, backache, lice, and syphilis (Bahmani et al., 2012). *P. harmala* has many other pharmacological properties, including analgesic, anticonvulsant, antihistamine, anti-inflammatory, antioxidant, antitumor (Astulla et al., 2008; Asghari et al., 2002), antibacterial, antifungal, and antiviral activities (Shonouda et al., 2008). *T. ramosissima*, commonly known as tamaris korsaltcedaris, called gaz in Persian, is a halophytic herb (Sattelmacher, 2001) that prefers alluvial soils but also grows well on saline and alkaline soils (Sultanova et al., 2001) and is widespread in the north, northwest, west, central, northeast, and east (Schiman-Czeika, 1964). Because of its unparalleled flavor, *T. Ramosissima* is used as a skewer (Ren et al., 2019). The *Tamarix* species are useful in leucoderma, spleen disorders, eye diseases (Sultanova et al., 2001), and wound healing (Watkins et al., 2012). *T. Ramosissima* (Zargari, 1996) has been shown to possess antibacterial (Ksouri et al., 2009; Watkins et al., 2012), antioxidant, (Ksouri et al., 2009) astringent, appetizing, and invigorating properties. *P. reptans* is a perennial plant from Caspian and Iranian-Turanian regions and is common in Afghanistan,

Europe, Iran, Iraq, the former Soviet Union, Turkey, and North Africa. This herb is common in Azerbaijan, Boyer-Ahmad, Gorgan, Gilan, Kohgiluyeh, Lorestan, Semnan, and Tehran provinces of Iran (Zielinski, 1982; Akhani, 2002). The genus *Potentilla* has been known for its therapeutic properties since ancient times. Extracts from the aerial or underground parts of *P. reptans* are used in traditional medicine against bacterial (Tumbariski et al., 2017), fungal, and viral infections, cancer, diabetes, diarrhea, inflammation, and wounds (Tomczyk et al., 2009). The leaves of *P. reptans* are also used to treat sore throat, stomach ulcers, and toothache (Zargari, 1996).

Against this background, the present study aims to compare the quality of the extracted total DNA (tDNA) from fresh and herbarium samples of *P. harmala*, *T. Ramosissima*, and *P. reptans* using five extraction methods: the main CTAB method (Doyle and Doyle, 1987), two modified CTAB methods, the modified Murray and Thompson method (Murray and Thompson, 1980), and the Gene All Kit method. To compare the quality of the extracted tDNAs for molecular studies, the internal transcribed spacer (ITS) region of 18S-26S nuclear ribosomal DNA (nrDNA) (King and Roalson 2008) and the *trnL* intron and *trnL*-F spacer in the chloroplast genome (Taberlet et al. 1991) were amplified by the polymerase chain reaction (PCR). Fragments of nrDNA ITS are the most important tool for phylogenetic studies in various taxonomy categories. The multigenes of eukaryotic nrDNA are organized tandemly into hundreds or thousands of repeated units. ITS 4 and ITS 5 primers amplify the nrDNA ITS fragments. In phylogenetic studies of plants, the *trnL*-F region is one of the most common chloroplast markers. *trnL*(c) and *trnL*(f) primers are used to amplify specific sequences of tRNA genes in the chloroplast (Pirie et al., 2007; Kan et al., 2007).

2 Methods

Fresh and dried leaves of *P. harmala*, *T. Ramosissima*, and *P. reptans* were studied.

2-1- Herbs

P. harmala was collected on July 17, 2018, a Tuesday, on our way from Shahrood to Azadshar (Semnan province, Iran). On the same day, *T. ramossissima*, known commonly as salt cedar, was collected on our way from Damghan to Sari above Cheshmeh Ali (Semnan province). Finally, *P. reptans*, commonly known as creeping baby's breath, was collected on August 3, 2018, a Friday, from field margins in Sari (Mazandaran Province, Iran). Dr. Atefe Amirahmadi (Ph.D. in Plant Biosystematics) identified the herbs. The specimens of *P. harmala* (Amirahmadi: 2048.), *T. ramosissima* (Amirahmadi: 2539.), and *P. reptans* (Amirahmadi: 2666.) were kept in the herbarium of Damghan University. The young leaves which were free of wounds and pests were isolated. Once they had been washed with distilled water, some of the leaves were frozen to -80 °C, and the rest were dried at room temperature.

2-2 DNA extraction

Using five methods, the total DNA was extracted from dried and fresh (stored in the freezer) leaves. The three main extraction methods included the CTAB method (Doyle and Doyle, 1987), the modified Murray and Thompson method (Murray and Thompson, 1980) and the kit method. In addition, modifications were made to the original CTAB method to make DNA extraction more cost-effective. DNA extraction from all samples was performed in triplicate.

2-2-1- Main and modified CTAB methods

In the main CTAB method (Doyle and Doyle, 1987), 0.25 g of fresh plant leaves or 0.125 g of herbarium leaves were crushed with 3000 µl of a preheated CTAB buffer (60 °C) in a preheated mortar, and 6 µl of beta-mercaptoethanol (BME; 0.2% extraction buffer) was added to the homogenate. The CTAB buffer contained 2% CTAB powder, 20 mM EDTA, 1.4 M sodium chloride, and 100 mM Tris-HCl (pH = 7.5). Subsequently, 400 microliters of homogenate were incubated in 1.5 ml microtubes for 30 minutes in a dry bath at 60 °C. The homogenate was gently mixed every 5 minutes by turning the microtubes upside down. After 30 minutes, two volumes of chloroform-isoamyl alcohol (24:1) were added to the microtubes and mixed 20 times by slow inverting. The microtubes were centrifuged at 13000 rpm at 4 °C for 15 minutes. The step was repeated when the supernatant was not clear. The upper aqueous phase containing tDNA was carefully transferred to new tubes, and two volumes of cold isopropanol were added to each microtube. The microtubes were centrifuged at 10000 rpm at 4 °C for 10 minutes after being kept at -20 °C for 20 minutes. The supernatant was discarded, and the pellet was washed with 250 µl of a wash buffer (76% ethanol and 10 mM ammonium acetate). The microtubes were centrifuged at 10,000 rpm for 2 minutes. The wash buffer was discarded, and the pellet was dried on a paper towel at room temperature. The pellet was dissolved in 30 µl of deionized water.

The first modified CTAB method differed from the main CTAB method in that no BME was used. The only difference between the second modified CTAB method and the main CTAB method was that no ammonium acetate was used in the wash buffer, and only 76% ethanol was used to wash the DNA pellets.

2-2-2- The modified Murray and Thompson method

In this study, the modified method of Murray and Thompson published by Riahi et al. in 2010 (Riahi et L., 2010) was used. The components of the extraction buffer were 2% CTAB powder, 100 mM Tris-HCl (pH = 7.5), 1.4 M NaCl, and 50 mM EDTA (pH = 8). Fresh (0.25 g) leaves or 0.125 g of herbarium leaves were crushed in a mortar with 1875 µl of a CTAB buffer and 7.5 µl of BME was added to the homogenate. Then, 750 µl of the homogenate was transferred to new microtubes and incubated at 60 °C in a dry bath for 1 hour, with careful mixing by inverting the microtubes every et al. 2010) 15 minutes. In this step, 700 µl of chloroform-isoamyl alcohol (24:1) was added to all microtubes, and the contents were mixed by gently inverting the microtubes several times. The microtubes were centrifuged at 10000 rpm, 4°C for 15 minutes. The upper aqueous phase containing the DNA was carefully transferred to new microtubes, and 0.33 volume of cold isopropanol was added to each microtube. The microtubes were kept at -20°C for 1 hour before centrifugation at 10000 rpm and at 4°C for 15 minutes. The supernatant was discarded, and 100 microliters of TE buffer (1 mM EDTA and 10 mM Tris), 0.1 volume of 2.5 M sodium oxaloacetate (NaOAc), and 2 volumes of 95% cold ethanol were added to the pellets. Samples were kept in the freezer at -20 °C for 30 minutes. The microtubes were centrifuged at 10,000 rpm for 5 minutes. The supernatant was discarded, and 1 ml of 70% ethanol was added to the precipitate. The microtubes were centrifuged at 10,000 rpm, 4°C for 4 minutes. The pellet was dissolved in 30 µl of the TE buffer.

2-2-3- Gene All kit

The total DNA was also extracted using the Gene All kit (Gene All Biotechnology Company, South Korea) as per the instructions of the kit.

2-4- Evaluation of the extracted DNA

2-4-1- Evaluation of quality and quantity assessment

The quantity and purity of the extracted tDNA were determined using a UV-Vis spectrophotometer (Eppendorf AG, Hamburg 22331, Germany) at 230, 260, 280, and 320 nm. The absorbance ratios of 260/280 and 260/230 were calculated for the evaluation of DNA purity and the detection of protein and non-protein contamination, respectively (van Wieren-de Wijer et al. 2009; Maniatis et al. 1982). The DNA samples were analyzed by electrophoresis using a 0.8% agarose gel (Khare et al. 2014) Experiments were performed in triplicate.

2-4-2- Polymerase chain reaction

The quality of the tDNA extracted from the fresh and dried leaves of the studied herbs was by the PCR of the ITS fragments in the ribosomal nuclear DNA and the *trnL*-F region in the chloroplast genome. Of the different *trnL*-F regions in the chloroplast (Taberlet et al., 1991), *trnL* (UAA) (c) and *trnF* (GAA)(f) were analyzed. Table 1 shows the sequences of the primers used and the size of the amplicons. In some cases, touchdown (TD) PCR was performed to increase the sensitivity and efficiency of the results (Korbie and Mattick, 2008).

Table 1- The sequence of universal primers used for amplification of nuclear internal transcribed spacer (ITS) and *trnL*-F (f and c) regions.

	Name	Sequence	Amplicon size (bp)	References
Nuclear region	ITS5m (F)	5'-GAAGGAGAAGTCGTAACAAGG-3'	700	Sang et al., 1995
	ITSF (R)	5'-TCCTCCGCTTATTGATATGC-3'		White et al., 1990
Chloroplast region	<i>trnL</i> (UAA)(c) (F)	5'-CGAAATCGGTAGACGCTACG-3'	1100	Taberlet et al. 1991
	<i>trnF</i> (GAA)(f) (R)	5'-ATTTGAACTGGTGACACGAG-3'		

F, forward; R, reverse; Of the different *trnL*-F regions in the chloroplast, *trnL* (UAA) (c) and *trnF* (GAA) (f) were examined.

Tables 2 and 3 show the main PCR and TDPCR programs used to amplify the nuclear ITS fragments, respectively. The PCR program for the chloroplast *trnL-F* region was the same as the main PCR program used for the nuclear region, except for the annealing temperature, which was 57°C for the chloroplast sequences. For amplification of the target sequence, 500 ng of DNA was used per 20 µl reaction volume containing 10 µl of 2X Ampliqon Master Mix (Ampliqon company, Denmark, 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) and 10 picomoles of each forward and reverse primer. Five microliters of the PCR products were electrophoresed on a 1.5% agarose gel. The size of the PCR products on the agarose gel was determined using a DNA molecular weight marker.

Table 2- The main polymerase chain reaction program for amplifying the nuclear internal transcribed spacer (ITS).

Steps	Temperature (°C) *	Time	Cycle(s)
Initial denaturation	94	2 min and 30 sec	1
Denaturation	94	50 sec	30
Annealing	55, 56, or 58	30 sec	30
Extension	72	50 sec	30
Final extension	72	7 min	1

The same PCR program was used to amplify the chloroplast *trnL-F* regions. However, the annealing temperature for the chloroplast sequences was 57°C.

* For the DNA extracted from fresh and dried leaves of the studied herbs, the following annealing temperatures were used: main CTAB (55 °C), first and second modified CTAB (56 °C), and modified Murray and Thompson methods and Gene All kit methods (58 °C). (For more information on the DNA extraction methods, please refer to Section 2-2-DNA Extraction.)

Table 3- Touchdown polymerase chain reaction programs (TD 1 and 2 PCR) were used to amplify the fragments of nuclear internal transcribed spacer (ITS).

TD 1 PCR				TD 2 PCR		
Steps	Temperature (°)	Time	Cycle(s)	Temperature (°)	Time	Cycle(s)
Initial denaturation	94	2 min and 30 sec	1	94	2 min and 30 sec	1
First Denaturation	94	50 sec	10	94	50 sec	10
First Annealing	56 - 58	30 sec	10	60 - 69	30 sec	10
First Extension	72	50 sec	10	72	50 sec	10
Second Denaturation	94	50 sec	32	94	50 sec	25
Second Annealing	57	30 sec	32	59	30 sec	25
Second Extension	72	50 sec	32	72	50 sec	25
Final extension	72	7 min	1	72	7 min	1

2-5- Statistical analysis

The results of the DNA extracted from fresh and dried leaves by five extraction methods were analyzed using nonparametric Mann-Whitney and Kruskal-Wallis tests. A comparison of the five extraction methods was performed using the Kruskal-Wallis test. The results of each extraction method for fresh and dried samples were compared using the Mann-Whitney test. Statistical analysis was performed using SPSS software (version 16) at a significance level of 0.05.

3 Results

3-1- Evaluation of quality and quantity

3-1-1- Spectrophotometric analysis

The quantity and purity of the tDNA extracted from the leaves of *P. harmala*, *T. ramosissima*, and *P. reptans* were determined spectrophotometrically at 230, 260, 280, and 320 nm. There was no significant difference between the tDNAs extracted from both fresh and dried leaves of the studied herbs using each of the five extraction methods (Table 4; the results of the Mann-Whitney test). When the results of all extraction methods were compared (Table 4; results of the Kruskal-Wallis test), no significant difference was observed between the studied extraction methods for the DNA extracted from fresh or dried leaves of the herbs in terms of the A260/A280 ratio. However, the difference between the DNA extracted from the fresh and dried leaves of *P. harmala* by the five extraction methods was significant in terms of the concentration of the tDNA and the A260/A230 ratio. For *T. ramosissima*, a significant difference between the extraction methods was found only in terms of the A260/A230 ratio for the dried leaves. In comparison, only the tDNA extracted from the dried leaves of *P. reptans* by the methods studied showed significant differences in terms of both the concentration of the tDNA and the A260/A230 ratio.

Table 4

Statistical analysis of the concentration of the total extracted DNA ([DNA]; ng/ μ l) and the ratio of optical absorbance in the studied herbs.

	<i>Peganum harmala</i> L.			<i>Tamarix ramosissima</i> Ledeb			<i>Potentilla reptans</i> L.		
	A260/A280	A260/A230	[DNA] (ng/ μ L)	A260/A280	A260/A230	[DNA] (ng/ μ L)	A260/A280	A260/A230	[DNA] (ng/ μ L)
The Kruskal-Wallis test for comparison of the DNA extracted from dried leaves by 5 extraction methods	0.079	0.044	0.043	0.191	0.244	0.180	0.204	0.055	0.051
The Kruskal-Wallis test for comparison of the DNA extracted from dried leaves by 5 extraction methods	0.096	0.041	0.041	0.104	0.032	0.324	0.068	0.034	0.034
The Mann-Whitney test for the comparison of the DNA extracted from fresh and dried leaves using the CTAB method	1.000	0.100	0.100	0.200	1.000	1.000	0.333	0.333	0.333
The Mann-Whitney test for the comparison of the DNA extracted from fresh and dried leaves using the first modified CTAB method	0.100	1.000	0.100	0.100	0.100	0.400	0.200	1.000	0.200

The numbers in bold indicate that the difference is significant. The significance level was set at $P < 0.05$.

	<i>Peganum harmala</i> L.		<i>Tamarix ramosissima</i> Ledeb				<i>Potentilla reptans</i> L.		
The Mann-Whitney test for the comparison of the DNA extracted from fresh and dried leaves using the second modified CTAB method	0.800	0.200	1.000	0.200	0.200	0.400	0.100	0.100	0.700
The Mann-Whitney test for the comparison of the DNA extracted from fresh and dried leaves using the modified Murray and Thompson method	0.100	1.000	0.700	1.000	0.700	0.700	0.100	0.100	0.700
The Mann-Whitney test for the comparison of the DNA extracted from fresh and dried leaves using Gene All kit	0.667	1.000	0.333	1.000	1.000	0.667	0.667	0.667	0.667
The numbers in bold indicate that the difference is significant. The significance level was set at $P < 0.05$.									

Table 4- **Statistical analysis of the concentration of the total extracted DNA ([DNA]; ng/μl) and the ratio of optical absorbance in the studied herbs.**

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In the next step, the results of the five extraction methods that showed a significant difference were compared to determine the best method for DNA extraction from fresh or dried leaves of each herb. As shown in Table 5, for the herb *P. harmala*, the highest and lowest concentrations of the tDNA extracted from the fresh leaves were obtained using the modified Murray and Thompson method and the Gene All kit, respectively. In comparison, the first and second modified CTAB methods gave the highest and lowest concentrations of the tDNA extracted from dried leaves, respectively. As for the A260/A230 ratio of the tDNA extracted from the fresh leaves, the second modified CTAB and the main CTAB methods resulted in the lowest and highest non-protein contaminants. However, for the dried leaves of *P. harmala*, the modified Murray and Thompson method and the main CTAB method ranked first and last, respectively.

Table 5

Ranking of the studied DNA extraction methods based on the concentration of the extracted total DNA ([DNA]) and optical absorbance ratios in the herbs *Peganum harmala* L., *Tamarix ramosissima* Ledeb, and *Potentilla reptans* L.

Fresh leaves		Dried leaves	
P. harmala	[DNA] (ng/ μL)	The modified Murray and Thompson method > the main CTAB > the first modified CTAB > the second modified CTAB > Gene All kit	The first modified CTAB > modified Murray and Thompson method > Gene All kit > the main CTAB > the second modified CTAB
	A260/A230	The second modified CTAB > the modified Murray and Thompson method > the first modified CTAB > Gene All kit > the main CTAB	The modified Murray and Thompson method > the first modified CTAB > Gene All kit > the second modified CTAB > the main CTAB
T. ramosissima	[DNA] (ng/ μL)	No significant difference was seen between the studied extraction methods.	The second modified CTAB > the first modified CTAB > the main CTAB > the modified Murray and Thompson method > Gene All kit
	A260/A230	No significant difference was seen between the studied extraction methods.	The modified Murray and Thompson method > the first modified CTAB > the main CTAB > the second modified CTAB > Gene All kit
P. reptans	[DNA] (ng/ μL)	No significant difference was seen between the studied extraction methods.	The modified Murray and Thompson method > the first modified CTAB > the main CTAB > the second modified CTAB > Gene All kit
	A260/A230	No significant difference was seen between the studied extraction methods.	The first modified CTAB > the second modified CTAB > the main CTAB > the modified Murray and Thompson method > Gene All kit

In *T. ramosissima*, the second modified CTAB method and the Gene All kit resulted in the highest and lowest quality of DNA, respectively, in terms of non-protein contamination (A260/A230). For *T. ramosissima*, the modified Murray and Thompson method gave the highest tDNA concentration, while the lowest non-protein contamination was obtained with the first modified CTAB method. However, the Gene All kit found the lowest tDNA concentration and the highest non-protein contamination (Table 5).

For the dried leaves of *P. reptans*, where the difference between the extraction methods was significant, the modified Murray and Thompson method and the Gene All kit resulted in the highest and lowest tDNA concentrations. The tDNA extracted from dried leaves of *P. reptans* using the Gene All kit also had the lowest A260/A230 ratio. However, the highest A260/A230 ratio was obtained with the first modified CTAB method (Table 5).

Table 6 shows the statistical comparison of the quantitative and qualitative parameters of the extracted tDNA between the studied herbs. The statistical comparison was performed based on the Kruskal-Wallis test, as explained in the data presented in Table 4.

Table 6

Statistical comparison of the quantitative and qualitative parameters of the extracted total DNA and PCR results of the fragments of the nuclear internal transcribed spacer (ITS) and *trnL-F* (f and c) regions between the herbs *Peganum harmala* L., *Potentilla reptans* L., and *Tamarix ramosissima* Ledeb.

	<i>P. harmala</i>		<i>T. ramosissima</i>		<i>P. reptans</i>	
	Fresh leaves	Dried leaves	Fresh leaves	Dried leaves	Fresh leaves	Dried leaves
The highest [DNA] (ng/μL)	The modified Murray and Thompson method (403.33 ± 261.74) *	The first modified CTAB (308.66 ± 71.16) *	The main CTAB method (236.00 ± 112.66)	The main CTAB method (223.33 ± 102.83)	The modified Murray and Thompson method (1131.33 ± 541.47)	The modified Murray and Thompson method (1477.66 ± 231.70) *
The lowest [DNA] (ng/μL)	Gene All kit (51.50 ± 3.53) *	The second modified CTAB (63.50 ± 45.96) *	Gene All kit (46.00 ± 28.28)	Gene All kit (60.00 ± 11.31)	Gene All kit (55.50 ± 7.77)	Gene All kit (56.50 ± 7.77) *
The highest A260/A230	The second modified CTAB method (0.72 ± 0.04) *	The modified Murray and Thompson method (0.64 ± 0.02) *	The second modified CTAB method (1.21 ± 0.300)	The second modified CTAB (1.77 ± 0.212) *	The first modified CTAB method (1.45 ± 0.742)	The first modified CTAB method (1.66 ± 0.113) *
The lowest A260/A230	The main CTAB method (0.54 ± 0.015) *	The second modified CTAB (0.45 ± 0.12) *	Gene All kit (0.46 ± 0.035)	Gene All kit (0.48 ± 0.070) *	Gene All kit (0.58 ± 0.084)	Gene All kit (0.56 ± 0.070) *
2 > A260/A280 > 1.8	-	-	-	The second modified CTAB method (1.77 ± 0.212)	-	The main CTAB method (1.50 ± 0.02) The first modified CTAB (1.66 ± 0.113) The second modified CTAB (1.51 ± 0.043)
The highest A260/A280	The second modified CTAB method (1.35 ± 0.06)	The second modified CTAB method (2.06 ± 1.04)	The modified Murray and Thompson method (1.48 ± 0.129)	The second modified CTAB method (1.67 ± 0.042)	The main CTAB method (1.33 ± 0.049)	The main CTAB method (1.51 ± 0.021)
The lowest A260/A280	The modified Murray and Thompson method (1.19 ± 0.045)	Gene All kit (1.21 ± 0.084)	Gene All kit (1.28 ± 0.070)	The main CTAB method (1.25 ± 0.170)	The modified Murray and Thompson method (1.17 ± 0.037)	Gene All kit (1.11 ± 0.106)
A260/A280 ≥ 1.8	-	The second modified CTAB method (2.06 ± 1.04)	-	-	-	-

The asterisk indicates the elements where the extraction methods differed significantly based on the Kruskal-Wallis test.

	<i>P. harmala</i>		<i>T. ramosissima</i>		<i>P. reptans</i>	
	Fresh leaves	Dried leaves	Fresh leaves	Dried leaves	Fresh leaves	Dried leaves
1.5 < A260/A280 < 1.8	-	The first modified CTAB method (1.70 ± 0.17)	-	The second modified CTAB method (1.67 ± 0.042)	-	The main CTAB method (1.51 ± 0.021) The 1st modified CTAB (1.50 ± 0.077)
The asterisk indicates the elements where the extraction methods differed significantly based on the Kruskal-Wallis test.						

Table 6- **Statistical comparison of the quantitative and qualitative parameters of the extracted total DNA and PCR results of the fragments of the nuclear internal transcribed spacer (ITS) and trnL-F (f and c) regions between the herbs Peganum harmala L., Potentilla reptans L., and Tamarix ramosissima Ledeb.**

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The concentrations of the DNA extracted from the fresh and dried leaves of *P. harmala* by the five extraction methods were significantly different (p-values = 0.043 and 0.041, respectively; Table 4). As shown in Table 6, the highest and lowest DNA concentrations were obtained from fresh leaves using the modified Murray and Thompson method (403.33 ± 261.74) and the Gene All kit (51.50 ± 3.53), respectively. The highest and lowest DNA concentrations for the dried leaves were extracted using the first modified CTAB (308.66 ± 71.16) and the second modified CTAB (63.50 ± 45.96), respectively.

The difference between the studied extraction methods was also significant with respect to the A260/A230 ratio between the extracted DNA from fresh and dried leaves of *P. harmala* (both p-values = 0.04; Table 4). The highest and lowest ratios of A260/A230 for the fresh leaves were obtained using the second modified CTAB method (0.72 ± 0.04) and the modified Murray and Thompson method (0.54 ± 0.02), respectively. For the dried leaves, the highest and lowest A260/A230 ratios were obtained using the modified Murray and Thompson (0.64 ± 0.02) method and the second modified CTAB (0.45 ± 0.12) method, respectively (Table 6).

Regarding the A260/A280 ratio, no significant difference was found between the extraction methods for fresh or dried leaves of *P. harmala* (p-values = 0.079 and 0.096, respectively; Table 4). As can be seen in Table 6, the highest A260/A280 ratios for both fresh and dried leaves were obtained using the second modified CTAB method (1.35 ± 0.06 and 2.06 ± 1.04 , respectively). However, the modified Murray and Thompson method and the Gene All kit gave the highest (1.19 ± 0.045) and lowest (1.21 ± 0.084) A260/A280 ratios for fresh and dried leaves, respectively.

For *T. ramosissima*, no significant difference was found between the extraction methods with respect to the parameters studied, except for the A260/A230 ratio in the dried leaves (p-value = 0.032; Table 4). The highest amount of the DNA extracted from both fresh (236.00 ± 112.66) and dried leaves (223.33 ± 102.83) was obtained by the main CTAB method. In comparison, the Gene All kit yielded the least amount of DNA from both fresh (46.00 ± 28.28) and dried leaves (60.00 ± 11.31).

The only parameter that changed significantly for *T. ramosissima* was the A260/A230 ratio in the dried leaves, yielding the highest (1.77 ± 0.212) and lowest (0.48 ± 0.070) ratios with the second modified CTAB method and the Gene All kit, respectively. For the fresh leaves, the highest (1.21 ± 0.300) and lowest (0.46 ± 0.035) ratios of A260/A230 were obtained with the second modified CTAB method and the Gene All kit, respectively.

The ratio of A260/A280 of the DNA extracted from *T. ramosissima* did not change significantly across the extraction methods (Table 4). The highest ratio in the fresh (1.48 ± 0.129) and dried (1.67 ± 0.042) leaves of *T. ramosissima* was obtained using the modified Murray and Thompson method and the second modified CTAB method. However, the Gene All kit and the main CTAB method gave the lowest A260/A280 ratio in the fresh (1.28 ± 0.070) and dried (1.25 ± 0.170) leaves, respectively.

For *P. reptans*, the five extraction methods differed significantly in the concentration and A260/A230 ratio of the DNA extracted from the dried leaves (both p-values = 0.034), but not for the fresh leaves (Table 4). According to our results, the concentration of the DNA extracted from *P. reptans* leaves was higher compared to the other two herbs studied. As shown in Table 6, the highest DNA concentration was obtained in both the fresh (1131.33 ± 541.47) and dried (1477.66 ± 231.70) leaves using the modified Murray and Thompson method. The Gene All Kit, on the other hand, gave the lowest DNA concentration in both fresh (55.50 ± 7.77) and dried (56.50 ± 7.77) leaves. The Gene All kit also gave the lowest A260/A230 ratio in both the fresh (0.58 ± 0.084) and the dried (0.56 ± 0.070) leaves. However, the highest A260/A230 ratio was obtained by the first modified CTAB method in both fresh (1.45 ± 0.742) and dried (1.66 ± 0.113) leaves.

Regarding the ratio of A260/A280 in the fresh or dried leaves of *P. reptans*, no significant change was observed across the extraction methods (p-values = 0.204 and 0.068, respectively; Table 4). The highest ratio in the fresh (1.33 ± 0.049) and dried (1.51 ± 0.021) leaves was obtained by the main CTAB method. In comparison, the highest A260/A280 ratios in the fresh (1.33 ± 0.049) or dried (1.17 ± 0.037) leaves were obtained by the main CTAB method and the modified Murray and Thompson method respectively.

3-1-2- Gel Electrophoresis

Figure 1. **The quality assessment of the extracted total DNA from (a) *Peganum harmala* L., (b) *Tamarix. ramosissima* Ledeb., and (c) *Potentilla reptans* L. using the studied extraction methods.** About 500 µg of DNA was analyzed by electrophoresis on a 0.8% agarose gel. 1) the main CTAB method - fresh leaves, 2) the main CTAB method - dried leaves, 3) the first CTAB method - fresh leaves, 4) the first CTAB method - dried leaves, 5) the second CTAB method - fresh leaves, 6) the second CTAB method - dried leaves, 7) the modified Murray and Thompson method - fresh leaves, 8) the modified Murray and Thompson method - dried leaves, 9) the Gene All kit- fresh leaves, and 10) the Gene All kit- dried leaves.

3-2- PCR

Table 7 shows the PCR programs used to amplify the ITS fragments in the DNA extracted from the leaves of the studied herbs. The suitability of the tDNA extracted from fresh and dried leaves of the studied herbs for molecular studies was evaluated by the PCR of the ITS fragments in the nuclear ribosomal DNA and *trnL-F* region in the chloroplast genome.

Tables 7- PCR programs used to amplify the nuclear internal transcribed spacer (ITS) in the DNA extracted from leaves of the herbs *Peganum harmala* L., *Potentilla reptans* L., and *Tamarix ramosissima* Ledeb.

	<i>P. harmala</i>	<i>T. Ramosissima</i>	<i>P. reptans</i>
DNA extraction method- plant sample	PCR program		
Main CTAB method (Doyle and Doyle, 1987) fresh and dried leaves	TD 2 PCR	Main PCR	Main PCR
The first modified CTAB method - fresh leaves	TD 2 PCR	TD 2 PCR	TD 1 PCR
The first modified CTAB method - dried leaves	Main PCR	Main PCR	Main PCR
The second modified CTAB method - fresh leaves	TD 2 PCR	TD 1 PCR	TD 1 PCR
The second modified CTAB method - dried leaves	Main PCR	Main PCR	Main PCR
Modified Murray and Thompson method (Murray andThompson, 1980 fresh and dried leaves	Main PCR	Main PCR	Main PCR
Gene All kit - fresh leaves	Main PCR	Main PCR	TD 2 PCR
Gene All kit - dried leaves	Main PCR	Main PCR	Main PCR
For more information on the main and touchdown polymerase chain reaction (TDPCR) programs, see Tables 2 and 3, respectively. For information on the extraction methods, see Section 2-2-DNA extraction.			

Figure 2 (a, b, and c) shows the results of the PCR of the ITS fragments of *P. harmala*, *T. ramosissima*, and *P. reptans*, respectively. The PCR products were amplified to the expected size (700 bp) from the tDNA extracted by all extraction methods studied.

Figure 2 (d, e, and f) shows the results of the PCR of the *trnL-F* region in the chloroplast genome of *P. harmala*, *T. ramosissima*, and *P. reptans*, respectively. AS EXPECTED, the PCR products were amplified at 700 bp with the tDNA extracted by all extraction methods examined. However, the PCR of the *trnL-F* region gave no results using the tDNA extracted by the main CTAB method, the first CTAB method, and the modified Murray and Thompson method from fresh or dried leaves of *T. ramosissima* when the main PCR programs (Table 5) or TD PCR (Table 6) were used.

4 Discussion

This is the first report on the extraction of the tDNA from medicinal plants suitable for molecular studies: *P. harmala*, *T. Ramosissima*, and *P. reptans*. High-quality DNA extraction is essential for molecular studies and identification of the genetic source of herbal medicine and helps in ethnopharmacological and ethnobotanical studies (Nesbitt, 2014). DNA is often harder to extract from medicinal herbs because these plants usually contain more secondary metabolites, biochemicals other than DNA that interfere with the extraction process (Sika et al., 2015; Sharma et al., 2017; Anerao et al., 2016). In addition, different plants have different biochemical compositions. Therefore, the same methods will not work with all of them (Sharma et al., 2017).

Young plant tissues are usually used for DNA extraction because they contain fewer polyphenols and polysaccharides compared with mature tissues. These substances interfere with DNA extraction and inhibit enzymatic digestion of DNA or PCR (Allen et al., 2006; Saboora et al., 2019; Khan et al., 2007; Križman et al., 2006). Drying can destroy cells and the cell wall, remove the cell membrane, and degrade the DNA. In addition, the sample of interest is sometimes a rare species, not easily found or is collected in remote areas. Therefore, it is kept in a dried or semi-dried state. Therefore, the usefulness of protocols for DNA extraction from dried samples cannot be overstated (Allen et al., 2006; Saboora et al., 2019; Khan et al., 2007).

In the present study, the tDNA was extracted from young fresh and dried leaves of the studied herbs using five methods: the main CTAB method (Doyle and Doyle, 1987) and its two modified protocols, the modified Murray and Thompson method (Murray and Thompson, 1980), and the Gene All kit. The first and second modified CTAB methods excluded BME and ammonium acetate, respectively, compared to the main method. The Murray and Thompson method was introduced in 1980 (Murray and Thompson 1980). In the current study, a modified protocol of the Murray and Thompson method developed by Riahi et al. (Riahi et al., 2010) was used, which does not require an ultracentrifuge.

For all herbs studied, there were no significant differences between the tDNAs extracted from fresh and dried leaves by each of the extraction methods examined in terms of concentration and optical absorbance ratios (Table 4; results of the Mann-Whitney test). Considering the A260/A280 ratio, all extracted total DNAs from the fresh and dried leaves of all studied herbs had the same quality in terms of protein contaminants. However, none of the extraction methods studied resulted in an A206/A280 ratio greater than 1.8, except for the DNA extracted from the dried leaves of *P. harmala* (Table 6). It can be concluded that all the extracted DNAs had protein contaminants (1001).

Significant differences were observed between the DNA extracted from the fresh or dried leaves of the studied herbs by the five extraction methods investigated in terms of DNA concentration and A260/A230 ratio (Table 4; results of the Kruskal-Wallis test). In the case of *P. harmala*, the tDNAs extracted from fresh and dried leaves differed significantly in terms of the A260/A230 ratio and concentration. The highest and lowest concentrations of the DNA extracted from fresh leaves were obtained using the modified Murray and Thompson method and the Gene All kit, respectively. In comparison, the DNA with the highest and lowest concentration was extracted from dried leaves using the first and second modified CTAB methods, respectively. The second modified CTAB method and the main CTAB method resulted in DNAs with the highest and lowest ratios of A260/230 extracted from the fresh leaves. However, the DNAs with the highest and lowest A260/230 ratios were extracted from the dried leaves using the modified Murray and Thompson method and the main CTAB, respectively.

A significant difference between the studied extraction methods was only observed in the A260/A230 ratio of the tDNAs extracted from the dried leaves of *T. ramosissima*, such that the second modified CTAB and the Gene All kit resulted in the highest and lowest A260/230 ratios, respectively.

For *P. reptans*, the concentration and A260/A230 ratio of the tDNAs extracted from only the dried leaves differed significantly across the methods studied. The modified Murray and Thompson method and the first modified CTAB method yielded the highest concentration and A260/230 ratio. However, the tDNA extracted using the Gene All kit had the lowest concentration and A260/230 ratio.

The appropriateness of the extracted tDNA was examined by the PCR of the ITS region of the nrDNA (King and Roalson 2008) and the *trnL-F* region in the chloroplast genome (Taberlet et al., 1991). Despite the protein and non-protein contamination of the DNA, the ITS fragments in the nuclear ribosomal region of all extracted DNA from the fresh and dried leaves of all studied herbs were amplified by PCR using all methods examined (Fig. 2a-c). However, only the *trnL-F* region in the chloroplast regions of the tDNA extracted from both the fresh and dried leaves of *P. harmala* by all methods examined was amplified by PCR (Figure 2d). In comparison, the *trnL-F* region in the cpDNA was amplified only in the DNA extracted from the fresh and dried leaves of *T. Ramosissima* using the second modified CTAB method and the Gene All kit (Figure 2e). In *P. reptans*, the *trnL-F* region in the cpDNA was amplified only in tDNAs extracted both from the fresh and dried leaves using the Gene All kit and from the fresh leaves using the main and modified CTAB methods (Figure 2f).

It appears that the removal of BME or ammonium acetate from the main CTAB as in the first and second modified CTAB methods, respectively, did not significantly affect the protein contaminants of the extracted DNAs, and the PCR results were acceptable.

BME is a reducing agent that can remove tannins and polyphenols and denatures proteins by reducing disulfide bonds (Jadhav et al., 2015). In many protocols, the addition of BME is an approach to improving the quality of the extracted DNA. Arruda et al. developed a modified protocol for DNA extraction from *Mimosa tenuiflora*. In this method, which resulted in intact DNA successfully amplified in PCR, the concentrations of CTAB, NaCl, polyvinylpyrrolidone (PVP), and BME were increased, phenol was used to eliminate proteins, and the incubation time was decreased at lower temperatures (Arruda et al., 2017).

Hammad and Qari (Hammad and Qari, 2010) extracted DNA from some herbs related to *P. harmala*, such as *Zygophyllum coccineum* L., *Zygophyllum album* L.F., and *Zygophyllum aegyptium* A. Hosny using a modified CTAB method similar to the main CTAB method used in this study. They successfully used the extracted DNA for random amplified polymorphic DNA (RAPD).

The chloroplast genome (cpDNA) can provide a wealth of information on plant phylogeny, molecular ecology, population genetics, and evolution. The extraction of cpDNA and the nuclear genome usually reduces the quality of the cpDNA (Atherton et al., 2010; Cronn et al., 2008; Shi et al., 2012). Therefore, the low quality or quantity of cpDNA in the extracted tDNA could be a possible reason for the observed PCR errors in the amplification of the chloroplast region in *T. Ramosissima* and *P. reptans*. Not all research teams,

though, have access to centrifuges or ultracentrifuges necessary for the isolation of intact chloroplasts and direct extraction of cpDNA from them.

In the present study, plant samples were homogenized without liquid nitrogen. Despite its great influence on the quality of DNA extraction, researchers performing DNA extraction often try to exclude liquid nitrogen from their protocols to save money, as tight research budgets do not allow such extravagances. Extreme care must also be taken when using this substance in a laboratory. It is highly toxic and poses a hazard to the user (Sharma et al., 2010; Saba Hasan et al., 2012; Ali et al., 2019). Qasim Ali et al., developed a method to extract DNA from *Polianthes tuberosa* using common laboratory equipment. The DNA was obtained in reasonable yield and was suitable for subsequent molecular studies (Ali et al., 2019).

4-1- Strengths and limitations of the study

This study is one of the few studies that have been statistically analyzed. However, it has several major shortcomings due to a lack of equipment and adequate research funding. The most important deficiency was the lack of detailed phytochemical analyses. We did not examine phytochemicals in any of the fresh or dried leaves. However, in other similar studies on these herbs, researchers have found useful information on *P. harmala* (Herraiz et al., 2010; Sharifi-Rad et al., 2012), *T. Ramosissima* (Mikaeili et al., 2018; Ren et al., 2019), and *P. reptans* (Radovanovic et al., 2013; Tomczyk and Latté, 2009). It is also worth noting that the nature of the remaining contaminants in the extracted DNAs was not studied. Protein and non-protein contaminants were evaluated only by calculating the ratios of A260/A280 and A260/A230, respectively. Accordingly, the exact reason for the observed PCR failures in some DNA samples of *T. ramosissima* and *P. reptans* was not determined. The second shortcoming is that only the extraction protocols were performed in three replicates. Therefore, it was impossible to analyze the results' normality and apply parametric methods. Despite all these shortcomings, this study is important for two reasons: 1) we statistically compared the results from five different extraction methods, and 2) the results apply to laboratories that have the same limitations as we do.

5 Conclusion

In conclusion, a single protocol cannot be proposed as a suitable method for DNA extraction for PCR from all plant species. Although the Gene All kit gave the lowest concentration of the tDNA in some samples studied, it was the best method to extract high-quality genomic DNA for PCR. In the absence of kits for DNA extraction, the main CTAB method and its modified protocols (with the elimination of BME or ammonium acetate) investigated in the current study are less time-consuming protocols and yield a DNA suitable for subsequent PCR compared with the modified Murray and Thompson method.

Declarations

Acknowledgment

The authors would like to thank Damghan University for the financial support of this study.

Ethical Approval

Not applicable.

Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Authors' contributions

This research was extracted from ZS's dissertation. AR, AA, and PF have made a substantial contribution to the conception and design of the study. All authors except for JGH have made contributions to performing the experiments. The analysis and the interpretation of data were done in collaboration with JGH. AR wrote the article. All authors were involved in the critical revision of the manuscript and approved the final manuscript.

Funding

The authors would like to thank Damghan University for the financial support of this study.

Availability of data and materials

Data are available upon request.

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Figures

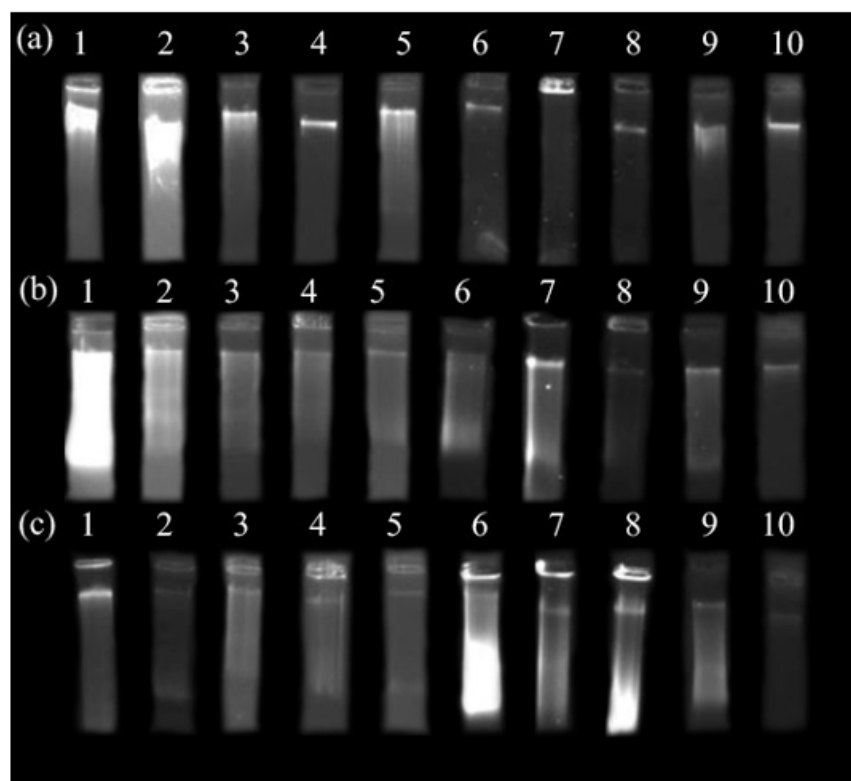


Figure 1

The quality assessment of the extracted total DNA from (a) *Peganum harmala* L., (b) *Tamarix. ramosissima* Ledeb., and (c) *Potentilla reptans* L. using the studied extraction methods. About 500 µg of DNA was analyzed by electrophoresis on a 0.8% agarose gel. 1) the main CTAB method - fresh leaves, 2) the main CTAB method - dried leaves, 3) the first CTAB method - fresh leaves, 4) the first CTAB method - dried leaves, 5) the second CTAB method - fresh leaves, 6) the second CTAB method - dried leaves, 7) the modified Murray and Thompson method - fresh leaves, 8) the modified Murray and Thompson method - dried leaves, 9) the Gene All kit- fresh leaves, and 10) the Gene All kit- dried leaves.

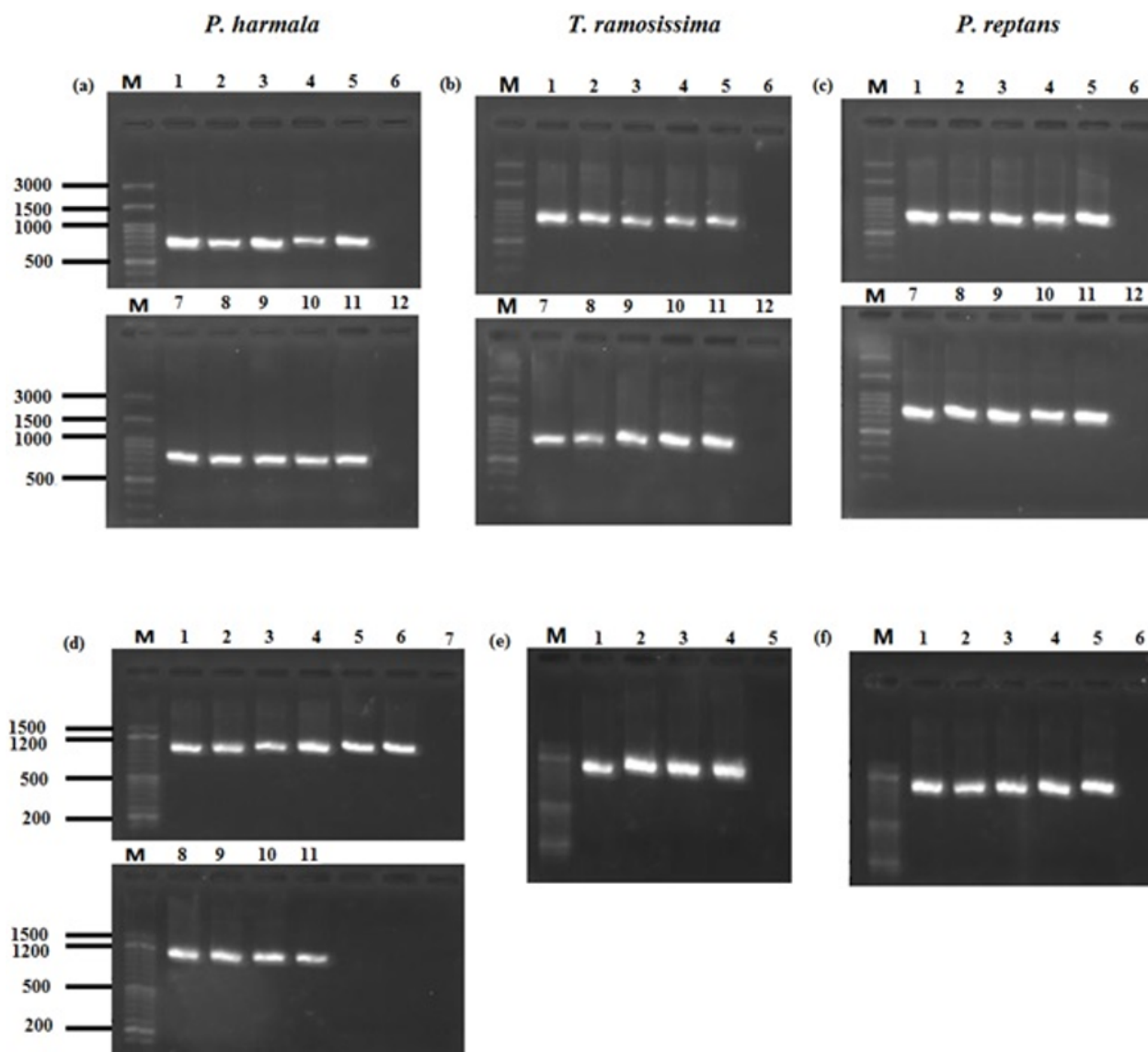


Figure 2

Approximately five microliters of the PCR products were electrophoresed on a 1.5% agarose gel. **(a, b, and c)** The results of the PCR of the ITS fragments in the nuclear ribosomal DNA of *Peganum harmala* L., *Tamarix ramosissima* Ledeb., and *Potentilla reptans* L. using the universal primers of ITS4 and ITS5m. M: DNA molecular ladder (DM2300), 1) the main CTAB method - fresh leaves, 2) the first CTAB method - fresh leaves, 3) the second CTAB method - fresh leaves, 4) the modified Murray and Thompson method - fresh leaves, 5) Gene All kit - fresh leaves, 6) negative control, 7) the main CTAB method - dried leaves, 8) the first CTAB method - dried leaves, 9) the second CTAB method - dried leaves, 10) the modified Murray and Thompson method - dried leaves, 11) the Gene All kit - dried leaves, and 12) negative control. **(d, e, and f)** The results of the PCR of the *trnL*-F region in the chloroplast genome of *P. harmala*, *T. ramosissima*, and *P. reptans*, respectively, using the universal primers of *trnL*(f) and *trnL*(c). M: DNA molecular ladder (DM2300); (d): 1) the Gene All kit - fresh leaves, 2) Gene All kit - dried leaves, 3) the second CTAB method - fresh leaves, 4) the second CTAB method - dried leaves, 5) the main CTAB method - fresh leaves, 6) the main CTAB method - dried leaves, 7) negative control, 8) the first CTAB method - fresh leaves, 9) the first CTAB method - dried leaves, 10) the modified Murray and Thompson method - fresh leaves, and 11) the modified Murray and Thompson method - dried leaves. (e): 1) Gene All - fresh leaves, 2) Gene All Kit - dried leaves, 3) the second CTAB method - fresh leaves, 4) the second CTAB method - dried leaves, 5) negative control. (f): 1) Gene All Kit - fresh leaves, 2) Gene All Kit - dried leaves, 3) the second CTAB method - dried leaves, 4) the first CTAB method - dried

leaves, 5) the main CTAB method - dried leaves, 7) negative control. To see which PCR products were obtained with which PCR program, please see Table 4.