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Comparative Different DNA Isolation Protocols from *Ziziphus spina-christi* (L.) Leaves through RAPD and ISSR Markers

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Abstract

Genomic analysis of plants relies on high quantity and quality of pure DNA. Extraction and purification of DNA from woody and medicinal plants, such as fruit trees present a great challenge due to accumulation of a large amount of co-purify with DNA, including polysaccharides, polyphenols and proteins. Therefore, it is necessary to optimize the extraction protocols to reduce these compounds to the lowest level. A study was conducted to compare six DNA extraction and precipitation methods for genomic analysis in *Ziziphus spina-christi* (L.) plant tissues. The results showed significant differences in DNA contents among the six methods. Quantity and quality of extracted genomic DNAs were compared by employing the spectrophotometer, Nano-Drop, agarose gel electrophoresis, digestion by restriction enzymes and polymerase chain reaction (PCR) methods and molecular marker such as RAPD and ISSR. The method of Vroh Bi et al., provided the best results (208.89 ng/μL) in terms of quantity and quality of DNA, and Doyle and Doyle method as second method for leaves sample were chosen. According to the results, the method of Bi et al. is recommended for DNA extraction from plant tissues having high level of polysaccharides and phenol compounds.

Keywords: *Ziziphus spina-christi*, DNA extraction, PCR, ISSR, RAPD, gen cloning, enzyme digestion

1. Introduction

Ziziphus spina-christi (L.) is a tree indigenous to the south of Iran. The leaves of this plant, which are locally known as "Sedr" and "Konar", have been used for washing the hair and body (Anthony, 2005). Plant leaves are also used in Iranian folk medicine as an antiseptic, antifungal and anti-inflammatory agent, and for healing skin diseases such as topic dermatitis (Nafisy, 1989). The Irainian *Z. spina-christi*, commonly known as evergreen tree, is a dominant component of the natural vegetation in the south of Iran. Aqueous leaf extract of *Z. spina-christi* may possess anti-nociceptive properties in the rat and have a calming effect on the central nervous system (Effraim et al., 1998). It has been described as ant cathartic, astringent, diuretic and tonic (Larsen, 1985). *Z. spina-christi* is a naturally distributed tree of subtropical, arid and semi-arid parts of Iran. The species is ecologically and economically important due to fruit nutrition, leaves and shoots saponin, and tannin substrates (Sudhersan & Hussain, 2003; Weinges & Schick, 1995).

DNA extraction is the most critical step for genomic analysis especially from plant materials due to accumulation of a large amount of interfering substances including polysaccharides, proteins, polyphenols and secondary metabolites, which interfere in downstream reactions such as DNA restriction, amplification and cloning (Zamboni et al., 2008). Several DNA extraction protocols have been successfully utilized to plant species (J. J. Doyle & J. L. Doyle, 1990; Reichandt & Rogers, 1994).

J. J. Doyle and J. L. Doyle method (1990) is applied to extract DNA from horticulture plants and fruit trees (Jenderek et al., 1997). The extraction technique of Lodhi et al. (1994) has been utilized for grape, apple, apricot, peach, cherry and snapdragon. Bi et al. (1996) method has been used by Sarkhosh et al. (2006) for some Iranian pomegranate (*Punica granatum* L.) genotypes. Zamani et al. (2005) reported that Bi et al. (1996) method was an effective DNA extraction method from pomegranate leaves. Kiani et al. (2008) successfully used Bi et al. (1996) method for DNA extraction from Damask rose (*Rosa damasena* Mill.) genotypes. Murray and Thompson (1980)

method was used for DNA extraction in cabbage, olive, and rose (Csaikl et al., 1998), sweet cherry (Khadivi-Khub et al., 2008). Talebi Baddaf et al. (2003) introduced Murry and Thompson (1980) method as the most appropriate one regarding to quality of DNA extracted from pomegranate leaves. Dellaporta et al. (1983) method was an effective DNA extraction method from leaf samples of spruce, white poplar, pine, oak, corn and anthurium (Buldewo & Jaufeerally-Fakim, 2002). Ziegenhagen et al. (1993) was an effective DNA extraction method from leaf samples of silver fir (*Abiesalba* Mill.) and pearl millet (*Pennisetum glaucum*) (Zidani et al., 2005). These methods were further improved by modification in compound and pH of functional buffers to extract high-quality and quantity DNA from plants (Cheng et al., 2003; Lodhi et al., 1994; Porebski et al., 1997), but they are not usable for all medicinal plants. Therefore, it is necessary to modify the extraction protocols. A perfect method should be highly reproducible and represent the highest yield and pure DNA with the lowest content of contaminants that could be amplified in the polymerase chain reaction (PCR). Therefore, the present study aims to evaluate six different DNA extraction methods to isolate high-quality DNA from *Ziziphus spina-christi* leaves. Random amplified polymorphic DNA (RAPD's) reactions and inter-simple sequence repeat (ISSR) amplification were also performed in order to evaluate the suitability of the DNA extraction methods for PCR-based techniques. As far as we know, this is the first report on DNA extraction from *Ziziphus spina-christi* leaves, and we expect that the suggested protocol can be an incentive to perform studies in order to investigate the genetic diversity among the *Ziziphus* genus, which is comprised of about 100 species of deciduous or evergreen trees and shrubs distributed in the tropical and subtropical regions of the world (Johnston, 1963).

2. Materials and Methods

2.1 Plant Materials

Ziziphus spina-christi leaves were collected from Dezful, Iran and were used in all experiments. One gram of young and mature leaf were collected and thoroughly washed with deionised water, and were then frozen in liquid nitrogen and stored at -80 °C until extraction.

2.2 DNA Extraction Methods

One gram of the frozen leaf samples of *Z. spina-christi* was ground into fine powder using pre-cooled mortar and pestle, and then homogenized with six different DNA extraction methods based on randomized complete block design (RCBD) with five replicates. The six extraction methods were; 1) Murry and Thompson (1980); 2) Lodhi et al. (1994); 3) Dellaporta et al. (1983); 4) J. J. Doyle and J. L. Doyle (1990); 5) Ziegenhagen et al. (1993); 6) Bi et al. (1996) methods. After DNA extraction and sedimentation, resulted pellet was rinsed with ethanol 75% and then was dissolved in 200 µL double distilled sterile water overnighted at 4 °C and stored at -80 °C until next treatments.

2.3 Concentration, Purity and Quality of the DNA Extracted

The quantity and quality of the DNA obtained in ratio of 1:49 (20 µL of DNA stock solution + 980 µL of double distilled sterile water) were assessed spectrophotometrically at 260 and 280 nm, and the A260/A280 ratio was used to assess contamination with proteins by employing the spectrophotometry (Hitachi U-2001 UV/VIS), Nano-Drop™ (Thermo Scientific) described by Brodmann (2008) and Wilmington (2008), and agarose gel electrophoresis, digestion by restriction enzymes such as *Eco.*RI and *Hin*d III, polymerase chain reaction (PCR) methods and molecular markers such as RAPD and ISSR. This spectrophotometric analysis was performed in triplicate on the samples of extracted DNA using spectrophotometer. In order to verify DNA integrity, 3 µL DNA were subjected to gel electrophoresis on 0.8% (w/v) agarose gel, stained with ethidium bromide, visualized photographed using the Gelodoc© under UV lighting.

2.4 RAPD and ISSR Amplifications

RAPD and ISSR analysis were used to test the quality and performance of the DNA extracted from six different methods. RAPD reactions were performed in a volume of 12 µL containing 5 µL of extracted DNA, 2 µL loading buffer (0.5% Bromophenol blue, 40% sucrose, 0.1 M EDTA pH = 8 and 0.5% SDS) and 5 µL double distilled sterile water. The final volume of 12 µL was tested in PCR reaction (2.5 µL PCR reaction buffer 10x, 0.875 µL $MgCl_2$ 50 mM, 0.5 µL dNTPs 10 mM, 1.0 µL primer 10 µM (5′-CCACGGTCAG-3′), 0.2 µL Taq DNA polymerase 5 Unit/µL, 2.0 µL template DNA (5 ng/µL). The ability of amplification of extracted DNA strands was proved by a randomized RAPD and ISSR molecular markers by PCR. The products of PCR amplifications were analyzed by electrophoresis at 70 V in 0.8% (w/v) agarose (Sigma Aldrich, USA) gels in the presence of a one Kb molecular weight marker (Sigma Aldrich, USA). PCR amplification was performed as follows: initial denaturation at 94 °C for 4 min, followed by 35 cycles of 1 min at 92 °C, 1 min at 37 °C and 2 min at 72 °C, and a final extension at 72 °C for 1 min using Bio-Rad® thermocycler, model "ICycler". The gel was stained with

ethidium bromide, visualized under a UV transilluminator and then photographed using the Gelodoc© under UV lighting. The experiment was repeated three times.

3. Results and Discussion

3.1 Comparison of Different DNA Extraction Methods on Agarose Gel Electrophoresis

The six DNA extraction methods were compared in terms of DNA yield and DNA purity. Agarose gel electrophoresis was subsequently used to check the integrity of the DNA and its average molecular weight, which can be calculated by using an appropriate standard DNA marker. The DNA band density was measured by using the gel imaging system. The results showed that the DNA isolated by the six methods was over 10 kb (Figure 1). The results confirmed that extracted DNA by Bi et al. (1996) method from leaves possess better quality and quantity in comparison with the other extraction methods as well J. J. Doyle and J. L. Doyle (1990) and Ziegenhagen et al. (1993) methods.

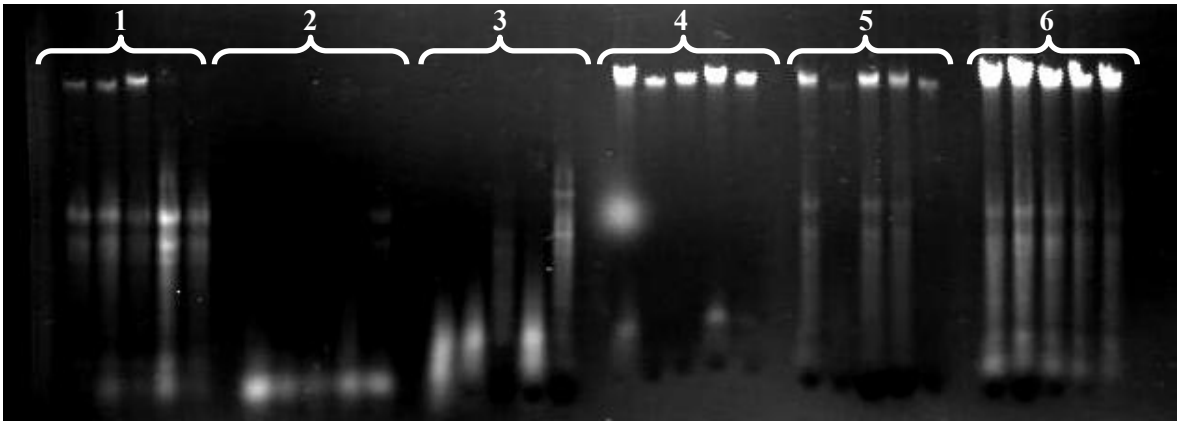

Figure 1. Electrophoretic pattern of DNA extracted by the six different methods from *Z. spina-christi* leaves

Note. The electrophoresis was performed in 0.8% (w/v) agarose gel. The extraction methods were: 1) Murry and Thompson (1980); 2) Lodhi et al. (1994); 3) Dellaporta et al. (1983); 4) J. J. Doyle and J. L. Doyle (1990); 5) Ziegenhagen et al. (1993); and 6) Bi et al. (1996).

3.2 UV Spectrophotometer

In spectrophotometer procedure, absorption of double-stranded DNA in wavelength of 260 nm is 50 μg/μL. In fact, the ratio of absorption amount resulted in 260 nm to 280 nm is range from 1.8 to 2. It shows the most absorption is done by nucleic acids and therefore extracted DNA is well-qualified and its purity is acceptable.

The results showed that the DNA yield and DNA Purity obtained from one g of the fresh leaf tissue in different methods using UV spectrophotometer was statistically significant ($P \leq 0.01$) (Table 1). A higher DNA yield was obtained with method 3 (783.5±140.7 ng/μL fresh weight), while the lowest was obtained with method 5 (107.5±12.6 ng/μL fresh weight) (Table 2). Therefore, the results confirmed that extracted DNA by Dellaporta et al. (1983) method from leaves of *Z. spina-christi* possess better quality and quantity in compare with the other methods. DNA sample was measured with a UV spectrophotometer for the ratio of OD260/OD280 using TE buffer. The ratio of OD260/OD280 was determined in order to assess the purity and concentration of the DNA sample. DNA concentration was calculated according to the equation of Wilmington et al. (2008).

$$\text{DNA concentration (ng/μL)} = OD_{260} \times \text{a (dilution factor)} \times 50 \quad (1)$$

Table 1. Analysis of variance of six different DNA extraction methods in terms of Yield and purity of DNA in *Ziziphus spina-christi* leaves

Source	df	Mean Square			
		Spectrophotometer		Nano-Drop	
		DNA Yield	DNA Purity	DNA Yield	DNA Purity
Methods	5	332401.38**	0.36**	208100.47**	0.01ns
Error	24	24382.08	0.06	20232.89	0.11
CV%		42.00	12.30	30.20	18.50

Note. ** and ns, refer to 1% and not significant, respectively.

Table 2. Comparison of means for efficiency of six different DNA extraction methods in leaf samples of *Ziziphus spina-christi* leaves using Duncan's multiple range test ($P \leq 0.01$)

Methods	Spectrophotometer		Nano-Drop	
	DNA yield (ng/µL)	DNA purity (ng/µL)	DNA yield (ng/µL)	DNA purity (ng/µL)
1	404±67.1^{ab}	2.02±0.03^{ab}	532.4±76.5^{bc}	1.74±0.18^{a}
2	199±33.9^{bc}	2.17±0.14^{a}	612.5±114.8^{ab}	1.78±0.09^{a}
3	783.5±140.7^{a}	1.4±0.01^{c}	320.3±9.9^{de}	1.82±0.05^{a}
4	192±53.4^{bc}	2.00±0.17^{ab}	399.5±37.4^{cd}	1.82±0.07^{a}
5	107.5±12.6^{c}	1.82±0.12^{b}	202.6±34.7^{e}	1.75±0.18^{a}
6	544.5±28.2^{b}	2.04±0.01^{ab}	761.6±50.4^{a}	1.83±0.23^{a}

Note. Different letters indicate significant difference between the values of pair of methods (Mean ± SE).

3.3 NanoDrop™ 1000 Spectrophotometer

The NanoDrop 1000 Spectrophotometer has the capability to measure highly concentrated samples without dilution (50X higher concentration than the samples measured by a standard covet spectrophotometer). The NanoDrop 1000 Spectrophotometer will accurately measure DNA samples up to 3700 ng/µL without dilution. To do this, the instrument automatically detects the high concentration and utilizes the 0.2mm path length to calculate the absorbance. The ratio of 260 and 280 nm absorbance is used to assess the purity of DNA and RNA. This ratio is between 1.8 and 2, and this rang is generally accepted as "pure" for DNA. If the ratio is appreciably lower in either case, it may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm. The 260/230 values for "pure" nucleic acid are often higher than the respective 260/280 values. They are commonly in the range of 1.8-2.2. If the ratio is appreciably lower, this may indicate the presence of co-purified contaminants.

The results showed that the DNA yield obtained from one g of the fresh leaf tissue in different methods using NanoDrop 1000 Spectrophotometer was statistically significant ($P \leq 0.01$), while there was no significant difference among different methods in terms of DNA purity (Table 1). Figure 2 represent the mean comparison for efficiency of DNA extraction methods by UV spectrophotometer and NanoDrop.

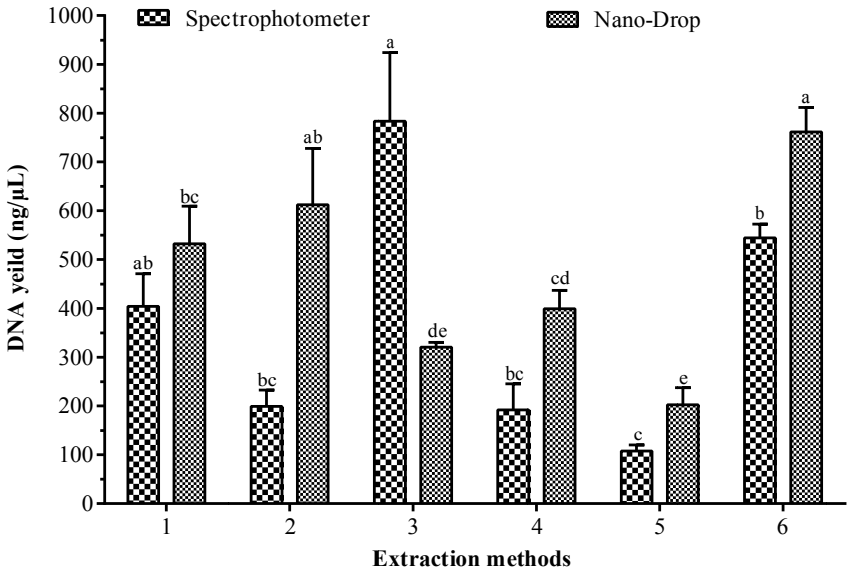

Figure 2. Mean comparison of DNA yeild of *Ziziphus spina-christi* under six different extraction methods by UV spectrophotometer and Nano-Drop using Duncan's multiple range test ($P \leq 0.01$)

Note. Different letters indicate significant difference between the values of pairs of extraction methods.

3.4 Restriction Enzyme Analysis

All restriction enzymes and the DNA markers used in this work were 1 kb DNA ladder and DNA digested with *Eco*.RI and *Hin*d III. Two µg genomic DNA was digested for 90 min with 5 U of restriction enzyme under optimal temperature and 2.5 µL buffer, as recommended by manufacturer (Fermentas, USA). DNA incubating was done by Dry Bath the reaction mixture at 37 °C for 90 min. This indicated that isolated DNA was amenable for further downstream applications. To determine DNA quality, undigested and *Eco*.RI and *Hin*d III digested DNA were run on agarose gel. The digested DNA fragments were fractionated on 0.8% agarose at 5 V/cm, 70 V for 120 min. A perfect migration pattern was observed without any signs of DNA degradation (Figure 3).

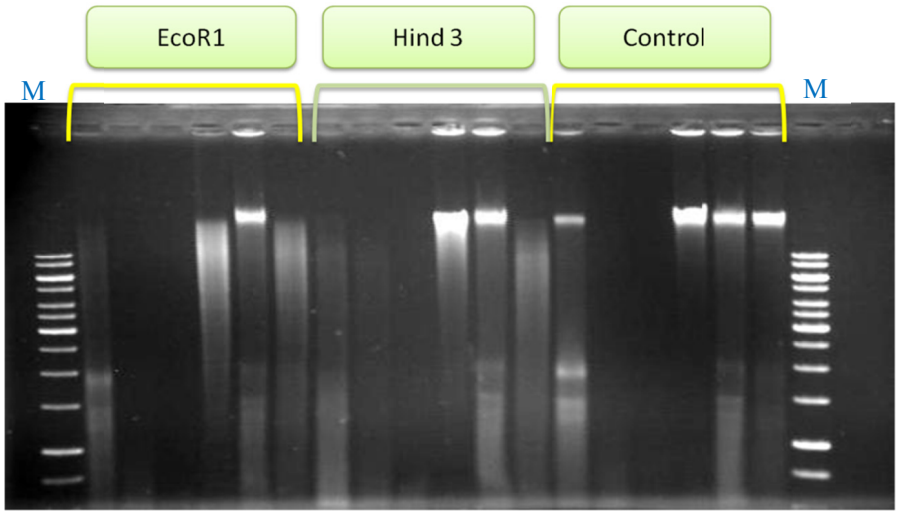

Figure 3. Restriction enzyme analysis of nuclear DNA isolated from Bi et al. (1996) digested with *Eco*.RI and *Hind* III

Note. The EtBr-stained DNA fragments in 0.8% agarose gel. M: 1 Kb molecular weight marker (SM0313, Fermentase) (DNA digested with *Eco*.RI and *Hin*d III).

3.5 PCR Amplification Analysis

PCR analysis, including RAPD and ISSR, was generally used previously to further verify the quality and purity of extracted DNA (Haugland et al., 1999, 2002). The ability of amplification of extracted DNA strands is proved by a randomized 10-nucleotide-primer TIBMBA-18 by PCR. The final volume of 25 µL is tested in PCR reaction (2.5 µL PCR reaction buffer 10x, 0.875 µL $MgCl_2$ 50 mM, 0.5 µL dNTPs 10 mM, 1.0 µL primer 10 µM (5′-CTCGGATGTC-3′), 0.2 µL *Taq* polymerase 5 Unit/µL, 2.0 µL template DNA (5 ng/µL). Thermo cycling condition was scheduled as 1 cycle in 94 °C for 4 min, 35 cycles including 1 min in 92 °C, 1 min in 37 °C and 2 min in 72 °C, and at last 1 cycle in 72 °C for 1 min and a hold temperature of 4 °C at the end using Bio-Rad® thermocycler, model "ICycler". PCR products were electrophoresed on 1% (w/v) agarose gels, in 1X TBE Buffer at 70 V for 2 h and then stained with ethidium-bromide (0.5 g/mL). The gels with amplification fragments were visualized and photographed under UV light.

Talebi et al. (2003) in a study on genetic diversity of pomegranate cultivars of Iran, using Random Amplified Polymorphic DNA (RAPD) using four different genomic DNA extraction procedures; Murray and Thompson (1980), J. J. Doyle and J. L. Doyle (1990), Ziegenhagen et al. (1993) and Lodhi et al. (1994) introduced Murray and Thompson's method as the most appropriate method in aspect of quality of DNA extracted from young leaves of pomegranate. Jenderek et al. (1997) have found the method of J. J. Doyle and J. L. Doyle as the best quality resulting method for DNA extraction form marshmallow, but its quantity was too low. Saha et al. (2016) in a study on genetic stability of *Morus alba* L. variety and Nadha et al. (2011) on genetic diversity of *Guadua angustifolia* Kunth, using RAPD and ISSR marker introduced Murray and Thompson (1980), and J. J. Doyle and J. L. Doyle (1990) methods as appropriate DNA extraction procedures, respectively. Bhatia et al. (2011) in a study on the genetic fidelity of *Gerbera jamesonii* Bolus using DNA-based markers were used Murry and Thompson (1980). PCR tests outcomes showed that the extracted DNA by Bi et al. (1996) method from leaf samples brings an acceptable quality forth for PCR, and the candescence of amplified DNA bands apperceives this truth of chemicals and shorter length of time to extract DNA and its higher quality and quantity of extraction as well, the method set up by Bi et al (1996) was the best extraction method for *Z. spina-christi* leaf samples (Figures 4 and 5).

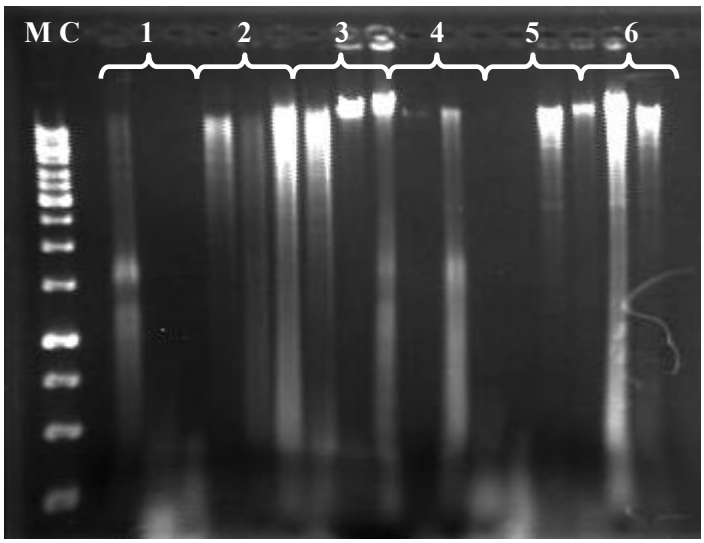

Figure 4. Amplification of DNA from *Ziziphus spina-christi* leaf using six different extraction methods by inter-simple sequence repeat (ISSR) amplification and the primer UBC-868

Note. M: 1 Kb molecular weight marker (SM0313, Fermentase); 1) Murry and Thompson (1980); 2) Lodhi et al. (1994); 3) Dellaporta et al. (1983); 4) J. J. Doyle and J. L. Doyle (1990); 5) Ziegenhagen et al. (1993); and 6) Bi et al. (1996). The electrophoresis was performed in 8% (w/v) agarose gel.

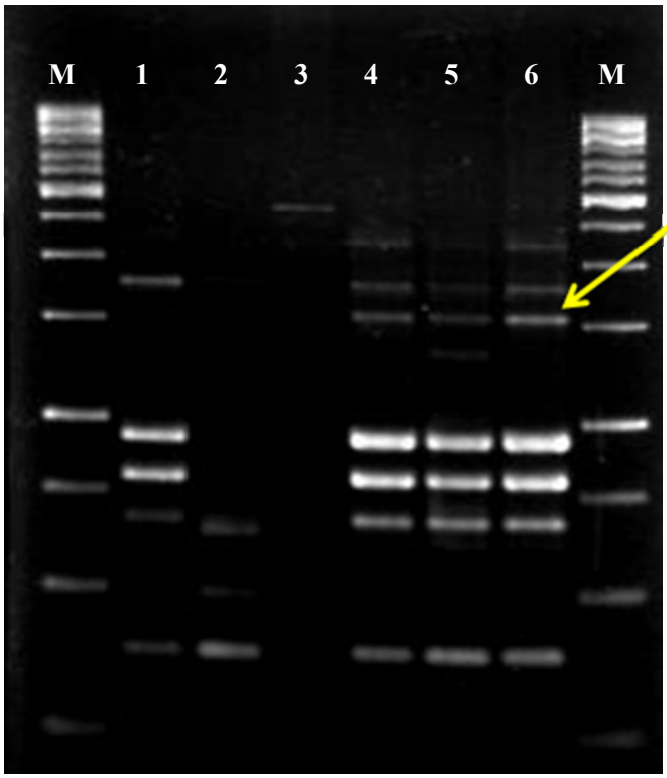

Figure 5. Amplification of DNA from *Ziziphus spina-christi* leaf using six different extraction methods by Random amplified polymorphic DNA (RAPD) and primer TIBMBA-18

Note. M: 1 Kb molecular weight marker (SM0313, Fermentase); 1) Murry and Thompson (1980); 2) Lodhi et al. (1994); 3) Dellaporta et al. (1983); 4) J. J. Doyle and J. L. Doyle (1990); 5) Ziegenhagen et al. (1993); and 6) Bi et al. (1996). The electrophoresis was performed in 8% (w/v) agarose gel.

4. Conclusions

To establish a routine procedure for the application of genomic analysis, considering the characteristics of *Ziziphus spina-christi*, the Bi et al. (1996) method was an effective DNA extraction method. The method efficiently improved DNA purity and quality and quantity of DNA and the candescence of amplified DNA bands. In addition the method was simple and consequently short time-consuming, success in gaining high-quality DNA. To our knowledge, no other studies report DNA extraction from this plant. The results obtained will form a strong beginning for future molecular characterization and genetic improvement works in this promising medicinal plant.

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