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Research Article

Comparison of DNA extraction methods from *Halocnemum strabilaceum* (Amaranthaceae)

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Abstract

Molecular techniques such as DNA extraction and DNA sequencing are playing an important role in studying the genetic makeup of the plant and identifying the evolutionary relationship using DNA barcoding. Extraction of DNA from plant tissue is often problematic, as many plants contain high levels of secondary metabolites that can interfere with downstream applications, such as PCR. Removal of these secondary metabolites usually requires further purification of the DNA using organic solvents or other toxic substances. In this study, we have focused on the DNA isolation process using three isolation techniques: the cetyl trimethyl ammonium bromide (CTAB) method that uses the ionic detergent hexadecyl trimethyl ammonium bromide and chloroformisoamyl alcohol, BioFACT, and GeneAll methods on desert/rangeland plant including *Halocnemum.* The quantity and quality of extracted genomic DNAs were compared by employing the spectrophotometer, Nano-Drop, agarose gel electrophoresis, polymerase chain reaction (PCR) methods, and molecular markers such as ISSR. Our results showed that the modified method of CTAB provided the best results than the BioFACT and GeneAll methods for extracting DNA from the tissues of *Halocnemum*. We present a safe and cost-efficient DNA purification procedure and recommend using this CTAB method to extract DNA from plant tissues and to use the young leaf for the highest DNA yields.

Keywords: Agarose Gel Electrophoresis, CTAB, PCR, Nano-Drop, plant tissue

Introduction

Halocnemum is quite variable and widely distributed. It includes many desert and seashore plants and halophytes, as well as plants in moist environments. Species within the genus are known for complex genetics, rapid evolutionary rates, and high tolerance to xeric, saline, and contaminated soils. Golestan Province is a vast land and very diverse in terms of vegetation, which has a different climate due to its special geographical location. The northern half of the province, the semi-arid and arid part, enjoys the least rainfall due to high water evaporation, saline lands, and low yields. *Halocnemum strobilaceous* belongs to the spinach subfamily, which is one of the key species in saline and alkaline rangelands in the north of Golestan Province (Hosseini & Shahmoradi, 2011). *H. strobilaceum* was formerly placed in the family Chenopodiaceae, but after the complete genome sequencing of its chloroplast and molecular-based APG system, it was transferred to the family Amaranthaceae. This plant is one of the succulent plants that act to protect itself and adapt to salinity conditions by dropping parts of its salt-rich leaves and stems (Toranjzar & Fathi, 2006). *H. strobilaceum* is a perennial plant, often shrubby, rarely shrubby, yellowish-green, purple, or between colors. (Assadi, 2001).

Molecular techniques are increasingly used for the detection and differentiation of plants, including taxonomy, genetics, and evolution (Moritz & Cicero, 2004). This type of molecular work requires DNA extraction from individuals (Allen *et al*., 2006). Molecular methods used for genetic studies of plant species are based on the extraction of intact, pure, and high-quality DNA (Shokatyari et al., 2020). DNA research, allow the organizing of the taxonomic identity of samples and reassembling of the obtained results (Gaudeul & Rouhan, 2013). Methods for DNA extraction vary in yield amount and quality (Chen *et al*. 2010). The DNA extraction method employed should be appropriate for varied samples, maximize yield, and minimize contamination, degradation, and costs of money and time (Lagisz *et al.*, 2010). The extraction of good quality DNA with a high yield is a limiting factor in plants' genetic analysis. DNA quality from each line should be consistent to allow a proper genetic analysis of several plant individuals. The high quality of DNA is characterized by predominantly high molecular weight fragments with an A260/280 ratio between 1.8 and 2.0 and the lack of contaminating substances, such as polysaccharides and phenols (Abdel-Latif & Osman, 2017). The extraction and purification of high-quality DNA from some species of *Halocnemum* is generally difficult due to the presence of polyphenols and other secondary metabolites and proteins. The presence of these compounds affects the quality and quantity of isolated DNA, and therefore, renders the sample

non-amplifiable (Abdel-Latif & Osman, 2017). Saghai-Maroof et al. (1984)'s method was used for DNA extraction in mangroves and salt marsh species (Sahu et al., 2012). Talebi-Baddaf et al. (2003) introduced Murray and Thompson's (1980) method as the most appropriate method to achieve high-quality DNA extraction from pomegranate leaves. Because plants contain high amounts of many different substances, it is unlikely that just one nucleic acid isolation method suitable for all plants can ever exist (Loomis, 1974). Therefore, it is necessary to modify the extraction protocols.

One of the most commonly used methods to extract DNA from plants uses the ionic detergent cetyltrimethyl ammonium bromide (CTAB) to disrupt membranes and a chloroform-isoamyl alcohol mixture that separates contaminants into the organic phase and nucleic acid into the aqueous phase (Tamari *et al*., 2013). However, many plants contain very high levels of secondary metabolites including lipids, phenolic compounds, and viscous polysaccharides that can be difficult to remove without further processing, often with organic solvents, such as phenol or other toxic compounds (Sahu *et al.,* 2012). If these contaminants are not removed, then they often inhibit subsequent downstream assays including PCR (Tamari *et al*., 2013).

One alternative to the CTAB DNA extraction method has been developed by the BioFACT method. The CTAB method uses the anionic detergent SDS to solubilize membranes, followed by precipitation of DNA with isopropyl alcohol. BioFACT's method (Daejeon, Korea) was used for DNA extraction in salt marsh species. Because these plants contain high amounts of many different substances, it is unlikely that just one nucleic acid isolation method suitable for all plants can ever exist (Loomis 1974). BioFACT's method is a quick and simple method for extracting DNA from plants, and the DNA can be used directly for PCR amplification without further processing (Loomis, 1974).

Tahmasebi and Nasrollahi (2021) studied the genetics of *Halocnemum strobilaceum* in rangeland ecosystems of Golestan Province total genomic DNA was isolated from fresh or dried material using the modified CTAB method of Doyle and Doyle (1987). In this paper, we tested the CTAB, BioFACT, and GeneAll methods to extract DNA from the leaf of *Halocnemum*. to acquire an appropriate and cost-effective procedure for DNA isolation by considering Sahu et al. (2012), the Cetyltrimethyl ammonium bromide (CTAB) protocol was modified. We assessed the yield and quality of DNA, as well as the time and cost required for each method. PCR was used

to assess the capacity for amplification and sequencing. The present study aims to compare three different DNA extraction methods to isolate high-quality DNA from *H. strobilaceum* leaves. In this study, we showed the results of tests from several DNA extraction protocols that were made to overcome the problems that mainly arise from polysaccharide contamination. We evaluate a highly effective method for high-quality DNA isolation from a young and mature leave of *H. strobilaceum*.

Materials and methods

Isolation of Genomic DNA

In the present study, 12 populations of *Halocnemum strabilaceum* were collected from different habitats in the North of Iran (Table 1) (Figure 1). Fresh and herbarium samples were selected based on the following materials and methods (Figure 2). For molecular studies, we used a different number of plant specimens, as they were required. Three methods were used to extract DNA from *Halocnemum strabilaceum* plant: a CTAB method, the BioFACT, and GeneAll methods.

Table 1. Investigated *Halocnemum strobilaceum* populations

Pop.	Locality	collector	Longitude	Latitude	Altitude (m)	Voucher no.
1	Golestan Province: Gonbad-e Kavus, Incheboron	Hosseini	54 72 06	37 45 51	1257	804350(GKUH)
2	Golestan Province: Gonbad-e Kavus	Hosseini	55 09 30	37 15 00	1100	804361(GKUH)
3	Golestan Province: East of Gonbad-e Kavus	Hosseini	55 18 02	37 17 14	1320	804357(GKUH)
4	Golestan Province: Gonbad-e Kavus, Dashli-	Hosseini	54 80 99	37 63 66	1245	804354(GKUH)

	Boroon					
5	Golestan Province: Gomish Tape	Mohamadi	54 07 66	37 07 01	1324	804366(GKUH)
6	Golestan Province: Gomishan	Naeini	54 06 24	37 16 23	1870	804364(GKUH)
7	Golestan Province: Gomishan to Incheboron	Naeini	54 53 12	37 21 02	1754	804372(GKUH)
8	Golestan Province: Aq Qala	Mohamadi	54 45 12	37 01 31	1640	804370(GKUH)
9	Golestan Province: West of Aq Qala	Mohamadi	54 15 13	37 14 02	1573	804351(GKUH)
10	Golestan Province: North of Bandar Torkaman	Naeini	54 09 39	36 89 80	1875	804353(GKUH)
11	Golestan Province: West of Bandar Torkaman	Naeini	54 12 02	36 53 12	1643	804379(GKUH)
12	Golestan Province: Gonbad-e Kavus, Agh Abad	Hosseini	54 20 70	37 12 26	1268	804379(GKUH)

Figure 1. Distribution map of the studied *Halocnemum strobilaceum* populations in Golestan Province. Populations are marked with numbers from 1-12 according to Table 1.

Figure 2. *Halocnemum strobilaceum* (Golestan Province, Gomishan. 20 April 2021)

CTAB DNA Isolation

CTAB DNA extraction (Doyle & Doyle 1987; Doyle & Dickson 1987; Cullings 1992; Nasrollahi et al., 2019) method 200 mg of fresh or dried tissues were ground in mortar and pestle using liquid nitrogen. The CTAB protocol was optimized for DNA extraction including a combination of β-mercaptoethanol, polyvinyl pyrrolidone (PVP), sodium N-lauroyl sarcosine, and sodium dodecyl sulfate (SDS). Add 0.2 g activated charcoal, and 300 µL extraction buffer

(50 mM EDTA), 150 mM Tris-HCl, 1.5 M NaCl,1.5% Triton X-100, 0.1% β-mercaptoethanol, 2% CTAB, and 100 µL 1% PVP (polyvinyl pyrrolidone, 100 µL 1% SDS (w/v) and 30 µL 2% sodium N-lauroyl sarcosine (w/v), incubate at 65 ºC for 40 min.Centrifuged at 12000 g for 5 min and the supernatant is transferred to new microfuge tubes then 250 µl of Chloroform: IsoAmyl Alcohol (24:1) were added. The extracts were mixed by inversion and the upper aqueous portions were collected. Added 50 µl of 7.5 M ammonium acetate and 500 µl of ethanol and kept on ice for 1 hour to precipitate the DNA. Centrifuged at 13000 rpm to precipitate the DNA. The DNA pellets were then washed by adding 500 µl of ice-cold ethanol and pelleted again by centrifugation. After centrifugation, the supernatant was carefully discarded, the pellet was dried for 1 h, and DNA was dissolved in 100 µL sterile deionized water. To develop an appropriate for DNA extraction in lower plants, the CTAB protocol was modified. Triton X-100, SDS, activated charcoal, and ammonium acetate were used for the elution of the contaminations instead of the hazardous and risky materials (Doyle & Doyle 1987).

BioFACT Plant DNA Extraction

BioFACT plant DNA isolation kit (Daejeon, Korea) was used by following the manufacturer's protocol. First, ground *Halocnemum strabilaceum* fresh or a dried leaf using liquid nitrogen in an appropriate bowl. Placed the sample in a tube. Added 350 µl of lysis buffer (GD1) to the prepared sample, 2 µl of RNase A and 5 µl proteinase K were also added to the tube and then vortexed. The sample was then incubated at 65 °C for 10 minutes with mixing, added 100 µl of PPB, and then vortexed and centrifuged. Transferred supernatant to a new tube and added 200 GB. Placed the spin column in a 2 ml collection tube and transferred 200 µl of supernatant to a new tube. Added 500 µl WB (80٪ Ethanol) to the tube and centrifuged and this step was repeated for washing. Discarded the tube and placed the spin column in a new 1.5 ml tube. Finally, added 70 µl DNA Hydration Solution and incubated for 1 minute at RT, and centrifuged (catalog number: 28706; BIOFACT Co., Ltd., Daejeon, Korea).

general Plant DNA Isolation

DNeasy Plant Mini Kit (GeneAll, Korea) was used to isolate the DNA by following the manufacturer's protocol. First, ground plant tissue to a fine powder under liquid nitrogen. Added 400 µl PL buffer, 4 µl RNase A, then vortexed, incubated at 65 °C for 10-15 minutes, and mix 3 times during incubation by vortexing. Added 140 µl of PD buffer to the lysate and kept on ice for 5 minutes. The extracts were then centrifuged at full speed and applied to the EzSep filter and

again centrifuged. Transferred the pass-through to a new 1.5 ml tube by pipetting and added 1.5 volume of buffer BD to the lysate and mixed by inverting. Applied 700 µl of the mixture to the GeneAll SV column sitting in the collection tube, centrifuged and discarded he pass-through. Applied 700 µl buffer CW to the SV column, centrifuged a,nd discarded the pass-through. Added 300 µl of buffer CW to the SV column, centrifuged ,and transferred the SV column to a new 1.5 ml tube. Added 60 µl of buffer AE directly onto the center of the SV column membrane and incubated for 1 minute at RT, and centrifuged (catalog number: 73652; GeneAll Biotechnology Co., Ltd., Korea).

Concentration, purity, and quality of the DNA extracted

The quantity (concentration and extraction efficiency) and quality (purity and intactness) of the DNA obtained in the 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-DropTM (Thermo Scientific) described by Brodmann (2008) and Wilmington (2008), and agarose gel electrophoresis, PCR methods and molecular markers, such as ISSR. This spectrophotometric analysis was performed in triplicate on the samples of extracted DNA using a spectrophotometer. To verify DNA integrity, 5 µL of DNA were subjected to gel electrophoresis on 0.8% (w/v) agarose gel, stained with a safe stain, and a constant voltage of 120 V for 90 min. The DNA bands were visualized and images were acquired using the Gel Doc imagining system (Bio Rad Laboratories Inc., Germany).

PCR amplification

ISSR amplification

The PCR amplification reaction was carried out with 12 samples and four ISSR primers (Table 2) in a 25 µL reaction volume containing 10 mM Tris-HCl, pH 8.3, 2.5 mM MgCl2 (Cinna GenCo, Iran), 1 mM dNTP mix (Cinna Gen Co, Iran), 0.2 µM of primer (Cinna Gen Co, Iran), 1 U of Taq DNA polymerase-500 (Cinna Gen Co, Iran), and 15 40 ng of template DNA. ISSR-PCR was performed in the thermocycler (Biorad, USA) for 40 cycles consisting of denaturation at 94 ºC for 60 sec, annealing varying from 52–55 ºC for 60 sec, extension at 72 ºC for 90 sec, and 72 ºC for 6 min for the final extension. PCR protocol is outlined in table 3. The amplified product was checked in 1% agarose gel electrophoresis.

Table 2. The used ISSR primers

Code	Sequences
ISSR13	(ACG) $_6$Ga
ISSR826	CCCGGATCC (CA)8
ISSR810	(GA) 8T
ISSR7	((AC) 8AG

Table 3. PCR thermocycler profile for ISSR primers

Step	Temperature	Time	Cycling
Initial denaturation	94 ºC	5 min	-
Denaturation	94 ºC	60 s	-
Annealing	52–55 ºC	60 s	40
Extension	72 ºC	90 sec	-
Final extension	72 ºC	6 min	1

Results

In this study, three methods of DNA extraction were compared: the CTAB method and the BioFACT and GeneALL methods. Additionally, DNA was extracted from tissues of 12 populations of *Halocnemum strabilaceum* using three methods. Several protocols of DNA isolation were used each giving different results for the amount of DNA obtained and its purity. The high quality of DNA was only obtained with our modified method.

Comparison of DNA Yields using the CTAB and the Edwards Methods

The quality of 12 extracted DNA samples was verified spectrophotometrically using a NanoDrop instrument and agarose gel electrophoresis. DNA purity and yield were compared between these three extraction methods. BioFACT method did not give proper results for *Halocnemum strabilaceum* species due to the presence of polysaccharides and proteins in the pellet and showing green or yellow DNA precipitate that presents the DNA gel image (Figure 3). In the GeneALL method of extraction, we encountered many difficulties from the cell lysis to DNA

separation phases in the supernatant and subsequent reactions. The presence of phenolic compounds caused a brownish pellet (Figures 3a, 3b). The results confirmed that extracted DNA by our modified method of CTAB from leaves possess is better quality in comparison with the other extraction methods as well as BioFACT and GeneALL methods (Figure 3). Moyo et al. (2008) indicated that, the optimization of the correct balance between buffer volume extraction and tissue amount is key for favored DNA extraction. Lade *et al*. (2014) increase the concentration of DNA by using of longer lysis time for some cells. Due to the elimination of polysaccharides or protein contamination,s DNA has been extracted with high quality. We believe that this method will be efficient for molecular studies of many other aromatic and herbal plants. PCR test outcomes in the ISSR study showed that the extracted DNA by CTAB method with significant modifications from leaf samples brings an acceptable quality for PCR, and is the most appropriate method in the aspect of quality of DNA extracted from young leaves of *Halocnemum strabilaceum.* The PCR-amplified DNA fragments of ISSR for 12 samples showed a clean single band product when examined on an agarose gel (Figure 3). The PCR products were of about 580 bp.

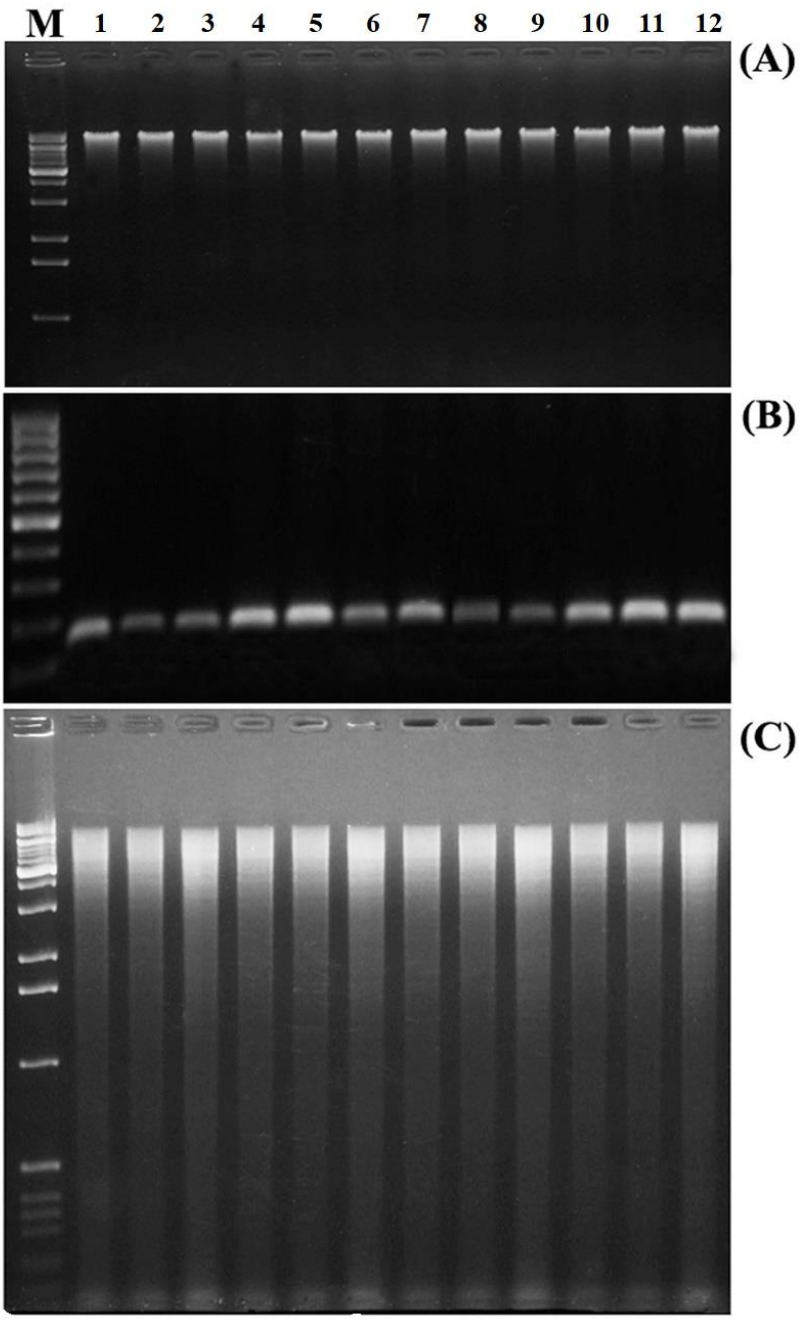

Figure 3. Amplification of DNA from *Halocnemum strabilaceum* leaf using three different extraction methods by ISSR amplification. (A): modified CTAB method. (B): BioFACT kit. (C): GeneALL kit.

UV spectrophotometer and NanoDrop™ 1000 spectrophotometer

In the spectrophotometer procedure, absorption of double-stranded DNA at wavelength of 260 nm is 50 μg/μL. The ratio of absorption amount resulting in 260 nm to 280 nm is range from 1.7

to 2. It shows the most absorption is done by nucleic acids and therefore extracted DNA is well-qualified and its purity is acceptable. 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 results showed that the DNA yield and DNA purity obtained from one gram of the fresh leaf tissue in different methods using a UV spectrophotometer were statistically significant ($P \leq 0.01$). A higher DNA yield was obtained with the method of CTAB (450±88.1 ng/µL fresh weight), while the lowest was obtained with the method of BioFACT (292±44.4 ng/µL fresh weight) (Tab. 4). Therefore, the results confirmed that extracted DNA by CTAB modified method from leaves of *Halocnemum strabilaceum* possesses better quality and quantity in comparison with the other methods. A DNA sample was measured with a UV spectrophotometer for the ratio of OD260/OD280 using TE buffer. The ratio of OD260/OD280 was determined to assess the purity and concentration of the DNA sample. DNA concentration was calculated according to the equation of Wilmington (2008). DNA concentration (ng/µL) = OD260 × a (dilution factor) × 50. Absorbance measurements made on a spectrophotometer, including any Thermo Scientific NanoDrop Spectrophotometer, will include the absorbance of all molecules in the sample that absorbs at the wavelength of interest. The ratio of absorbance at 260 nm and 280 nm is used to assess the purity of DNA and RNA. A ratio of ~1.8 is generally accepted as "pure" for DNA; a ratio of ~2.0 is generally accepted as "pure" for RNA. 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. Some researchers encounter a consistent 260/280 ratio change when switching from a standard cuvette spectrophotometer to a NanoDrop spectrophotometer.

The NanoDrop absorbance is useful for the detection of contaminants such as protein, salts, and polysaccharides, which can inhibit and interfere with DNA sequencing. The NanoDrop 1000 spectrophotometer can measure highly concentrated samples without dilution. The ratio of 260 and 280 nm absorbance is used to assess the purity of DNA and RNA. This ratio is between 1.7 and 1.8, and this range is generally accepted as "pure" for DNA (Table 4 and Figure 4).

Table 4. Comparison of means for efficiency of three different DNA extraction methods in leaf samples using Duncan's multiple range test ($P \leq 0.01$) of leaves *Halocnemum strobilaceum*

Methods	Spectrophotometer		Nano-Drop	
	DNA yield (ng/µL)	DNA purity (ng/µL)	DNA yield (ng/µL)	DNA purity (ng/µL)
CTAB	450±44.4	2.1±0.19	590±26.6	1.84±0.09
BioFACT	292±88.1	1.7±0.15	455.4±86.5	1.58±0.15
GeneALL	323±33.8	1.8±0.18	505±76.4	1.68±0.07

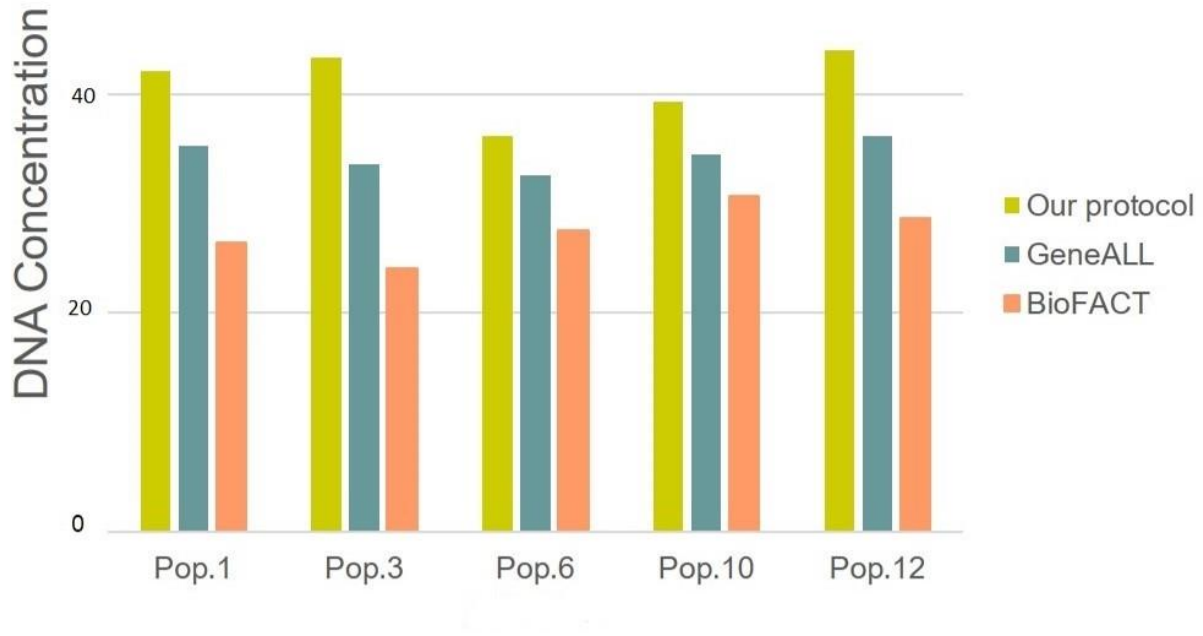

Figure 4. Comparing DNA concentration (µg/ml) in different studied methods in selected species (see Table 4).

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 *Halocnemum*. Yellow and brownish colored DNA pellets

indicate contamination by phenolic compounds (Weishing et al., 1995) were the biggest challenge we faced during DNA extraction from fresh and dried *H. strabilaceum* leaf. The modified method of CTAB 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 reduced the level of impurity and thus resulting in clear PCR bands. As a result, the presented method is efficient enough to amplify the PCR reaction.

A suitable choice of leaf tissue is considered to obtain high-quality DNA. Mature tissues have high quantities of polysaccharides, polyphenols, and tannins (Dabo et al., 1993) so it is an offered that, plant samples should be fresh and young. The fresh samples produced high DNA concentration and purity, more than the herbarium samples. It was observed that the herbarium samples resulted in lower DNA concentrations and yield than the fresh samples (there was a significant effect in both purity and concertation (p-value < 0.005). There were studies reported on CTAB DNA extraction, which supports our findings. These studies suggested the use of the CTAB extraction method for DNA isolation from mucilaginous herbarium samples and fresh samples, which resulted in good quality DNA. 20 to 1050 ng was the range of DNA concentration in herbarium while, 300 to 2500 ng was the range in fresh specimens. The isolated samples showed reliable amplification of the chloroplast gene from specimens that are 50-60 years old. Overall and regarding the significant effect between fresh and herbarium, there are noticeable differences in values in both purity and concentration.

Conclusion

In this study, three DNA extraction methods were compared to isolate high-quality DNA that can be efficiently amplified using PCR. The BioFACT and GeneALL methods resulted in green or yellow DNA precipitate that could not be reliably amplified by PCR. Therefore, we used our protocol of the CTAB method that produced good quality DNA. This method helped us to provide pure DNA with high efficiency in *Halocnemum strabilaceum* species. Advantages of the present method for studying plants with secondary metabolites are as follows including omission of liquid nitrogen, decrease of toxic effects, hazardous and lower amount of dried or fresh plant material, without any conservation specific condition. We present a safe and cost-efficient DNA purification procedure. The results indicated the relevance of applying this method for DNA extraction in *Halocnemum strabilaceum*. High efficiency and lack of toxic organic solutions make our protocol an optimal substitute for the commercial kits. Furthermore, this

protocol with high-quality DNA produced was proposed as an alternative in the analysis of the molecular marker and also in any other applications based on the polymerase chain reaction, sequencing technologies, and bioinformatics tools.

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