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Evaluation of five genomic DNA extraction methods for downstream molecular applications in fresh and herbarium leaves of *Astragalus fridae*

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Zahra Alidoosty Shahraky: MSc Graduate in Biochemistry, School of Biology, Damghan University, Damghan, Iran

Atefe Amirahmadi✉: Assistant Prof., School of Biology and Institute of Biological Sciences, Damghan University, P.O. Box 36716-45667, Damghan, Iran (a.amirahmadi@du.ac.ir)

Parisa Farrokh: Assistant Prof., School of Biology and Institute of Biological Sciences, Damghan University, Damghan, Iran

Arezou Rezaei: Assistant Prof., School of Biology and Institute of Biological Sciences, Damghan University, Damghan, Iran

Javad Ghasemian: Assistant Prof., School of Mathematics and Computer Sciences, Damghan University, Damghan, Iran

Abstract

The protection of genetic resources is crucial for safeguarding national assets as natural resources face various threats such as climate change and changing land use. To conserve endangered species such as *Astragalus fridae*, an endemic species of gypsum soils of Semnan province (Iran) which is on the brink of extinction due to limited distribution and habitat destruction, establishing a gene bank is essential. To ensure the survival of this species, it is necessary to extract high-quality and high-quantity DNA. The present survey, has evaluated five genome extraction methods including Protocol A (main CTAB), Protocol B (CTAB without β-mercaptoethanol), Protocol C (CTAB without ammonium acetate), Protocol D (the modified Murray & Thompson method), and Protocol E (GeneAll Plant Kit), to determine their ability to extract DNA from fresh and herbarium leaves of *A. fridae*. The quality and quantity of DNA were assessed using gel electrophoresis, spectrophotometry, and usability of the purified DNA tested by PCR of the ITS region of nuclear ribosomal DNA. Protocol D yielded the highest quality of the extracted genome from fresh leaves, and Protocol A, extracted the highest DNA concentration from fresh leaves. Although Protocols B and C extracted a reasonable amount of genome of acceptable quality with dry leaves, the ITS region was not amplified in these samples, rendering them unsuitable for DNA extraction of *A. fridae*.

Keywords: Climate change, CTAB, endangered species, endemic, *Fabaceae*, threats

ارزیابی پنج روش استخراج DNA ژنومی برای کاربردهای مولکولی پایین دست در برگ‌های تازه و هرباریومی *Astragalus fridae**

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زهرا علیدوستی شهرکی: دانش‌آموخته کارشناسی ارشد بیوشیمی، دانشکده زیست‌شناسی، دانشگاه دامغان، دامغان، ایران

عاطفه امیراحمدی✉: استادیار دانشکده زیست‌شناسی و پژوهشکده علوم زیستی، دانشگاه دامغان، دامغان، ایران (a.amirahmadi@du.ac.ir)

پریسا فرخ: استادیار دانشکده زیست‌شناسی و پژوهشکده علوم زیستی، دانشگاه دامغان، دامغان، ایران

آرزو رضایی: استادیار دانشکده زیست‌شناسی و پژوهشکده علوم زیستی، دانشگاه دامغان، دامغان، ایران

جواد قاسمیان: استادیار دانشکده ریاضی و علوم کامپیوتر، دانشگاه دامغان، دامغان، ایران

خلاصه

حفاظت از ذخایر ژنتیکی جزو حیاتی حفاظت از سرمایه ملی است. از آنجایی که منابع طبیعی توسط عوامل مختلفی از جمله تغییرات آب و هوایی و تغییر کاربری اراضی تهدید می‌شود، بنابراین ایجاد بانک ژن برای حفاظت از گونه‌های در خطر انقراض ضروری به نظر می‌رسد. *Astragalus fridae* Rech.f. (باقلاییان) گونه بومی خاک‌های گچی استان سمنان است که به دلیل پراکنش محدود و تخریب زیستگاه در حال انقراض می‌باشد. برای اطمینان از بقای این گونه، باید تلاش‌هایی برای استخراج DNA با کیفیت و کمیت بالا انجام شود. به این منظور، پنج روش استخراج ژنوم شامل پروتکل A (CTAB اصلی)، پروتکل B (CTAB بدون بتا-مرکاپتواتانول)، پروتکل C (CTAB بدون استات آمونیوم)، پروتکل D (روش اصلاح شده موری و تامسون) و پروتکل E (کیت گیاهی GeneAll)، از نظر توانایی آن‌ها در استخراج DNA از برگ‌های تازه و هرباریومی مورد ارزیابی قرار گرفتند. کیفیت و کمیت DNA توسط ژل الکتروفورز و اسپکتروفتومتری بررسی شد و قابلیت استفاده DNA خالص شده توسط PCR ناحیه DNA ITS ریبوزومی هسته‌ای مورد آزمایش قرار گرفت. بالاترین کیفیت ژنوم استخراج شده به پروتکل D با برگ‌های تازه اختصاص یافت. بیشترین غلظت DNA از برگ‌های تازه (پروتکل A) به دست آمد. اگرچه مقدار معقولی از ژنوم با کیفیت قابل قبول توسط پروتکل‌های B و C با برگ‌های خشک استخراج شد ولی منطقه ITS در این نمونه‌ها تکثیر نشد، بنابراین پروتکل‌های B و C برای استخراج ژنوم *A. fridae* مناسب نبودند.

واژه‌های کلیدی: باقلاییان، بومی، تغییرات آب و هوایی، تهدید، در معرض خطر انقراض، CTAB

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Introduction

Considering the recent increase in the number of biodiversity studies utilizing plant molecular data (Vinson *et al.* 2018), it is crucial to establish efficient and optimized DNA extraction methods that yield high-quality DNA samples suitable for molecular genetic analyses such as PCR and real-time PCR analysis, Southern blotting, restriction enzyme digestion, fingerprinting, and other genotyping techniques (Pipan *et al.* 2018). However, extracting suitable DNA samples from plants is challenging due to the presence of large amounts of secondary metabolites, the concentration of which varies depending on the plant species. Thus, extraction protocols must be optimized for each plant material to ensure optimal results (Sahu *et al.* 2012). High-quality DNA is primarily characterized by high molecular weight fragments with A260/280 ratios between 1.8 and 2.0, as well as the absence of contaminants (Kasem *et al.* 2008).

In order to evaluate the entire genome, PCR analysis should also be performed. Because non-coding nuclear DNA (nrDNA) is inherited from both parents, there is higher genetic variability in comparison to maternally inherited chloroplast DNA (cpDNA) (Tsitrone *et al.* 2003, Petit & Excoffier 2009, Hollingsworth 2011). As a result, nrDNA sequences have become widely used for phylogenetic studies at various levels (Amirahmadi *et al.* 2014, Kaveh *et al.* 2019). The primary impurities found in plant DNA extractions are polysaccharides, which tend to adhere to DNA and anionic impurities that inhibit restriction enzymes, thereby affecting enzymatic analyses (Abdel-Latif & Osman 2017). Phenolic compounds can also bind to nucleic acids and make them resistant to various ameliorating enzymes, thereby, leading to DNA degradation and low efficiency (Manoj *et al.* 2007, Azmat *et al.* 2012, Souza *et al.* 2012). Large-scale phylogenetic projects are often not feasible through field collections alone, so herbaria provide a way to overcome these limitations. They contain centuries of collected genetic material that cover broad areas and timeframes (Kates *et al*. 2021).

Astragalus L., the largest genus of *Fabaceae* is distributed worldwide in temperate zones, with approximately 3000 species (Ghahremaninejad *et al.* 2022). Over 800 *Astragalus* species have been identified and studied in pastoral and mountainous regions of Iran (Mozaffarian 2003). However, there have been few phytochemical investigations of this genus, even though saponins, flavonoids, and polysaccharides are its primary metabolites. Despite their widespread distribution in Iran, there have been few studies on the chemical constituents of *Astragalus* species (Ghasemian-Yadegari *et al.* 2017). Many *Astragalus* species have long been used in traditional medicine, especially in Asia, for various diseases (Siwicka *et al*. 2011). For example, some species in the genus have been used for anticancer (Yesilada *et al.* 2005), immunostimulant (Bedir *et al.* 2000), antihypertensive (Castillo *et al.* 1993), neuroprotective (Luo *et al.* 2004), hepatoprotective (Jia *et al.* 2012), antimicrobial (El-Sebakhy *et al.* 1994, Song *et al.* 1996, Pistelli *et al.* 2002), antiviral (Huang *et al.* 2008), cardioprotective (Zhang *et al.* 2006), and anti-aging (Rios & Waterman 1997, Fathiazad *et al.* 2012) purposes.

Astragalus fridae Rech.f. is a plant species native to the gypsum hills of Semnan province, Iran. It grows along with other endangered species such as *A. moussavii* Maassoumi, F.Ghahrem. & Ghahr. and *A. semnanensis* Bornm. & Rech.f. (Ghahremaninejad *et al.* 2020), which have been classified as endangered by the IUCN (Jalili & Jamzad 1999). To conduct genetic studies on this endangered plant species, obtaining high-quality DNA is critical, but extracting pure total DNA with a high yield can be challenging (Abdel-Latif & Osman 2017). While various methods for DNA extraction are available, no comprehensive study has been conducted to optimize the extraction protocol for *A. fridae*. Additionally, using a simple and cost-effective method that minimizes the need for specialized equipment is essential for achieving optimal results. Hence, this paper aims to evaluate rapid and efficient methods for isolating pure total DNA from *A. fridae.*

Materials and Methods

- Plant materials

Astragalus fridae specimens were collected from Semnan province (Iran) in June 2018, from gypsum soils located after a village called "Aftar". One of the specimens was deposited in the herbarium of Damghan University, Damghan, Iran (DU: 2448). From the collected sample, young and undamaged leaves were isolated, washed with distilled water, and divided into two parts. One part was immediately frozen at -80 °C to preserve as fresh sample, while the second part was air-dried at room temperature to utilize as herbarium sample.

- DNA extraction methods

Protocol A (Main CTAB, Doyle & Doyle 1987):

Genomic DNA extraction with three replicates was performed using a six-step protocol based on Doyle & Doyle (1987) as follows:

1) 3000 µl of preheated CTAB buffer [2% CTAB powder, 20 mM EDTA, 1.4 M sodium chloride, and 100 mM Tris-HCl (pH = 7.5)] was added to 0.25 g of the crashed fresh leaves or 0.125 g of the herbarium leaves.

2) 6 µl of β-mercaptoethanol (0.2% v/v) was added to the microtubes. Each replicate containing 400 µl of homogenate was incubated in a dry bath (60 °C) for 30 min while swirling occasionally.

3) 800 µl of chloroform-isoamyl alcohol (24:1) was added to the microtubes and slowly mixed several times. The microtubes were then centrifuged at 13000 rpm (4 °C) for 15 min.

4) The upper aqueous phase was transferred to new tubes and 2 volumes of cold isopropanol were added to each microtube. The microtubes were stored at -20 °C for 20 min. They were then centrifuged at 10000 rpm (4 °C) for 10 min.

5) The supernatant was discarded and the pellet was washed with 250 µl of washing buffer (76% ethanol and 10 mm ammonium acetate). The microtubes were then centrifuged at 10000 rpm for 2 min.

6) The supernatant was discarded and the pellet was dried at room temperature. Finally, the pellet was dissolved in 30 µl of deionized water.

Protocol B (modified CTAB1, Alidoosty Shahraky *et al.* 2023):

All steps were similar to the main CTAB (2.1 part), except that, step 2 (6 µl of β-mercaptoethanol) was deleted in this method.

Protocol C (modified CTAB2, Alidoosty Shahraky *et al.* 2023):

All steps were similar to the main CTAB (2.1 part), except that, in this method, ammonium acetate was removed from the washing buffer (step 5).

Protocol D (modified by Murry & Thompson, Riahi *et al.* 2010):

1) 1875 µL of CTAB buffer [2% CTAB powder, 100 mM Tris-HCl (pH = 7.5), 1.4 M NaCl, and 50 mM EDTA (pH = 8)] was added to 0.25 g of the crashed fresh leaves or 0.125 g of herbarium leaves.

2) 7.5 µl of β-mercaptoethanol (0.4% v/v) was added to the microtubes. Each replicate containing 750 µl of homogenate was incubated in a dry bath (60 °C) for 60 min while swirling occasionally.

3) 700 µl of chloroform-isoamyl alcohol (24:1) was added to the microtubes and slowly mixed several times. The microtubes were then centrifuged at 10000 rpm (4 °C) for 15 min.

4) The aqueous phase was transferred to a new tube and 0.33 volume of ice-cold isopropanol was added and stored at -20 °C for 60 min. and then centrifuged at 10000 rpm for 15 min at room temperature.

5) The supernatant was discarded without disturbing the pellet. The pellet was resuspended in 100 µL TE (1 mM EDTA and 10 mM Tris), and 0.1 volume of 2.5 M NaOAc and 2 volumes of ice-cold 95% ethanol were added and stored at -20 °C for 30 min. The pellet was then centrifuged at 10000 rpm for 5 min. Finally, the supernatant was discarded.

6) The pellet was washed with 1 mL of 70% ethanol and then centrifuged at 10000 rpm for 4 min, and finally, the ethanol was poured off.

7) The pellet was dried at room temperature. Finally, the pellet was dissolved in 30 µl of deionized water.

Protocol E (GeneAll Plant Kit):

DNA extraction from both the fresh and herbarium samples was performed using the GeneAll Plant Kit (manufactured by the GeneAll Biotechnology Company, South Korea). The extraction process was conducted in accordance with the protocol provided by the kit.

- Evaluation of the quality and quantity of the extracted DNA

After the extraction of total DNA, the sample quality was visualized on a 0.8% EtBr agarose gel. An equal amount (150 ng/µL) of each DNA sample was added to the wells. DNA quantity was estimated also determined by measuring the A260/280 and A260/230 absorbance ratios using a spectrophotometer (Eppendorf AG, Hamburg 22331, Germany). The quality and performance of the isolated DNA were assessed by PCR amplification of the internal transcribed spacer (ITS) of nuclear ribosomal DNA. Primers ITS5 m and ITS4 were used to amplify an approximately 700-bp fragment of the ITS region. The ITS was amplified using the primers described by Sang *et al*. (1995) and White *et al*. (1990), respectively. PCR amplification was performed in a 20 µl volume containing 10 µl 2x Taq DNA Polymerase Master Mix Red (Amplicon), 0.5 µl of reach primer (10 pmol/µl), 500–700 ng of extracted DNA, and the remainder sterile water. PCR cycles consisted of pre-denaturation at 94 °C for 3 min, followed by 30 cycles each consisting of denaturation at 94 °C for 50 sec, annealing at 55–56 °C for 30 sec, and elongation at 72 °C for 50 sec, followed by a final elongation step of 7 min at 72 °C. To assess the quality of the PCR products, electrophoresis was performed using a 1.5% (w/v) agarose gel. The gel buffer used was 1X TBE. After electrophoresis, the gel was stained with ethidium bromide.

- Statistical analysis

To analyze the results of the DNA extraction from both fresh and herbarium leaves, five different extraction methods were used. The data obtained from these methods were subjected to statistical analysis using the Mann-Whitney & Kruskal-Wallis tests. The SPSS software (Ver. 24) was utilized for this analysis with a significance level of 0.05.

Results and Discussion

- Evaluation of DNA quality and quantity

The quality of the extracted DNA was assessed through gel electrophoresis. A suitable DNA sample was indicated by the appearance of a clear, elongated single band with no smearing. In contrast, the presence of smearing on the gel, which indicates the breakdown of DNA fragments during the extraction process, is considered a sign of lower-quality DNA. Figure 1 displays the results of the DNA quality assay on agarose gel. The analysis revealed that, all five extraction protocols (A–E) were able to recover high molecular weight genomic DNA from *A. fridae*. However, only six methods, including Protocols A–C in fresh samples, as well as Protocols B–D in herbarium samples, were successful in recovering a good amount of genomic DNA (Fig. 1). The majority of the extracted DNAs showed a smearing pattern, which could be attributed to various factors such as high salt concentration in the sample, the presence of carbohydrates or phenolic compounds, or DNA fragmentation, as reported by Zamani *et al*. (2005).

The plot in figure 2 displays the amount of total DNA extracted using different methods. The modified Murray & Thompson method yielded the highest concentration of DNA, with a value of 185.66 ng/µL for herbarium samples (D). On the other hand, the modified CTAB2 method yielded the lowest concentration of DNA, with a value of 37.5 ng/µL for fresh samples (C). The other extraction methods resulted in DNA concentrations within this range. It is important to note that, there were no significant differences between the two methods depicted in the graph.

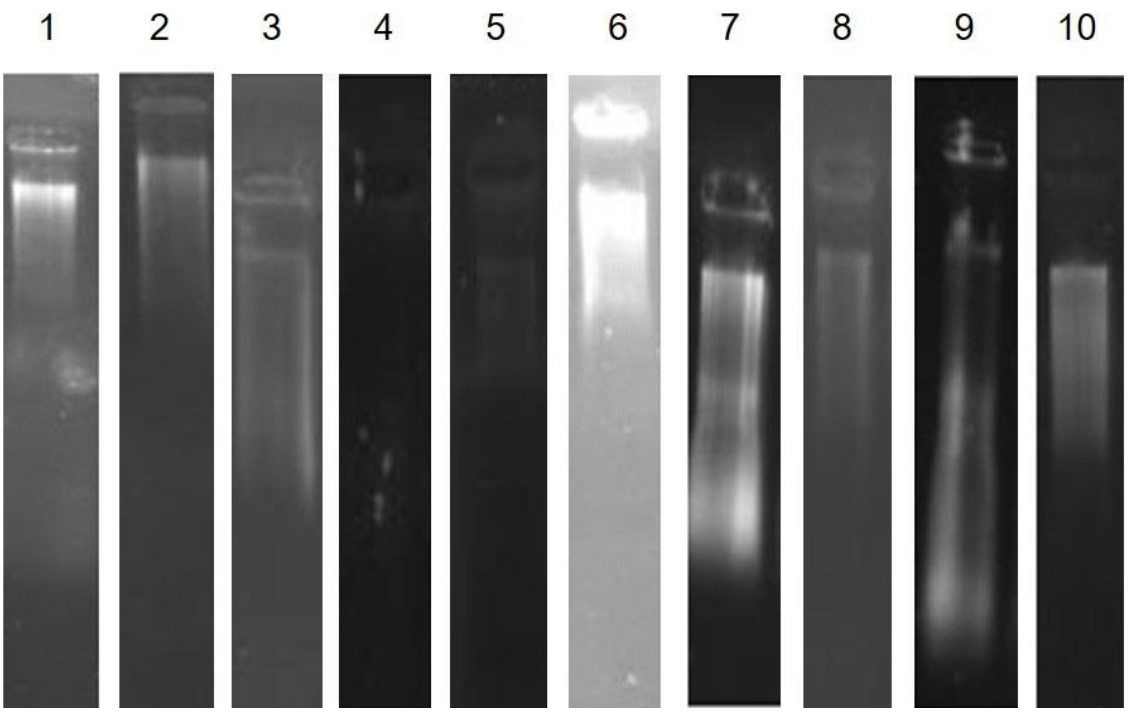

Fig. 1. Electrophoresis of total genomic DNA of *Astragalus fridae* on 0.8% agarose gel by the following methods: 1, 2- Protocol A with the fresh and herbarium sample; 3, 4- Protocol B with the fresh and herbarium sample; 5, 6- Protocol C with the fresh and herbarium sample; 7, 8- Protocol D with the fresh and herbarium sample; 9, 10- Protocol E with the fresh and herbarium sample.

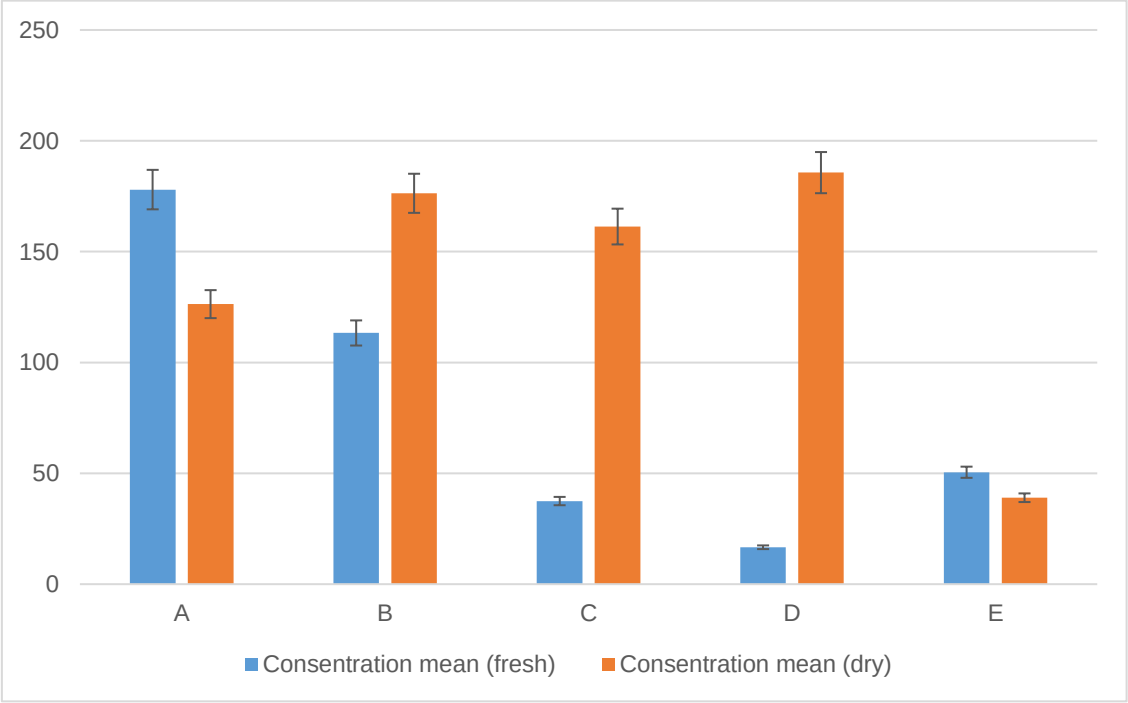

Fig. 2. The quantity means of total DNA extracted from *Astragalus fridae* by five different methods (Protocols A–E). Data are presented as the mean ± standard error of the mean.

- Spectrophotometry

In DNA spectrophotometry, an absorbance of one at 260 nm corresponds to 50 mcg/ml of double-stranded DNA. If the 260/280 nm ratio is in the range of 1.7–2.0, this indicates that, the nucleic acids absorb more, and the quality of the DNA obtained is desirable and has the necessary purity (Farsi & Zolala 2003). The spectrophotometric result showed that, among the five methods, the highest absorption in A260/280 is associated with Protocol D with the fresh sample (1.52), and the lowest absorption is associated with Protocol B with the herbarium sample (1.2) and Protocol C with the fresh sample (1.21), respectively. Among the methods, Protocol D with the fresh sample and Protocol C with the fresh sample, one the one hand, and Protocol D with the fresh sample and Protocol B with the herbarium sample, on the other hand, differ significantly (Fig. 3). The spectrophotometric results also showed that, the amount of total DNA extracted does not differ significantly among the five methods in A260/230.

Figure 3 illustrates the absorption results of the extracted DNA using different methods. The highest absorption was observed in Protocol A with the fresh sample, having a value of 0.725. On the other hand, the lowest absorption was observed in Protocol D with the fresh sample, having a value of 0.38. A low 260/230 ratio is an indicator of contamination with salts or some solvents, such as carbohydrates, peptides, phenols, or aromatic compounds. A pure sample typically has a 260/230 ratio of about 2.2 (Aboul-Maaty *et al*. 2019). The spectrophotometric measurements were performed on three replicates for each type of procedure.

- PCR amplification

The results of PCR amplification of genomic DNA isolated from *A. fridae* using the five different extraction methods were shown in figure 4. All samples showed positive amplification except for Protocols B and C with herbarium specimen extractions (Lanes 10 & 11). The extracted DNA samples appeared as distinct bands separated on gel electrophoresis at their corresponding high molecular weight. The PCR products were approximately 700 bp in size. During the drying process, some metabolites concentrate in plant tissues and can negatively affect the DNA quality of herbarium samples. These compounds can interfere with several steps of genome extraction and inhibit the PCR reaction during the PCR process (Costa & Robert 2014).

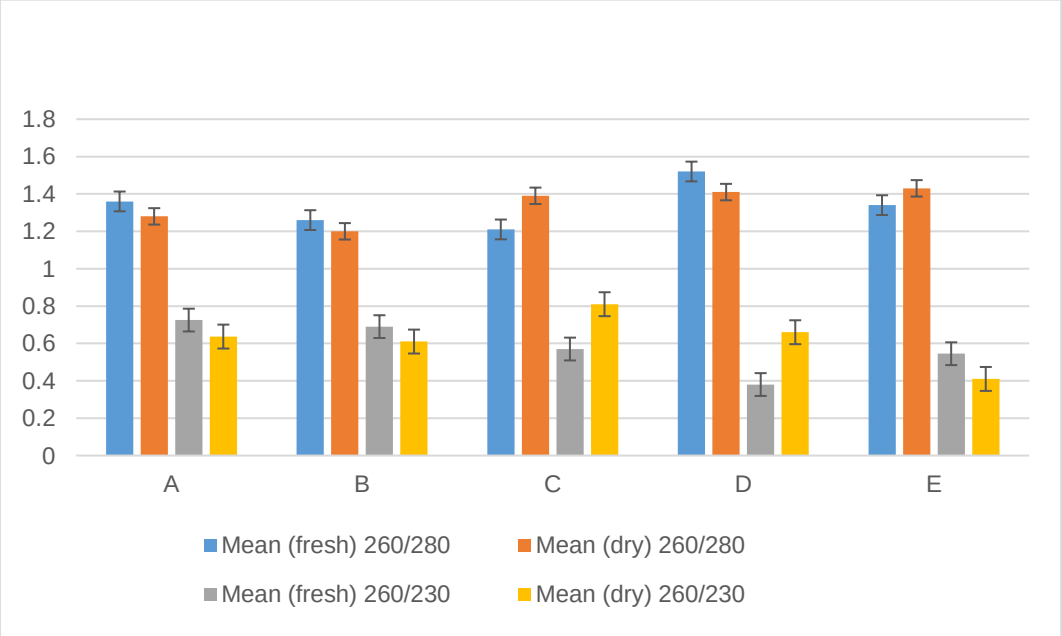

Fig. 3. Absorbance of average A260/A280 and A260/A230 ratios for DNA extracted by five methods (Protocols A–E). The columns are averages of three replicates and the bar is the standard deviation.

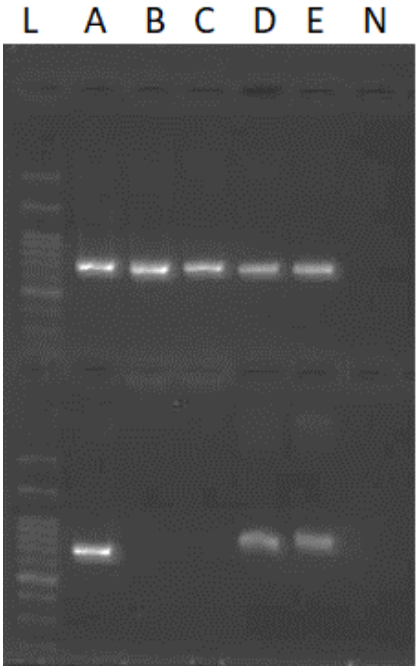

Fig. 4. The PCR amplification of nrDNA ITS for the Protocols A–E. The top row is based on the fresh specimen, and the bottom row on the herbarium specimen. L denotes DNA Ladder, and N denotes Negative. PCR products were run on a 1.5% agarose gel.

To obtain high-quality DNA from medicinal plants which are rich in phenolic compounds; these metabolites should be removed. β-Mercaptoethanol is a potent reducing agent for purifying tannins and other polyphenols in the crude plant extract which also reduces the disulfide bonds of the protein, resulting in protein denaturation. The role of sodium acetate as a salt in the extraction protocol is to neutralize the charges on the sugar-phosphate backbone of the DNA. Sodium acetate, with a pH of 5.2, is typically used with ethanol to precipitate nucleic acids. This is affected by either ethanol or isopropanol, making it much easier for Na^+ to interact with the PO^{3-}, shielding its charge and making the nucleic acid less hydrophilic, causing the DNA to precipitate out of solution (Heikrujam *et al*. 2020). In Protocols B and C, β-mercaptoethanol and sodium acetate were removed. Perhaps the absence of the components increased the PCR inhibitor compounds in the reaction, and PCR was stopped. As previously mentioned, genomic DNA extraction protocols vary in efficiency for different plant species due to the diverse chemical compounds present in each species (Saboora *et al.* 2019, Jalali Roudsary *et al.* 2022). For instance, in *Echium amoenum* Fisch. & C.A. Mey., the elimination of β-mercaptoethanol and ammonium acetate from the primary CTAB method does not interfere with PCR, making it a viable method for genome extraction (Alidoosty Shahraky *et al.* 2023). Jalali Roudsary *et al*. (2022) reported that, the CTAB method is not suitable for DNA extraction of the *Bellevalia* species, whereas Riahi *et al*. (2010) demonstrated that, the modified CTAB method is the most suitable method for DNA extraction in *Fabaceae*. Findings of the present study suggest that, the modified Murray & Thompson method, GeneAll Plant Kit, and primary CTAB method are all suitable for total genomic extraction in *A. fridae.*

Conclusion

The study found that, all five tested DNA extraction methods successfully extracted DNA from *Astragalus fridae* leaves. Protocol D, which involved the use of a fresh sample, yielded the purest product with the lowest level of DNA degradation and contamination. Protocol D with the herbarium sample resulted in the highest concentration of total DNA. However, it should be noted that, small amounts of DNA are sufficient for PCR. Based on the findings, the modified Murray & Thompson

method (D), the GeneAll Plant Kit (E), and the herbarium-based Protocol D were the most appropriate techniques for total genome extraction from *A. fridae* among the five tested methods.

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