Journal of Experimental Biology and Agricultural Sciences, December - 2023; Volume – 11(6) page 940 – 946

Journal of Experimental Biology and Agricultural Sciences

http://www.jebas.org

ISSN No. 2320 – 8694

# Genomic DNA extraction from the medicinal plant *Crocus sativus* : Optimization of Standard Methods

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Received – August 06, 2023; Revision – December 18, 2023; Accepted – December 27, 2023
Available Online – December 31, 2023

DOI: http://dx.doi.org/10.18006/2023.11(6).940.946

KEYWORDS

Genomic DNA extraction

Cetyltrimethylammonium bromide

CTAB

SDS

*Crocus sativus*

**ABSTRACT**

High-quality genomic DNA is essential for genomic and molecular investigations such as next-generation sequencing. However, DNA extraction from medicinal plants like *Crocus sativus* can be challenging due to their high secondary metabolite content, which can interact with nucleic acids and affect the quality and yield of extraction. This study aimed to optimize the quality and yield of DNA using the cetyltrimethylammonium bromide (CTAB) extraction method from the leaves, stigma, and saffron corm. This new method is easy to use and can be performed using standard equipment and inexpensive reagents. The modifications made to the CTAB lysis buffer in this study, with the addition of SDS, resulted in a yield of 4233 ng/µl of DNA per sample of saffron corm (100 mg). This protocol is efficient and cost-effective for DNA extraction for studies with large samples and limited resources. This method is expected to be widely used for large-scale plant extraction and has a broad application in PCR-based sequencing studies.

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Peer review under responsibility of Journal of Experimental Biology and Agricultural Sciences.

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## 1 Introduction

*Crocus sativus* L. is a bulbous plant that belongs to the Iridaceae family. It is also known as saffron and is used as a spice and food coloring due to its expensive dried red stigmas. Saffron has gained popularity recently due to its numerous medicinal and pharmaceutical properties, such as anti-inflammatory, antifungal, antimicrobial, antibacterial, and anticancer properties (Boskabady et al. 2020; Shahrajabian et al. 2021). Saffron is mainly cultivated in Iran, India, Greece, Morocco, Spain, and Italy. Worldwide saffron production is estimated at 418 tons per year, and Morocco is the fourth-largest producer of saffron in the world after Iran, India, and Greece, with an estimated production of 5.9 tons per year (El Caid et al. 2020). However, due to the high economic value and unique properties of saffron, there is a rising demand for its production. Saffron production is limited due to its prolonged vegetative reproduction and sterility (Alavi-Siney et al. 2022). Saffron production is based only on vegetative propagation by corms, and continuous vegetative propagation leads to low genetic diversity, which limits different genetic breeding and production improvement programs. However, increasing efforts have been dedicated to boosting saffron yield production with superior product quality and conservation of genetic viability. Therefore, several scientific investigations have been conducted to improve the yield and corm production of saffron, in particular, conducted studies involving agro-morphological (Ghanbari et al. 2019a; El Caid et al., 2020), soil, and fertilization management for superior quality saffron production (Ghanbari et al. 2019b; Esmaeilian et al. 2022). Other complemented molecular studies have been extensively performed for saffron to assess genetic diversity and to evaluate the saffron ecotypes for conservation purposes (Anabat et al. 2020; Alavi-Siney et al. 2022) and used for saffron quality control by identification of the purity and detection of adulteration of saffron (Villa et al. 2016; Bansal et al. 2019; Zhao et al. 2019). To conduct molecular-based studies on saffron, high-quality genomic DNA extraction is a critical prerequisite, as it is the primary key to a successful polymerase chain reaction (PCR) assay (Aboul-Maaty and Oraby 2019). However, DNA purification can be hindered due to the high content of phenolic compounds and other secondary metabolites in *C. sativus*. DNA degradation during extraction is also a common problem (Moratalla-López et al. 2019). Further, polyphenols and polysaccharide compounds interfere with total DNA isolation. DNA degradation during extraction is another encountered problem during purification due to the endonuclease's enzymes (Rezadoost et al. 2016; Nath et al. 2022; Singh et al. 2023)

Several DNA extraction protocols based on cetyltrimethylammonium bromide (CTAB) have been widely used (Schenk et al., 2023). However, a single DNA extraction protocol is unsuitable for all plant tissue due to the variety and complexity of secondary metabolite content. Furthermore, commercial extraction kits can be expensive and inappropriate for laboratories with limited funds (Yu et al. 2019). Therefore, a simple, reliable, and cost-effective CTAB-based method has been developed in this study for extracting high-quality total DNA from *C. sativus* and other medicinal plants with high phenolic content.

## 2 Materials and Methods

### 2.1 Plant material

This study extracted DNA from frozen leaves, dried frozen leaves, stigmas, and saffron corms. The plant materials were collected from saffron plants grown in the experimental field of the Faculty of Sciences at Ibn Zohr University in Agadir, Morocco, during the growing season. The leaves were collected and stored at -20 °C, while the stigma was dried before extraction.

### 2.2 DNA extractions

The saffron genomic extraction was performed using Doyle's (1991) CTAB-based method with modifications. DNA saffron extraction was performed in quadruplicate for all samples.

#### 2.2.1 Protocols 1

100mg of different parts of saffron were ground using a pestle and mortar. Then, 1ml of extraction buffer containing 2% (w/v) CTAB, 1% (w/v) PVP 400, 100 mM Tris–HCl (pH 8.0), 1.4 M NaCl, 20 mM Na-EDTA (pH 8.0), and 0.3% (v/v) β-mercaptoethanol was added. The mixture was incubated at 60°C in a water bath for 1 hour with continuous gentle agitation. Next, one ml of chloroform: isoamyl alcohol [24:1, v/v] was added, and the mixture was centrifuged at 20,000g for 15 minutes. After that, the supernatant was transferred to a new microtube (1.5 ml). Then, 1/3 of 6 M NaCl and 2/3 of cold isopropanol were added to the aqueous phase and gently mixed. The mixture was incubated at -20 °C overnight to allow DNA precipitation. The microtubes were again centrifuged at 20,000g for 10 minutes, and then the DNA pellet was rinsed with 1 mL washing buffer (75% ethanol, 10 mM ice-cold ammonium acetate). After that, centrifugation was carried out at 20,000g for 5 minutes, and double washing of DNA pellets with 75% ethanol was performed. Finally, the DNA pellet was air-dried at room temperature and resuspended in 50µl of TE buffer (1 mL 1 M Tris–HCl, 20 mL 0.5 M 2Na2EDTA (pH 8.0), and 79 mL Milli-Q water).

#### 2.2.2 Modified Protocol 2

After an initial DNA extraction method, small tubes were mixed with 2% CTAB extraction buffer and incubated for an hour. Following this, the tubes were allowed to sit at room temperature for 5 minutes and then 1 ml of phenol: chloroform [1:1] was added to them. The tubes were gently mixed and centrifuged at 20,000g for 15 minutes. The upper phase was extracted with an equal amount of chloroform: isoamyl alcohol [24:1, v/v] and centrifuged

at 20,000g for 15 minutes. The genomic DNA was then precipitated using NaCl/isopropanol and washed, and the obtained DNA pellet was resuspended in 50µl of 1% TE buffer.

**2.2.3 Modified Protocol 3**

100mg of various plant parts were ground in a mortar. Instead of using the standard 2% CTAB lysis buffer, a modified extraction buffer with SDS was used. To make the mixture's final concentration of 0.2% SDS, 10% SDS was added to the 2% CTAB buffer. The ground samples were then homogenized with 1 ml of the extraction buffer with SDS and incubated at 60°C for 1 hour. After this, all subsequent extraction steps followed protocol 1.

**2.3 Assessing the quality and quantification of the DNA genomic extracted**

The quality of the genomic DNA extracted from different parts of saffron using three different protocols was evaluated. Electrophoresis was performed to run the DNA on a 1% agarose gel at 100V for 60 minutes, using 1 × TBE as the electrophoresis buffer. The gel was then stained with ethidium bromide and analyzed under UV light. The DNA concentration was also determined spectrophotometrically at 260 nm using 5µl of DNA diluted in 1500µl of TE buffer. The purity of the samples was also assessed by checking for contamination from proteins and polysaccharides based on the A260/A280 ratio of the absorbance.

**2.4 PCR amplification analysis**

The PCR reaction was carried out in an Eppendorf tube. The reaction mixture consisted of 1 µL of template DNA, 1 × PCR buffer, 0.5 U Taq DNA polymerase (Bioline Meridian Bioscience Inc, USA), 0.06 µM of ITS2 primer (Jiang et al., 2014), forward primer (5' ATGCGATACTTGGTGTGAAT), and 0.06µM reverse primer (5' GACGCTTCTCCAGACTACAAT). The PCR process involved a denaturation step at 95°C for 2 minutes, followed by 36 cycles of denaturation at 94°C for 30 seconds, annealing at 58°C for 30 seconds, and extension at 72°C for 1 minute. The process concluded with a final extension step at 72°C for 7 minutes. Subsequently, the 10 µL PCR product was loaded and run on an agarose gel. The result was recorded and visualized using a UV detector after the run.

**3 Results**

This study evaluated a straightforward and efficient method for extracting genomic DNA from various parts of the saffron plant. DNA extraction protocols such as extraction kits can be time-consuming, expensive, and inefficient, especially for plants rich in secondary phytochemicals like phenolic compounds (Yu et al., 2019).

The CTAB-based protocol is the most commonly used option for labs with limited resources and repetitive extraction. However, this protocol still needs to be optimized for plants with high levels of polysaccharides and polyphenols. In this study, the total genomic DNA of saffron was extracted from frozen and dried leaves, stigmas, and saffron corms (Figure 1) using a CTAB buffer. Three extraction protocols were evaluated, and approximately 100mg of samples were used for each extraction method. The concentration and yield of the extracted DNA varied depending on the different parts of the saffron plant and the type of protocol employed in the extraction procedure (Table 1).

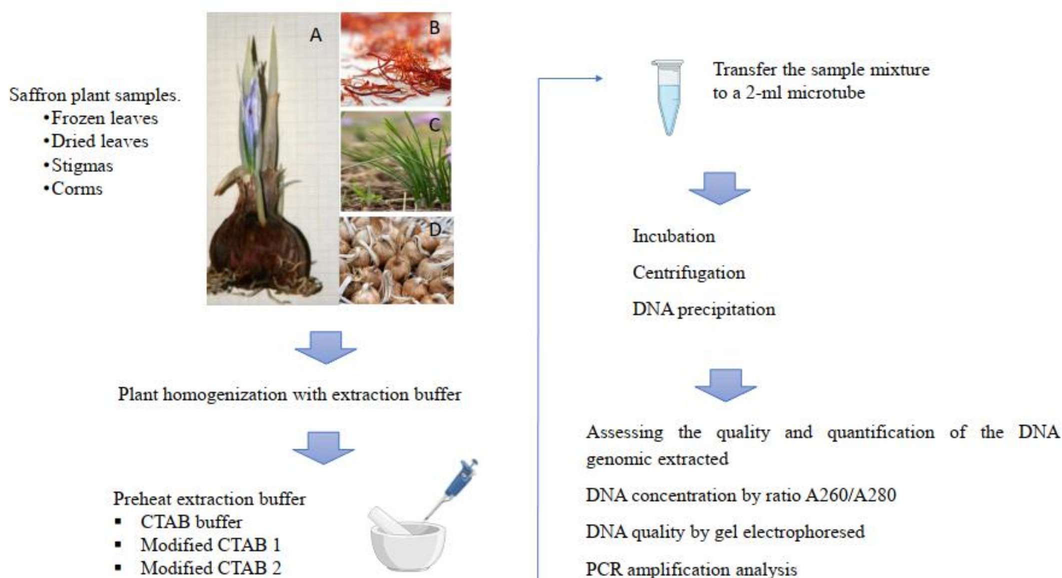


Figure 1 Flow diagrams of the three CTAB methods for saffron DNA extractions. A: *Crocus sativus* L. plant, B: stigmas, C: leaves and D: corms

Table 1 The purity and concentration of DNA extracted from saffron samples with three protocols

| Samples | Protocols | A260/A280 (Purity) | DNA Concentration (ng/µl) |
|---|---|---|---|
| Frozen leaves | P1 | 1.58 | 1641 |
| | P2 | 1.71 | 1992 |
| | P3 | 1.80 | 2538 |
| Dried leaves | P1 | 2.02 | 1797 |
| | P2 | 1.69 | 723 |
| | P3 | 1.02 | 1597 |
| Stigmas | P1 | 1.77 | 3153 |
| | P2 | 1.66 | 2856 |
| | P3 | 1.75 | 3072 |
| corms | P1 | 1.81 | 2709 |
| | P2 | 1.85 | 3069 |
| | P3 | 1.68 | 4233 |

P1: traditional CTAB, P2: Modified protocol, and P3: CTAB with 10% SDS

The study found that the DNA extracted from the leaves, stigmas, and corm using three different extraction buffers had a purity between 1.7 to 2.0 at the A260/A230 ratio. The ratio A260/A280, measured to determine DNA quality, ranged from 1.02 to 2.02. In the traditional CTAB method (protocol 1), the highest ratio of 2.02 was obtained from the dried leaves, while the DNA purity was recorded at 1.77 and 1.81 in the case of stigma and corm, respectively. In protocol 2, using phenol-chloroform, the highest purity ratio was obtained from corm, which was 1.85. Lastly, in protocol 3, where SDS was combined with CTAB buffer, the ratio A260/A280 ranged from 1.68 to 1.80 for corm and frozen leaves, respectively.

The overall DNA concentration obtained with protocol 1 based on the traditional CTAB buffer ranged from 1641ng/µl to 2709ng/µl, obtained from frozen leaves and corm. Protocol 2 successfully increased the DNA genomic yield and got 3069 ng/µl from the corm. However, modification in the CTAB buffer by adding the SDS substantially increased genomic DNA concentration to 4233ng/µl from Corms samples. Among the samples tested, the highest DNA crude purity and concentration were obtained from corm samples by protocol 3 with SDS addition. The quantitatively efficient protocol was CTAB with 10% SDS buffer to extract frozen leaves and corms.

The saffron DNA genomics extracted from various samples using multiple buffer protocols were analyzed for background compounds and potential degradation during extraction. Gel electrophoresis separation revealed some intense bands near the gel wells for DNA extracted by the CTAB buffer with SDS from frozen leaves and corms (Figure 2). Similarly, the most effective protocols for leaves were CTAB with SDS. PCR amplification

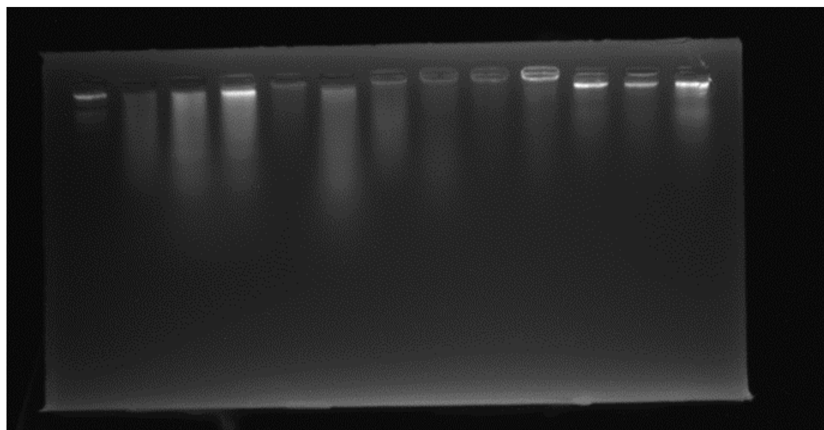

Figure 2 Electrophoretic patterns of *Crocus sativus* genomic DNA; Lanes 1 to 3: DNA extracted from frozen leaves using protocols 1, 2, and 3, respectively; Lanes 4 to 6: DNA extracted from dried leaves; lane 7 to 9: DNA extracted from stigmas, and lanes 10 to 12: DNA extracted from corm

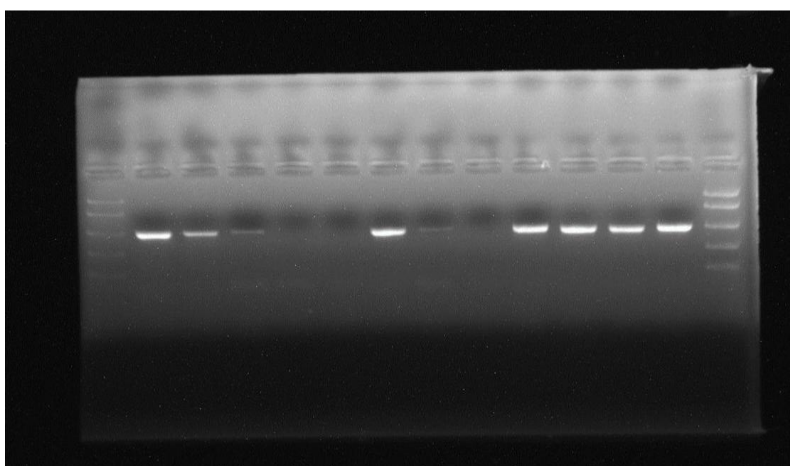

Figure 3 Agarose gel electrophoresis of ITS-PCR amplification products of *Crocus sativus* genomic DNA extracted by three protocols; Lanes 1 to 3: DNA extracted from frozen leaves using protocols 1, 2, and 3, respectively; Lanes 4 to 6: DNA extracted from dried leaves

with ITS primer showed a well-differentiated band pattern in the case of DNA extracted by modified protocols 3 for all saffron samples (Figure 3). The 560 bp length band was consistently observed in all DNA extracted from corm with the three protocols tested.

**4 Discussion**

Extracting high-quality genomic DNA is crucial for plant biology research, including genetic diversity, genetic improvement, and detection of adulteration through PCR-based analysis (Yu et al. 2019; Schenk et al. 2023). However, obtaining high-quality DNA can be challenging, especially for medicinal and aromatic plants with high levels of phenols and other secondary metabolites that can interfere with DNA purity. While several protocols for genomic DNA isolation are available, some are expensive or may not yield good DNA quality for certain plant species (Li et al. 2013; Yu et al. 2019). This study describes a simple and efficient method for extracting genomic DNA from saffron that can be applied to plants with high amounts of secondary metabolites. The method involves using a CTAB-SDS buffer as a lysis buffer with detergent, which proved to be more effective than other methods tested. This buffer promotes plant cell wall lysis, prevents RNAse activity and polyphenol precipitation, and consistently produces a pure and high-quality DNA yield that can be used in subsequent molecular analysis techniques. The protocol involves using a high concentration (0.3%) of 2-β-mercaptoethanol in a CTAB buffer to remove polyphenols successfully and generate a clear translucent DNA pellet (Heikrujam et al. 2020; Schenk et al. 2023). The extraction buffer also includes a chloroform-isoamyl alcohol step, which removes proteins, lipids, and cellular debris by binding with non-aqueous compounds as precipitate. Although the CTAB-based extraction buffer is the most widely used for plant DNA extraction, this study obtained better results using CTAB-SDS as a lysis buffer with detergent. The addition of SDS to the classic CTAB buffer had a significant effect on the extraction process. The CTAB/SDS buffer helps break down the plant cell wall and prevents RNAse activity and polyphenol from precipitation (Sika et al. 2015). Among the three tested protocols, the modified CTAB protocol with SDS proved more effective than the other two. This result is consistent with Mançano et al. (2022), who reported that using 20% SDS combined with CTAB yields high DNA (700ng) from Chrysobalanus icaco. Sabriu-Haxhijaha et al. (2020) used 2% SDS for soybean genomic extraction and obtained a high DNA yield. Moreover, Wang et al. (2012) obtained the highest DNA yield from soybean (25.89±3.2 µg) using the SDS-based extraction method compared to the classic CTAB method and commercial kits. The main modification for saffron DNA extraction involves adding SDS to the lysing buffer. This modification consistently produced a pure and high-quality DNA yield, which can be used in subsequent molecular analysis techniques such as PCR and sequencing. This study's simple and efficient extraction protocols are essential for assessing food safety by detecting adulteration and further biodiversity conservation. They are particularly useful for large analyses involving a large number of plant samples.

**Conclusions**

Research on molecular aspects of saffron plants lags behind other crops. This means that the information on saffron plants is limited and needs further exploration for crop improvement. Some studies have been conducted to examine genetic diversity, genome sequencing, and identification of markers for crop development. However, genomic DNA isolation is required for all molecular and genomic sequencing studies. Although several protocols have been developed for plant genomic DNA extraction, they are often expensive and less effective for certain plant species. The CTAB method is commonly used but still has limitations such as low quantity and purity, especially in plant species with high secondary metabolite content. This study has attempted three different

protocols to extract saffron DNA and found that adding 10% SDS to the CTAB buffer significantly increased the quantity and quality of extracted DNA. This method is simple, rapid, low-cost, and does not require special equipment. Therefore, it efficiently extracts high-quality genomic DNA from plant species with high secondary metabolite content. However, further studies are necessary to standardize this method for different plant species under varying conditions.

**Authors' Contributions**

SEM Conceptualization; SEM, IB, HEF, ML, methodology; SEM, IB, HEF, ML, KL, validation; SEM, IB, HEF, KH, ML, writing—original draft preparation; KL, MBEC, REL, writing—review and editing; REL, MAS supervision. All authors read and approved the final manuscript.

**Acknowledgments**

This work was supported by the Hassan II Academy of Sciences and Technology (SafranVal project), the National Centre for Scientific and Technical Research (PPR/2015/33 project), and the University of Ibn Zohr.

**Conflict of Interests**

The authors declare no conflicts of interest related to this article.

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