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دستورالعمل ساده و کارآمد استخراج DNA برای برگ‌های قدیمی و هرباریومی تمشکین (تیره مارچوبه‌ایان)

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چکیده. DNA استخراج شده با کیفیت بالا در بدست آمدن باندهای واضح در مرحله الکتروفورز و نتایج صحیح در تعیین توالی نقش مهمی ایفا می‌کند. معمولا CTAB یکی از روش های رایج برای استخراج DNA است که برای برگ‌های قدیمی نمونه‌های هرباریومی سرده تمشکین (*Bellevalia*) که متابولیت های ثانویه با اثر بازدارندگی دارد مناسب نسیت. برای حل این مسئله، تغییراتی در مراحل مختلف CTAB اعمال شد و ترکیبات زغال فعال، فنل، استات پتاسیم و RNase استفاده شد و آب دیونیزه جایگزین Tris EDTA شد تا ناخالصی‌ها حذف شده و DNA با کیفیت تری بدست آید. داده‌های میزان جذب DNA روش جدیدبا روش CTAB در طول موج‌های ۲۶۰ و ۲۸۰ نانومتر مقایسه شد. نتایج، تغییر ۱۵تا۸۰ برابری در غلظت DNA را نشان داد. این مطالعه روش اصلاح شده‌ای را پیشنهاد می‌کند که امکان استخراج DNA با کیفیت بالاتری نسبت به روش CTAB را از برگ‌های قدیمی نمونه های هرباریومی *Bellevalia* امکان‌پذیر می‌کند.

واژه‌های کلیدی تمشکین، خالص سازی DNA، زغال فعال، فنل، نانودراپ

A simple and efficient DNA extraction protocol for old herbarium leaves of *Bellevalia* (Asparagaceae, Scilloideae)

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Abstract. High-quality DNA extraction plays an important role to make sharp bands in the gel electrophoresis and also produces clean chromatograms. Usually, DNA extract is delivered using the modified CTAB method but this method cannot obtain high-quality DNA for molecular analysis from old dried leaves of *Bellevalia* due to having different chemical compounds which inhibit to obtain a clear DNA extraction. To solve the problem, in different phases of a modified CTAB protocol, activated carbon, phenol, potassium acetate, RNase and deionized water (instead of TE) were applied to eliminate contaminants and improve obtained high-quality DNA. The DNA absorption data from the modified method were compared with CTAB method in 260 and 280 nm wavelengths. The results showed a 15-80-fold change in the DNA concentration. This study suggests the modified method of extraction is more efficient in the quality of DNA obtained from the little amount of herbarium old leaves of *Bellevalia* than that of the CTAB method.

Keywords: activated charcoal, *Bellevalia*, DNA purification, phenol, nanodrop

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INTRODUCTION

DNA extraction methods from tissues are often used to obtain a qualified genome and isolate other compounds from the cell. If compounds such as lipids, polysaccharides, proteins, minerals, and other contaminants are extracted with DNA, those would interfere in later stages of PCR (Huang et al., 2000; Rat et al., 2014). Thus, these compounds must be eliminated during the extraction process completely. There are several DNA extraction methods which can be selected based on the type of fresh or herbarium tissues (Gaudeul & Rouhan, 2012; Zabeti, 2015; Riahi et al., 2019; Saboora et al., 2019; Drábková, 2021**)**. The principles of DNA extraction protocol are the same in plants, however, different secondary metabolites in the various genera of the same family such as Asteraceae Bercht. & J Persl., Lamiaceae Martinov and Myrtaceae Juss. families can be interfere and affect DNA extraction **(**Michiels et al., 2003; Agostini, 2011; Borges et al., 2017).

Therefore, it is necessary to modify the methods and introduce fast and cost-effective protocol to obtain qualified and pure nuclear and cytoplasmic DNA. Several protocols have been introduced to isolate DNA from plants (Murray & Thompson, 1980; Doyle & Doyle, 1987; Rogers & Bendich, 1988; Zamani, 2005). In many cases, with a few modifications in the DNA extraction method, the quality and quantity of the DNA can be enhanced up to several times. These changes have been made in various plants, including *Ornithogalum* L. (Rat et al., 2014).

The genus *Bellevalia* with 75 species belongs to the Scilloideae Burnett. subfamily, Asparagaceae Juss. family, and Asparagales Link. order (APG IV, 2016). This genus is distributed in Africa, Europe, and West and Central Asia (Jafari & Maassoumi, 2008; Govaert, 2021). Based on the recent infrageneric classification according to four plastid genes, the genus was divided into four sections (Jafari et al., 2021). During our phylogenetic analysis on *Bellevalia* species, DNA was extracted from old and dried silica-gel leaves based on CTAB method. Low DNA concentration between 2.1 to 19.9 ng/100µl was obtained following NanoDrop measurements. Moreover, the quality of DNA was not appropriate for the next processes. Due to existing different secondary metabolites such as Saponin, Terpenes, Flavonoids, Tannins, Anthraquinone, Alkaloids, homoisoflavonoids, ferulic acid-derived acrylamide, methylthioacrylate bellegimycin in the leaves of *Bellevalia* species (Alali et al., 2016), extraction of the high-quality DNA was difficult so that it was necessary to be introduced a specific protocol for DNA extraction of different *Bellevalia* species. Therefore, we decided to find a rapid and cost-effective method to obtain high-quality DNA from a little amount of leaves of *Bellevalia* spp. Here, we proposed a new modification of the CTAB method specified for the herbarium old and dried silica-gel leaves of *Bellevalia tabriziana* Turrill, *B. paradoxa* Boiss, and *B. macrobotrys* Boiss. We compared our new method with the classic CTAB method proposed by Doyle and Doyle (1987).

MATERIALS AND METHODS

Plant tissue sample preparation. The experiments conducted on the leaves of *B. tabriziana* and *B. paradoxa* (sect. *Bellevalia*), and *B. macrobotrys* (sect. *Conicae*). The samples of the first two species were sent from IRAN (Iranian Research Institute of Plant Protection) and P (Muséum National d'Histoire Naturelle. Paris) herbaria respectively, while *B. paradoxa* had been collected from its known locality (Table 1). The specimens of the last species were preserved in the IAUM (Islamic Azad University, Mashhad Branch) herbarium**.**

DNA isolation procedure

According to the CTAB method, EDTA, Tris HCl/NaCl, PVP and CTAB are used (Doyle & Doyle 1987, 1990) as separate powder compounds in the first stage of extraction. While, in our proposed method, phenol, activated charcoal, potassium acetate, and glucose were applied and the deionized water was used instead of Tris-EDTA Buffer. The extraction steps are as follows:

1. Powder 10 mg of herbarium leaf with liquid nitrogen in 1.5 µl microtubes.
2. Add activated charcoal powder as much as the weight of the leaves to the microtubes.
3. Add 10 mg PVP to 390 µl CTAB in eppendorf tube and incubate at 65 °C for 45 minutes.
4. Add 1.5 µl of beta mercaptoethanol, 25 µl of 5 M potassium acetate dissolved in glacial acetic acid and a quarter of the volume of glucose-EDTA to half of the prepared CTAB.
5. Incubate at 65 °C for 60 minutes and invert 2 or 3 times.
6. Add the phenol in a volume equal to the buffer while it is on ice and vortex
7. Add isoamyl alcohol/chloroform solution (1:24) to each sample, invert gently for 10 minutes and centrifuge for 10 minutes at Max rpm.
8. Separate the aqueous phase and mix it again with an equal volume of isoamyl alcohol/chloroform (1:24). 15 minutes

centrifuge at Max rpm and separate the aqueous phase again.
9. Add 10 µl of 4 M NaCl and cold isopropanol and invert gently.
10. Put at - 20°C for at least 3 hours.
11. Centrifuge for 20 minutes at Max rpm, remove supernatant and add 70% ethanol in two steps.
12. Dislodge the obtained DNA and centrifuge for 10-minute at Max rpm
13. Remove ethanol and dry DNA at ambient temperature.
14. Add 100 µl of deionized water, 0.2 µl of RNase and place for 1 hour at room temperature and transfer to refrigerator.

PCR reaction. Genomic DNA was obtained from *Bellevalia* samples using the specific and general primers: TOPO6 (forward 8F: AGGCACTTATHTGGTCAAAGATGAG), reverse11R (AGGAGGCATAACATCTGTC) (Blattner, 2016) and BELITS 18S (forward: AAGTCGTAACAAGGTTTCCGTAG), BELITS 25S (reverse CTTCTCCTCCGC TTATTGATATG) (Borzatti et al., 2013). For the first primers, a PCR was performed at 94°C for 4min followed by 35 amplification cycles at 94°C for 80 s, 59°C for 1min and 72°C for 30 s and a final extension reaction at 72°C for 10 min, while for the second primers, PCR was done at 94°C for 4min followed by 40 amplification cycles at 94°C for 80 s, 56°C for 1min and 72°C for 30 s and a final extension reaction at 72°C for 10 min.

Gel Electrophoresis. Gel electrophoresis was carried out using 0.8% agarose gel. The gel solution consisted of TBE buffer pH 8.0 and 1 µl of ethidium bromide per 10 µl of agarose solution.

Statistics. The nuclear DNA extract based on the proposed method was evaluated using NanoDrops 2000/2000c. Software Statistical Analysis System (SAS) version 9.1 (SAS Institute, Cary, NC, USA) (Taheri et al., 2014) was used to compare DNA yield, quality and concentration obtained from samples using CTAB and the proposed protocol.

Table 1. The list of studied species with voucher number based on Jafari et al., 2021.

Section	Species	Locality	Voucher number
Bellevalia	*B. tabriziana*	IRAN, Azarbayejan province, Tabriz and Ahar, Daneshpazhuh	IRAN29677
Bellevalia=Oxydontae.	*B. paradoxa*	IRAN, Kordestan province, 70 km Sanandaj to Divandere road, Kharkeh station, 2160 m, 2011.05.28, Jafari, Dezyanian & Kaffash	IAUM10340
Conicae=Nutantes	*B. macrobotrys*	SYRIA, Idlib, Idlib to Armenza road (northern Syria). Louis [Marist Brothers of Aleoppo]	P1775006

Table 2. Recipes for materials used for the protocol.

Material	Volume
Dried leaves	10 mg
Activated charcoal	10 mg
PVP	10 mg
CTAB	390 µl
Phenol	390 µl
Potassium acetate solution 5M	25 µl
Glucose solution	25 µl
ß –mercaptoethanol	1.5 µl
Isoamyl alcohol	780 µl
NaCl 4M	100 µl
Isopropanol	260 µl
Ethanol 70%	500 µl
Deionized water	70-100 µl
RNase	0.2 µl
Tris solution 1M (pH 8.0)	108 gr
Boric Acid	55 gr
EDTA 0.5 M (pH. 8.0)	4.6 gr

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RESULTS

For the first time we used a new DNA extraction protocol for *Bellevalia* species which remove impurities more than that proposed in the CTAB. Gel-electrophoresis DNA bands in the electrophoresis gel were not sharp and optimal in the CTAB method, while sharp and clear gel-electrophoresis DNA bands were appeared in the new method (Fig. 1A, B). DNA extract obtained from the modified method had an obvious effect on PCR and gel electrophoresis. In comparison of the modified method with the CTAB protocol, a great increase was observed in the dissolved DNA concentration per 100µl of deionized water e.g., 15.1 to 234 ng in *B. tabriziana*, 18.2 to 357 ng in *B. paradoxa* and 4.1 to 310.2 ng in *B. macrobotrys*, respectively. Also, isolated DNA purity for *B. tabriziana*, *B. paradoxa*, and *B. macrobotrys* was reported 1.90, 1.92, and 1.91 based on absorption ratio in A260/A280 wavelengths. A significant difference was observed in absorption rate in 260 nm, 280 nm and absorption ratio 260/230 between new and CTAB method (Figs. 2-4.). Although, there is no significant difference in the absorption ratio of 260/280 between the couple methods ($p \leq 0.05$) (Table 3).

DISCUSSION

As a result, a 15-80-fold improvement was observed in the yield of obtained DNA from the studied *Bellevalia* specimens based the proposed method compared to the CTAB method. Regarding to the DNA purity, Page (2010) and Arif et al. (2010) explained the best absorption rate varies 1.8-2. They mentioned the values less than 1.8 indicate protein contamination and other UV absorbent, while the values more than 2 show a high concentration of RNA in the sample. DNA purity of the three studied species of *Bellevalia* in our method was reported 1.90, 1.92, and 1.91 which are optimal according to Page (2010) and Arif et al. (2010). A qualified DNA could be obtained using activated charcoal and phenol (Rat et al., 2014) with some changes in the materials of the CTAB method. In our proposed method, at the first stage of extraction, liquid phenol was added to remove more contamination and to denature proteins in the leaves and separate cellular proteins and histones attached to DNA (Rice, 2017). Liquid phenol also removed compounds bound to DNA which makes it brown (Hiesinger et al., 2001). After centrifuging, denatured proteins remained in the organic phase while nucleic acid including chloroform remained in the aqueous phase. In fact, chloroform was used to remove phenolic residues. The use of chloroform with isoamyl alcohol was caused to dissolve proteins and fats, destroyed pigments and fell into the organic phase (Sahu et al., 2012). In addition, PVP was applied to separate polyphenol substances from DNA. Subsequently, a decrease in phenolic residues was observed in the product (Pich & Schubert, 1993; Permingeat et al., 1998). Since the activated charcoal and PVP were not effective alone to homogenize *Bellevalia* leaf tissues, adding the liquid phenol improved the DNA amount. On the other hand, the role of activated charcoal in the proposed method was to absorb dark brown, black, unknown pigments (semi-phenolic and melanin compounds) and toxic substances during the DNA extraction process (Maliyakal, 1992; Porebski et al., 1997; Martellosi et al., 2005). It could destroy the cell walls more. Moreover, potassium acetate and EDTA solution helped to remove metal atoms from the cell at pH 8.0. Added deionized water instead of TE also prevented DNA damage in examined *Bellevalia* species. The results showed the use of the above-mentioned materials in certain stages of the extraction process increased the DNA concentration 15-80-fold more than the CTAB method.

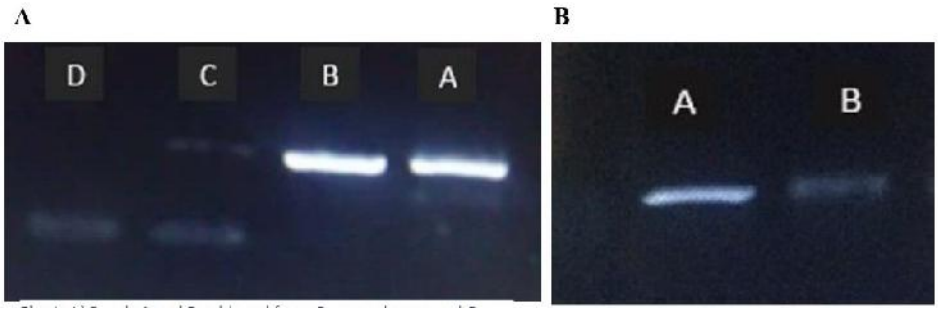


Figure 1. A. Gel electrophoresis following PCR for *B. macrobotrys* and *B. paradoxa*. Bands A and B obtained from *B. macrobotrys* and *B. paradoxa* respectively based on the proposed method and bands C and D based on CTAB method. **B.** Gel electrophoresis following PCR for *B. tabriziana*. Bands A and B based on the proposed method and CTAB respectively.

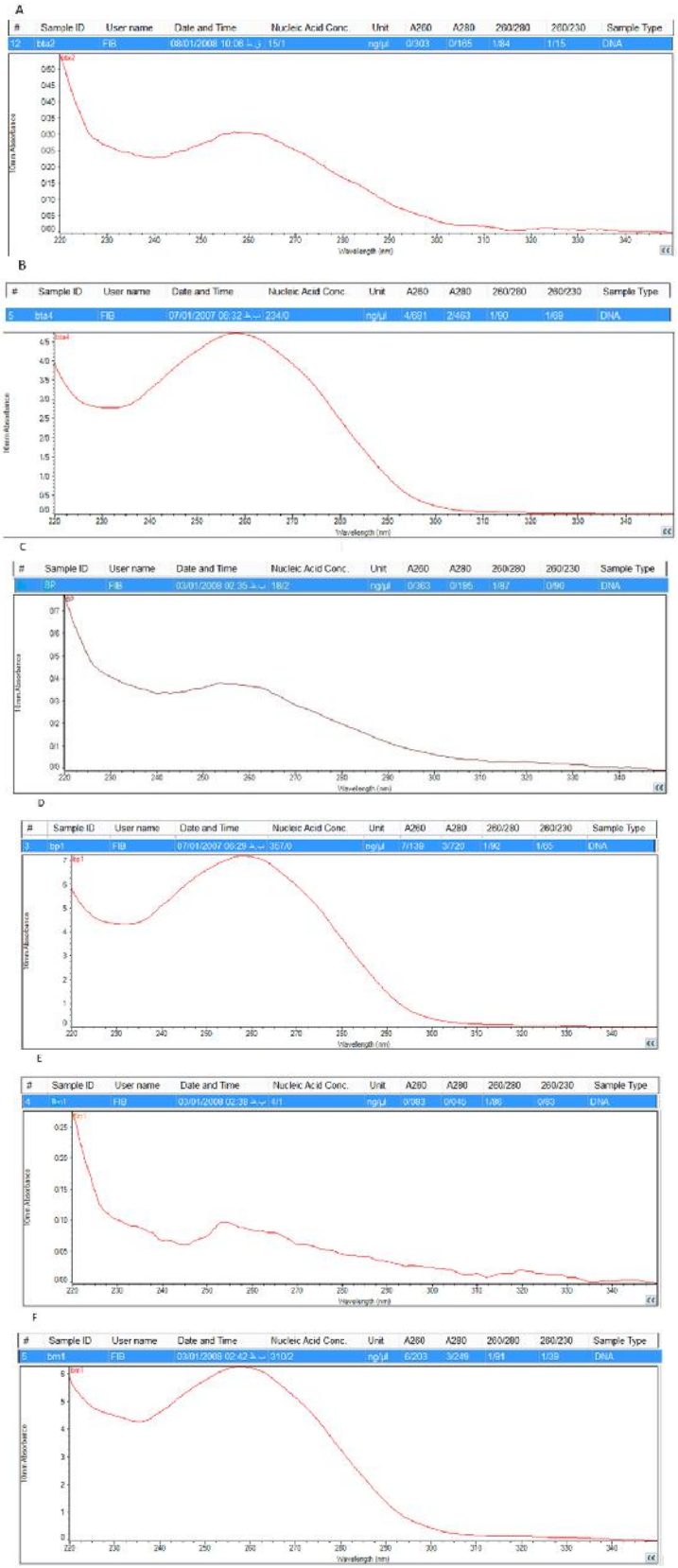


Figure 2. Diagram of absorbance rate in different wavelengths for DNA obtained from three studied *Bellevalia* species based on CTAB and the proposed methods using NANODROPS 2000/2000c software. **A, B.** *B. tabriziana.* **C, D.** *B. paradoxa*. **E, F.** *B. macrobotrys*.

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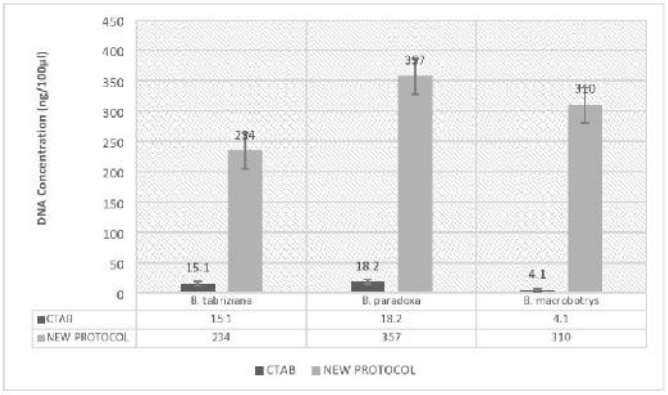


Figure 3. Comparison of DNA concentrations and absorption ratio adapted from NANODROP 2000/2000c.

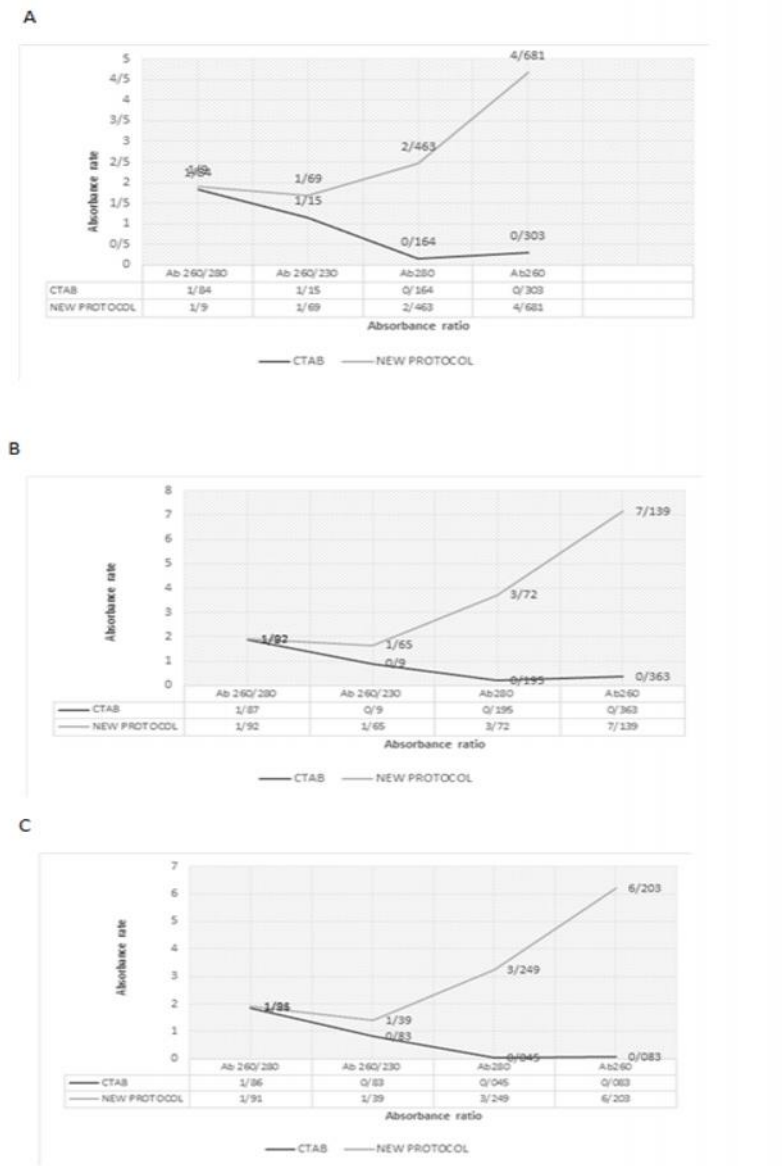


Figure 4. Diagram of absorption rate of obtained DNA by the couple methods in 260, 280, 260/280 and 260/230 wavelengths. **A.** B. tabirziana, **B.** B. paradoxa, **C.** B. macrobotry

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Table 3. Comparison of DNA concentration in different wavelengths in the three studied *Bellevalia* species based on the CTAB and the new method.

Species	extraction method	Concentration (ng/100 µl)	A260	A280	A260/A280	A260/A230
B. tabriziana			0.303d	0.164c	1.84a	1.15c
B. tabriziana	New	CTAB	15.1e	2.463b	1.90a	1.69a
B. paradoxa	CTAB	18.2d	0.363d	0.195c	1.87a	0.90d
B. paradoxa	New	357.0a	7.139a	3.720a	1.92a	1.65a
B. macrobotrys	CTAB	4.1f	0.083e	0.045d	1.86a	0.83d
B. macrobotrys	New	310.2b	6.203ab	3.249ab	1.91a	1.39b

Means in the same column with different superscripts are significantly different ($p \leq 0.05$)

CONCLUSION

Although the CTAB is a common method to extract DNA from plants, it remains some impurities, which inhibit the PCR and fail to obtain a high quality of the DNA. The new DNA extraction protocol provided a high efficiency of genomic DNA from the old and fine leaves of *Bellevalia* and probably the allied genera for example *Muscari* and *Hyacinthus* species without contamination, which can be easily implemented for future analyzes such as population genetics and phylogenetic studies.

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