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Original article

Optimization of an Efficient Method for Genomic DNA Extraction from (*Vicia faba* L) Leaves with High Contents of Inhibitor Compounds

Ahmed Shaaban[1], Wagdi Ghaleb[1]*, Hanan Abosalah[2], Masoud Alsheebani[1], Ghada Elragaihy[1], Salem Hammud[1], Elmundr Abughnia[1]

[1]*Department of Plant Tissue Culture, Libyan Biotechnology Research Centre, Tripoli, Libya*
[2]*Alasmarya Islamic University, Zliten, Libya*
Corresponding email. *ghalebwagdi@gmail.com*

Abstract
The extraction of high-quality plant DNA in large quantities is considered a difficult task in all plant molecular studies, which makes the extraction of high-quality DNA from plants containing high amounts of inhibitor compounds the main challenge for researchers. Therefore, three different DNA extraction methods (CTAB, SDS, and a mixture of CTAB and SDS) were used and tested for the purpose of obtaining the best method for DNA extraction from Faba bean (*Vicia faba* L) plant leaves at different plant ages (7, 14, and 30 days). The estimation of DNA quality and purity was conducted by the Nanodrop spectrophotometer and gel electrophoresis system. The results of the present study showed that among the three DNA extraction methods used in this study CTAB method was the best method for extracting DNA from leaves in different plant ages specially leaves at the age of 7 days, followed by the SDS and CTAB methods. The absorbance ratio of extracted DNA from leaves with 7 days' age using the CTAB method was above 1.9. CTAB performance was good in the elimination of the effects of inhibitor compounds such as protein; this technique has the potential to be an effective protocol for DNA extraction using leaf tissue. The result of this study also proved that the mixture of SDS and CTAB methods produced DNA with acceptable purity, whereas the A260/A280 ratio of absorbance for extracted DNA from leaves with 7 days' age by the SDS+CTAB method treatment was 1.89. The results showed that the purity levels of extracted DNA from plant leaves at the age of 7 days were significantly higher than the other two treatments (14 and 30 days) in all the used methods, which explains that extracting DNA from young leaves is clearly successful. According to our result SDS method has an A260/A280 ratio of absorbance ranging from 1.53 to 1.87 in the treatment of leaves at 7 days' age, whereas this range was lower than that of the CTAB method.
Keywords. DNA Extraction, Plant Leaves, Inhibitor Compound, CTAB, SDS.

Introduction

Recently, plant genetic engineering has been receiving increasing attention these days to improve plant productivity by obtaining new plant varieties more suitable for environmental conditions. Moreover, the most important task in any molecular genetics study is the isolation of high-quality plant DNA with high yield [1]. Plant improvement is still the major mission for researchers, which requires a large quantity of DNA [2]. Plant molecular and plant tissue culture studies lead to improvement of plant breeding in general [3]. On the other hand, the extraction of high-quality DNA with satiable yield is a limiting factor in all plant genetic analysis [4]. Furthermore, the extraction of pure high-quality DNA is very substantial for any plant biological studies [5]. Extraction and purification of DNA are a crucial step in DNA molecular techniques used in plant studies for the identification of genotypes, economic traits associated with genes of interest, and genetic diversity [6,7]. In fact, for more specifically, an efficient extraction and purification protocol should provide pure, intact, and functional with satiable yield [8].

DNA molecular studies are mainly based on poly-merase chain reaction (PCR) assay that requires the extraction of plant DNA of suitable purity [6]. Molecular studies reveal the phylogenetic structure and function of plant species, enabling growers to improve productivity in changing environments [9]. Three main steps take place in DNA extraction from all plant tissue, lyses of tissue, separation of DNA from other cellular components, and extraction of DNA [10]. In fact, the structure of the cell wall, through the presence of polysaccharides and other inhibitors, largely affects plant DNA extraction and makes it a difficult task. The concentration of such compounds is often different from plant to plant and also differs in the plant tissues of the plant itself. Particularly, polysaccharides are the most common contaminants found in plant extracts and can make DNA pellets slimy and difficult to handle [11]. However, many difficulties have been found in extracting high-quality DNA from many plants [6].

Extraction of DNA from plants largely contaminated by the presence of secondary metabolites [12]. Therefore, the method of DNA extraction will differ between crops, and from tissue to tissue, and a modification is extremely needed in their extraction methods [13]. Normally, breaking the cell wall through the use of SDS (sodium dodecyl sulfate) or CTAB (Cetyltrimethyl ammonium bromide), protection of DNA from the endogenous nucleases with EDTA, removal of protein from buffer/tissue, and separating the protein from DNA are the main steps in all plant DNA extraction methodologies [3]. Several methods are used for the extraction of plant DNA from different plants, especially woody and rich polysaccharide plants. Commercial Kits allow faster extraction, but commercial Kits are normally expensive [14]. Most of these methods are used to extract DNA from plant tissues that are easy to extract, such as young leaves [15].

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Additionally, the type of plant tissue and growth stage are significant considerations that should be meticulously analyzed in the selection of a plant DNA extraction method. Type of plant tissue greatly affects the quality and quantity of extracted DNA [16], and various types of plant tissue, such as seedlings, leaves, cotyledons, seeds, endosperm, tissue culture callus, roots etc. are commonly used for plant DNA extraction [17]. However, the selection of the DNA extraction method and plant tissue is considered the most important factors affecting the extraction of high-quality DNA. Therefore, this study aimed to develop an efficient method for extracting plant DNA from leaves tissue samples of different ages that contain a high amount of inhibitor compounds. On the other hand, commercial DNA extraction Kits are convenient and usually safe, but their availability to certain developing countries and high cost can be limiting [1]. Meanwhile, there is an increasing need for developing a simple, rapid, and inexpensive technology for plant genomic DNA extraction [18]. In fact, the extraction of high-quality plant DNA is still a difficult task due to the presence of some compounds, such as polyphenols and polysaccharides, in plant tissue, which makes the extraction of plant DNA a complicated task [19].

Methods

Plant material

Faba bean (*Vicia faba* L) grains were obtained from the Agriculture Research Center, Tripoli, Libya. These plants were selected for inclusion in this study due to their differing levels of polysaccharides. Seeds of eight Faba bean varieties were planted in green house in order to obtain plant leaves for the DNA extraction process. This step is applied mainly to select the best DNA extraction method suitable for high-quality DNA from plant leaves. However, leaves from plants at different developmental stages (7, 14, and 30 days) were utilized for isolating DNA. DNA isolation from the leaves was performed using two previously established methods, namely the CTAB method [20] and the SDS method of [21]. In this study, a unique protocol that integrates both CTAB and SDS methods was utilized to achieve the most efficient DNA extraction method.

Reagents:

- 3× extraction buffer containing: 2% CTAB (w/v), 5 M NaCl, 1M Tris-HCl pH 8.0, 0.5M EDTA pH 8.0 (autoclaved)
- 2% 2-Mercaptoethanol (BME)
- Chloroform/isoamyl alcohol 24:1(v/v)
- 70% ethanol
- 1×TE buffer (10mM Tris-HCl, pH 8.0; 1mM EDTA, pH 8.0) autoclaved.

DNA Isolation

The plant DNA extraction stage started with the preparation of three main solutions, which are Nacl 5M, Tris 1M (PH 8.0), and EDTA 0,5 M. For total genomic DNA extraction, about 70-100 mg of leaves tissue was crushed by mortar and pestle to a fine powder using pure sand. Plant DNA was isolated using the CTAB, SDS, and mixture of CTAB and SDS methods from plant leaves. Samples were transferred to an Eppendorf tube for the next steps of the experiment. In fact, samples were divided into three groups according to their ages (7 days, 14 days and finally 30 days), then every treatment supplemented with 2% CTAB, 0.5% SDS and mixture of CTAB and SDS (1:1). For each sample 600 µl of 2% CTAB, and 0.5% SDS was add separately to Eppendorf tube with a good intermittently mixture using vortex. Samples were incubated in a water bath at a temperature of 55°C for 60 minutes. Moreover, 60 µl Poroteinase k,12µl of (ß-mercaptoethanol) and 600 µl chloroform: isoamyl alcohol (24:1) were added to all samples and mixed well (inverting and spinning). Then place the samples in a centrifuge for 5 minutes at a speed of 13000 rpm under a temperature 24 °C. Transferred 400µl from the supernatant solution (aqueous phase) to a new centrifuge tube and eliminated the remaining organic residue (organic phase). Re –add 400µl of chloroform isoamyl alcohol by (24:1 v/v) and spin as previously, then move to a new tube, then the samples are moved to the centrifuge. Add Ethanol (800 µl), then spin for 5 minutes. Repeat the washing process three times by using ethanol 70% with a volume of 600µl. Add (50µg/ml) RNase and place the samples in a water bath 37°C for 30-60 minutes, dry the samples using soft paper and cold air. TE buffer was added to the pellet with a volume of (20-50µl) depend on the size of the pellet, then the samples were stored at -4 °C or in the freezer at – 20 °C.

Gel electrophoresis test

DNA was analyzed by agarose gel electrophoresis, contains 0.7% agarose. The DNA was run on a gel electrophoresis to check its quality. Gel electrophoresis was run for all treatments in the present study. 1X (electrophoresis buffer) was put in the gel electrophoresis system until the prepared gel was already covered, then 4µl loading buffer was added to every DNA sample. Plant DNA samples were subjected into electrophoreses 1x TEA buffer for 1h at 70 v on 0,7 % agarose gel matrix. Marker will be injected in the first hole for comparison, followed by the rest of the samples. Images of gel were taken immediately under UV light using a gel document system to report the results.

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Nanodrop spectrophotometer analyses

Finally, samples were moved to the spectrophotometer system for measuring the purity of extracted DNA for each tested extraction protocol (CTAB and SDS). The results were recorded for all barley varieties in this study. The analysis with the Nanodrop spectrophotometer started by calibrating the system with 5 µl of TE buffer. The samples were subsequently placed into the system and mixed thoroughly until the solution was fully integrated. The purity ratio for the samples was taken according to the standard ration which is (A260/A280), and absorbance ratio at 260–280 (A260/A230 ratio), while the results were measured with a Thermo Scientific Nanodrop.

Spectrophotometric analyses of DNA

The concentration and purity (A20/ A280 ratio). Absorbance ratio at 260 – 280 was measured using a Nanodrop spectrophotometer and 1µl of each sample.

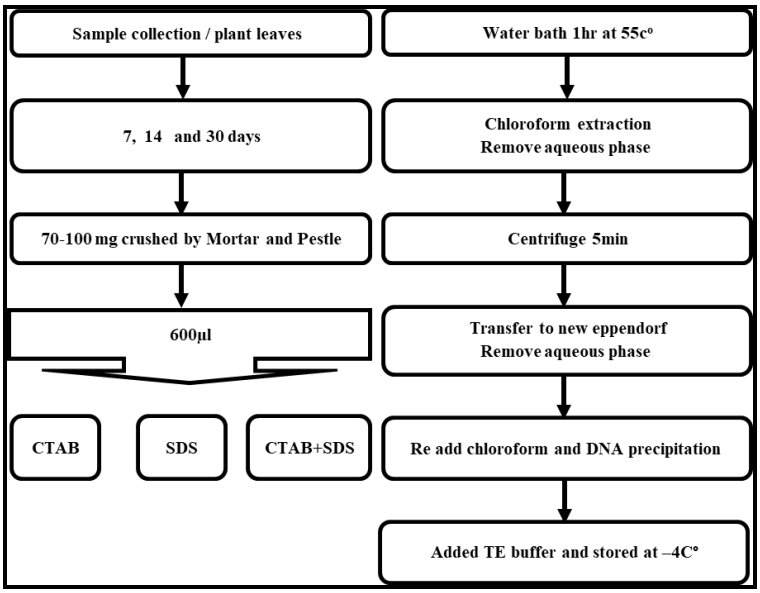

Figure 1. Plant DNA extraction steps

Results and discussion

Gel electrophoresis analysis

In this study, plant DNA was extracted from faba bean plant leaves at different ages (7, 14, and 30 days). While plant genomic DNA was extracted through the use of CTAB, SDS, and a mixture of CTAB and SDS. The presence of DNA was observed by gel electrophoresis system (Figure 2). Gel image of each treatment proved that the DNA was successfully extracted from the faba bean plant leaves in all the treatments. The results of this study also proved that CTAB, SDS, and mixture CTAB + SDS isolation buffers were successfully able to extract plant genomic DNA from plant leaves. The results of our study were in agreement with [22], who found a successful extraction of DNA from Yam plant leaves, already rich in polysaccharide compounds, using a gel electrophoresis system. Our results were also in agreement with [12], who reported that the genomic DNA was isolated from plant leaves, resolved under 8% agrosegel.

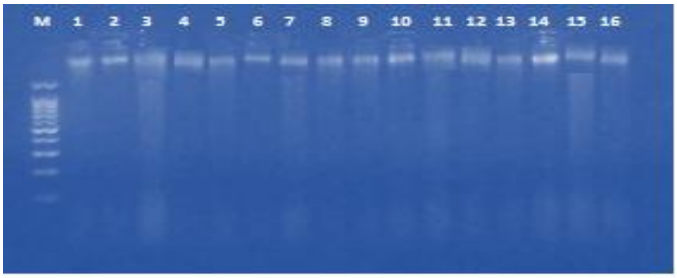

Figure 2. Gel electrophoresis picture of DNA isolated from different plant leaves

Comparison of different DNA extraction methods

DNA extraction methods

Since plant DNA has extracted using several methods discovered and modified over time in order to obtain high-quality DNA. In fact, several studies proved that the use of the CTAB method for extracting high-quality DNA is successful, and several DNA extraction methods and protocols are tested for obtaining high-quality DNA with satiable yields, which is important for other molecular studies. Particularly among several DNA extraction methods the CTAB method tends to be the best choice for plant DNA extraction from different

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plant tissues. Currently, the cost of a lab process is one of the main criteria for choosing the most suitable analysis method. It is obvious, then, that the time and cost of processing are key factors [15]. In this study, three different methods (CTAB, SDS, and their mixture) were used to extract plant DNA with high quality. However, the results of this study proved that the purity of plant DNA extracted from plant leaves by CTAB was significantly higher than the other two treatments, which explain that CTAB was extremely suitable for extracting high-quality DNA, followed by the mixture method. CTAB was able to remove the effect of the inhibitor compound (Figure 3). The A260 /A280 nm ratio of the extracted DNA ranged from 1.7 to 1.97. The present results were in complete agreement with those indicated in studies of [22], who reported that DNA yields and purity of the CTAB method were higher compared with other methods [23]. Furthermore, the results of this study showed that the CTAB method was able to extract high-quality DNA from all the used plant leaves, at ages (7, 14 and 30 days). These findings were in line with research that the CTAB method was able to extract DNA with quality suitable for DNA amplification, as shown by microsatellite amplification in the Oak plant. The protocol described provided DNA of good quality by the CTAB method from leaves of tree species, which have been a problem regarding the extraction of their DNA [24].

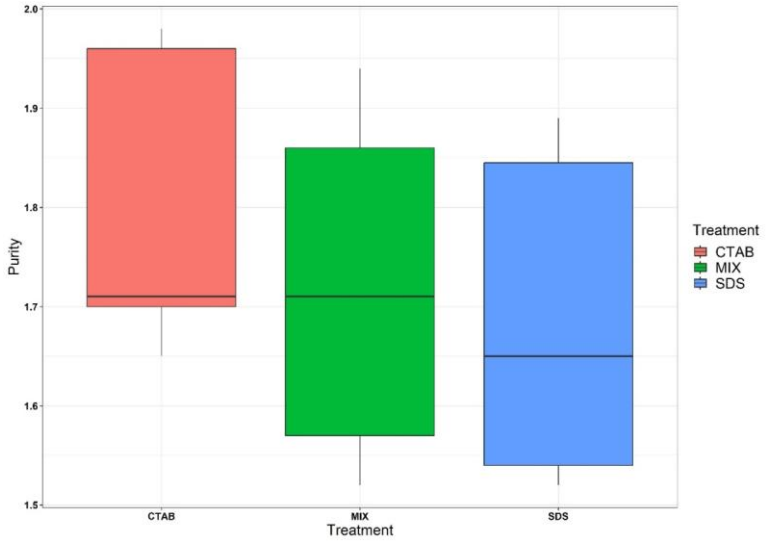

Figure 3. Comparison of three methods for DNA extraction

Plant leaves age

High-quality DNA is considered one of the most important goals in all plant molecular studies. Furthermore, plant genetic studies are based on the high efficiency of purified DNA samples [25]. In general, plant DNA is mainly extracted by procedures derived from the hot CTAB and SDS methods of [20,21], along with many others that are modified to be suitable for DNA extraction with high quality [26]. In this study, (CTAB, SDS, and their mixture) DNA extraction buffers were used and tested for isolating plant genomic DNA from leaves of Faba bean (*Vicia faba* L). The results showed that the purity of DNA extracted from plant leaves with an age of 7 days was significantly higher than the other two treatments, which are 14 and 30 days, that mean that extraction of plant DNA from fresh leaves is better than from old leaves. This might result from a reduction in the quantity of the inhibitor compound found in young leaves. The results of this study showed that the purity of DNA extracted from leaves at the age of 7 days was significantly higher compared to 14 and 30 days (Figure 4); this proves that extracting DNA from young leaves is very successful.

In general, the purity of DNA using the CTAB method was the highest compared to other methods (SDS & CTAB+SDS). This indicates the CTAB extraction method is the best solution for plant DNA extraction. Whereas, CTAB at 7 days leaves age had an overall better A260/A280 ratio above 1.9, followed by 14 days and finally 30 days leaves age, which indicated that the purity of extracted plant DNA was at high levels and extracted DNA can be used successfully in other PCR analyses. The findings indicate that DNA purity levels from treatments of 14 days and 30 days were quite similar, and the CTAB method achieved a superior average A260/A280 ratio of about 1.75 for both periods. The results also showed that there were no significant differences between leaves at 14 and 30 days' age, which indicated that CTAB was able to extract DNA from 14 and 30 days' treatment with the same efficiency. The study revealed that the CTAB method produced significantly greater amounts of DNA from fresh plant leaves samples than from etiolated leaves when compared to alternative methods. Furthermore, [22] found that the optimized CTAB method was able to extract high-quality DNA from 100mg fresh leaves tissue of Yam, banana and tomato leaves. Another study found that DNA produced by the CTAB method could be used in PCR-based techniques on a wide range of organisms and in laboratories, which proved that the CTAB method was able to extract large quantities of genomic plant DNA with high purity [27].

Our results also agreed with those in the studies of [24], who used fresh mature leaves for genomic plant DNA extraction by the CTAB method. On the other hand, the SDS method is usually used in plant DNA

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extraction studies for the purpose of making comparisons with other methods, which is often a modified CTAB method. One study found that there were no differences between CTAB and SDS when used to extract DNA from a wheat cultivar, and both methods obtained high-quality DNA. A number of studies reported that, when SDS is compared with the CTAB method usually gives less quality of extracted plant DNA [3]. The outcomes of our research demonstrated that the SDS method was successful in extracting DNA of satisfactory quality from the leaves of Faba bean (*Vicia faba* L.) at ages of 7, 14, and 30 days.

According to our results, treatment of 7 days leaves age under the SDS method gave DNA purity that was significantly higher than the other two treatments (14 and 30 days), which indicated that SDS was able to extract DNA from Faba bean leaves with an age of 7 days better than 14and 30 days. The SDS method has an A260/A280 ratio ranging from 1.53 to 1.87 in the treatment of leaves at 7 days, which explained that SDS performance was acceptable to extract DNA with suitable purity in this treatment. Furthermore, the present results indicated to those purity levels of extracted DNA by the SDS method from leaves aged 14 days were significantly higher than 30 days' plant leaves. Our results were in complete agreement with those indicating that SDS and CTAB methods were effective in the DNA extraction, which can be used successfully in PCR amplification, and DNA extraction methods presented similar color and concentration, and then purity [28]. The same was true with results in the study that found that the SDS method was able to extract plant DNA from dry seeds of barley crop, but the purity levels of the extracted DNA were lower than the CTAB method, the same has been found in our results [29]. In the same line, a study reported that after comparison of different DNA extraction protocols based on the use of CTAB and SDS from mango plant leaves [30]. These results were in agreement with our results except for the treatments with leaves of 7 days, which gave acceptable levels of DNA purity through our study. For the mixture of CTAB +SDS treatment.

In this study mixture of CTAB and SDS buffer was used and tested for obtaining new methods for extracting high-quality DNA. Mixture (CTAB + SDS) was also conducted to observe the ability of CTAB and SDS to extract high-quality DNA when they were used together as detergent tools and the ability of those compounds to remove the inhibitor compound from the extracts. The present results showed that the mixture CTAB + SDS method consistently resulted in higher DNA purity in 7 days' plant leaves samples, followed by treatment of 14 days' leaves, which explains that the SDS + CTAB method was able to extract DNA with satisfactory quality, indicating that the SDS + CTAB method was able to eliminate the effect of inhibitor compounds. The results also showed that the purity of extracted DNA by the SDS + CTAB method from Faba bean leaves with an age of 7 days was significantly higher than 14 and 30 days' treatments. The A206/A280 ratio of extracted DNA from leaves at 7 days' age was above 1.85. Furthermore, the purity of extracted DNA from14 days' leaves was significantly higher than 30 days' treatment. While the A206/A280 ratio in all the treatments ranged from 1.54 to 1.898. Our results are in complete agreement with those reported that the SDS + CTAB method consistently resulted in higher DNA yields compared to the Qiagen Kit for buffalo grass [1]. On the other hand, [9] developed a new SDS + CTAB method for RNA or DNA extraction from macadamia, avocado, and mango tissues. While the results of the study showed that the A260/A280 ratios of extracted RNA from Avocado, Mango, and Macadamia were 2, 2.03, and 1.39, respectively which proved the use of the SDS+CTAB method was successful, and the purity of extracted RNA from Avocado, Mango, and Macadamia was at acceptable levels [9]. DNA extraction from mature leaves is considered more complicated than from young leaves because of the higher presence of inhibitor compounds [15]. While DNA yield from young leaves was higher than from mature leaves, seeds and stems in most of the plant DNA extraction methods, this has been validated through our findings.

In our study, comparisons among three DNA extraction methods (CTAB, SDS, and SDS + CTAB) were made, and evaluations of the performance of each treatment were conducted to select the best DNA extraction method for isolating high-quality DNA from plant leaves of Faba bean (*Vicia faba* L) plant with different stage different ages. The present study (Figure 4) showed that for the purity of extracted DNA, the best results were obtained from leaves with an age of 7 days' treatment followed by 14 days' treatment. All of the used DNA extraction methods (CTAB, SDS, and SDS + CTAB) agreed on one point, which is that in every method, the treatment of 7 days leaves age, the purity of extracted DNA was significantly higher than the other two treatments (14 and 30 days).

According to our results, CTAB method was the best method for high quality DNA extraction from plant leaves in general and CTAB was able to produce high quality DNA products, which explain that CTAB performance was perfect in elimination of effects of inhibitor compounds such as protein and finally produced DNA with acceptable levels of purity. CTAB yields DNA with adequate purity indices (A 280/260) = 1.97 in the treatment of 7 days leaves age.

However, no significant differences were recorded among SDS and mixture SDS + CTAB treatments in the case of 7 days' age of leaves, which explains that the performance of both methods was at the same level for extracting DNA. The A260/A280 ratio of extracted DNA from both methods ranged from 1.8 to 1.89, but the CTAB method remains the best compared to the SDS and SDS + CTAB. Our findings are consistent with those who indicated that the modified CTAB method provides a rapid and effective protocol for extracting DNA from various polysaccharide-rich plant tissues [31]. Our findings are in alignment with those who demonstrated that CTAB could effectively isolate DNA from D. abolngifolia and D. lanceolata, yielding high-

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quality DNA and indicating that the CTAB method is promising [32]. The successful use of the CTAB method for tree species has been reported [33].

The A260/A280 ratio for DNA extracted from Psittacus hybrid by CTAB was clearly higher than DNA extracted by SDS method and the A260/A280 ratio was 2.05 which, proved that the extracted that of DNA by the CTAB method was of high-quality levels, whereas DNA yield by CTAB method was 854.90ng/µl [34]. The CTAB method of DNA extraction was found to be effective for complex plant tissues like corms, which are rich in polysaccharides and polyphenol inhibitor compounds [34]. Our findings are in the same line with those who reported that the CTAB technique has the potential to be an effective protocol for DNA extraction using mature leaves tissue for strawberries and perhaps for other species in the family Rosaceae with high inhibitor compounds [35]. When sufficient young leaves tissue is not available, this result indicates that the CTAB method can extract DNA from plant leaves of different ages, which has been proven in our study in the case of the CTAB method [35,28]. Furthermore, another study found CTAB extraction method provides high-quality DNA from recalcitrant mangroves and salt marsh plants containing elevated concentrations of polysaccharide compounds [31]. This method (CTAB method) eliminates the need to use liquid nitrogen and environmentally hazardous phenol to obtain high-quality DNA. CTAB gives better results than SDS in the particular case of recalcitrant tissue [36].

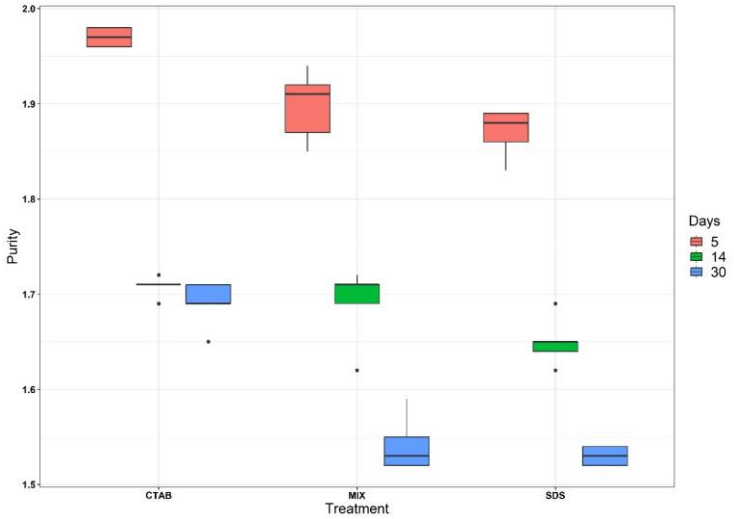

Figure 4. Comparison of different DNA extraction methods, CTAB, SDS, and SDS + CTAB methods from plant leaves with different ages (7, 14, and 30 days)

Conclusion

Our findings demonstrated that we could successfully extract DNA from the Faba plant (*Vicia faba* L) using the CTAB approach, even though this plant is challenging to work with because of its polysaccharide content. The young leaves are typically the best tissue utilized in plant DNA extractions, especially when using the CTAB buffer. This technique makes it possible to extract DNA from plants that are rich in inhibitory compounds more safely and affordably.

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Conflicts of Interest

Nil

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