**DNA extraction from different parts of lemon (*Citrus limon*)using the Chelex method**

**Running Title: DNA extraction from lemon (Citrus limon)**

Mehrnoush Aminisarteshnizi1\*

1Aquaculture Research Unit, School of Agricultural and Environmental Sciences, University of Limpopo (Turfloop Campus), Private Bag X1106, Sovenga, 0727, South Africa

\*Email: [mehrnoush.aminisarteshnizi@ul.ac.za](mailto:mehrnoush.aminisarteshnizi@ul.ac.za)

**Novelty statement**

Most plant DNA extraction has been done based on the CTAB method. However, in some woody fruit crops that contain high polysaccharide levels, such as Citrus spp., the DNA isolated was not of high enough quality to be used in PCR and RFLP analyses. Hence, the Chelex method was used in this study, which was simple, efficient, non-toxic, inexpensive reagents and required only a few steps. Therefore, in this study, the Chelex method was used for extracting DNA from different parts of *C. limon*. This study showed that the Chelex method could be used for genetic diversity and phylogenetic purposes and is highly recommended for DNA extraction from *C. limon*, especially in the stem part.

**Abstract**

The *Citrus limon*, popularly known as the lemon tree, is native to Asia. This species is cultivated commercially worldwide in tropical, semitropical, and warm temperate countries, including the Mediterranean region. Lemon cultivation and production play an essential role in the economy of many countries in which genomic DNA is critical for genetic diversity and molecular analysis of this tree. In this study, we compared three different parts of the lemon tree to extract DNA. The DNA from the lemon tree was extracted using the Chelex method from the fresh leaves, stems, and fruit. For this comparison, we used three samples of *C. limon* from South Africa in 2021. The quantity and purity of DNA were measured using a spectrophotometer. To confirm and evaluate the extracted DNA, we used PCR amplification with 26S primers.The spectrophotometer results showed the highest quantity of the Nucleic Acid extracted was for stem (63.18±2 mg/ml). However, the ratio of 260/280 for quality of DNA extraction in the stem (1.8±0.01) was in the range. The quantity of protein in the fruit (5.5±1.2 mg/ml) was high. The PCR reaction showed that the DNA extracted using the stem part had better quality than the other parts. The amplification of the samples showed the stem and leaf had high purity and concentration of DNA.We had DNA from all parts of the lemon tree, but the stem gave us better results regarding high-quality DNA.

**Keywords:** DNA extraction; *Citrus limon*; Spectrophotometer; PCR.

**Introduction**

Most studies based on plants are performed at the molecular level, which needs reliable, quick DNA extraction protocols. However, contamination with polysaccharides in tree DNA extraction is a common problem. Sometimes, the samples for DNA are contaminated with medicare colloidal hyalosome, which is almost insolvable in water or TE buffer. This can affect manipulation, inhibit enzyme reactions (Schlink and Reski 2002), and hinder the downstream work in molecular biology research. However, in some woody fruit crops that contain high polysaccharide levels, such as *Citrus spp.*, the DNA isolated was not of high enough quality to be used in PCR and RFLP analyses (Cheng *et al.* 2003).

Purity extracted DNA is a foundation for further study in the molecular field. Therefore, some methods, such as chloroform-based DNA extraction, is not safe for human. Additionally, chloroform-based DNA extraction needs toxic chemicals, magnetic separation, and silica-based DNA extraction is expensive. Several researchers reported the Chelex method for extraction of DNA for PCR amplification or a wide range of experiments has proven efficient. The Chelex method is rapid, simple, safe, and cheap (Turan *et al.* 2015).

The lemon tree grows up to 6 m tall, and the leaves are dark green, leathery, evergreen, oblong, elliptical, or oval (Mamede *et al.* 2020). Lemon fruits can be highly acidic and have a high citric acid, vitamin C, and other key nutrients, providing health benefits. Lemons are an interesting source of phenolic compounds, vitamins, minerals, dietary fibers, essential oils, organic acids, and carotenoids (Gonza´lez-Molina *et al.* 2010). Lemon is an acritical and high-demand fruit. In 2014 the worldwide commercial production of lemons was around 16.3 million metric tons harvested from 1.1 million hectares. Lemon cultivation and production play an essential role in the economy of many countries, and genomic DNA is critical for genetic diversity and molecular analysis of this tree (Mamede *et al.* 2020).

The 26S ribosomal DNA is a commonly used DNA marker in DNA barcoding analysis recommended in plant DNA barcodes. However, the purity of the extracted DNA is mainly sample-dependent. Furthermore, the chemical-physical composition affects DNA extraction. For example, several plant species, including the Citrus family, produce secondary metabolites like phenolic compounds, tannins, flavonoids, and alkaloids (Wang *et al.* 2007), which in the extract solution can interfere with DNA analysis and inhibit the PCR processing (HwangBo *et al.* 2010). Therefore, knowing which part of the plant is more suitable for extracting DNA and giving us high-quality DNA is good.

Therefore, this study aimed to evaluate the Chelex efficiency for DNA extraction in leaves, stems, and fruit of lemon with an overnight incubation at 56 ° C and discover which part could give us high-quality DNA.

**Materials and Methods**

**Sample collection**

Three samples of *C. limon* were collected from the Greener Tidings Garden Centre seedling in Polokwane (23°52'24.6"S 29°30'44.1"E) in 2021. All samples are transferred to the molecular lab for DNA extraction and molecular analysis.

**Molecular analysis**

DNA extraction was done using the Chelex method (Shokoohi 2021). Specimens of *C. limon* were hand-picked and transferred to a 1.5 ml Eppendorf tube containing 20 μL double distilled water. The lemon leaves, stem, and fruit separately in the tube were vortexed. Eighty microliters of Chelex® 20% were added to each microcentrifuge tube containing the crushed lemon and mixed. The tubes were incubated for 12 hrs at 56 °C overnight. Finally, it spun for 2 min at 16000 rpm. The DNA was stored at -4 °C. For PCR analysis, the forward and reverse primers, D2A (5'-ACAAGTACCGTGAGGGAAAGTTG–3') D3B (5'-TCGGAAGGAACCAGCTACTA–3'), was used for partial amplification of the 26S rDNA. The polymerase chain reaction was conducted with 8 μL of the DNA, 12.5 μL of 2X PCR Master Mix Red (Promega, USA), one μL of each primer (10 pmol/ μL), and 8 nucleotide free water for 30 μL volume. The polymerase chain reaction processing was done using an Eppendorf master cycler gradient (Eppendorf, Germany), with the following program: one cycles 94 °C for 3 min, then 37 cycles of denaturation for 45 s at 94°C; then 56 °C annealing temperatures for 26S rDNA; extension for 45 s to 1 min at 72 °C, and final step 6 min at 72 °C followed by a temperature on hold at four °C. 4μL of PCR product from the tube was loaded on a 1.5% agarose gel in TBE buffer to evaluate the DNA bands. The PCR products were evaluated using RedGel dye and visualized and photographed using a UV transilluminator.

**Spectrophotometric analyses**

We usedThermo Scientific NanoDrop™ One Spectrophotometer (Thermo Scientific, Germany) machine to determine sample quality and quantity of DNA ratio at 260–280 nm (A260/A230 ratio). These were measured using 2 μL of each sample. We measured each sample five times.

**Results**

**DNA quality and quantity assessment**

The purification of extracted DNA samples was evaluated using a NanoDrop instrument. In this study, the ratio of 260/280 was in a range of 1.52–2.01. The DNA extracted should be free of contaminants, such as phenols, salt, and polysaccharides. These compounds affect the purification and quantity of DNA. The stem part produced DNA with purity ratios in a range of 1.79–1.82, whereas the purity ratio of DNA extracted in the fruit part was between 1.98-2.01. (Table 1).

Approximately 20-200 ng/uL of DNA concentration is necessary for the polymerase chain reactions. The DNA concentration for the stem part was in a range of 60-66 ng/uL, whereas for the fruit part, it was in a range of 18-21 ng/uL.

The NanoDrop device measured the amount of total protein extracted from the samples (Fig.1). The results indicated that total protein in the stem and fruit parts differed. Furthermore, we observed a higher total protein in the fruit part than in the stem part.

**PCR amplification for DNA detection**

The DNA of a fresh sample of *C. limon* used for 26S rDNA primer for PCR amplification. The size of the fragment was approximately 680 base pairs (Fig. 2), consistent with the expected result*.* The result showed a clear band in the PCR products for leaf and stem parts. However, the amplified bands obtained from the fruit part were not neat and clear compere to the leaf and stem parts.

**Discussion**

For having high-quality DNA, the ratio of 260/280 nm must be 1.8. A purity ratio higher than 1.9 indicates the presence of RNA in the extracted DNA sample (Abdel‑Latif and Osman 2018). However, in the fruit part, the ratio of 260/280 was more than 1.9. In some samples of DNA extraction by the leaf, the ratio was less than 1.7. The result showed the presence of proteins in those samples.

This study showed that the quality of DNA extraction from leaves, stems, and fruit was different. This is because the leaves contain high polysaccharide levels; therefore, they cannot yield high-quality DNA. Cheng *et al.* (2003) showed woody fruit crops such as citrus have high polysaccharide levels. For this crop type, extraction of DNA from the leaves needs some special treatment. Angel *et al.* (2014) showed the best method for extracting DNA from citrus leaves; removing polysaccharides from the leaves. So the presence of polysaccharides on the leaves was a challenge for extracting DNA. The presence of polysaccharides was the main reason in this study that we could not achieve high-quality DNA from the leaves.

In citrus fruits, we have phenolic compounds that include flavonoids and phenolic acids. Flavonoids are secondary plant metabolites, and phenolic acids occur mainly as caffeic acid, chlorogenic acid, ferulic acid, sinapic acid, and ρ-coumaric acid (Wang *et al.* 2007). All these compounds in the extract solution can interfere with DNA analysis and inhibit PCR processing. In this study, the main reason that we could not have high-quality DNA from the fruit, and even in PCR processing, we had not cleared the line in electrophorese gel was these compounds. In this study, the results showed that the part of the stem was the best part for extracting DNA. A small part of the fresh stem gave us enough DNA for PCR processing.

Most of the study on DNA extraction from the plant is based on the CTAB method. Hence in this study, we used the Chelex method, which was easy to use and cheap. Several studies reported Chelex-100 method was better than the CTAB method (Turan *et al*. 2015). In addition, they said the purity of DNA isolated using the Chelex method was better than the CTAB method (HwangBo *et al.* 2010; Liu *et al.* 2015). On the other hand, the Chelex method was simple, efficient, non-toxic, inexpensive reagents, and required only a few steps (Sajiba *et al*. 2017; **Singh *et al.* 2018**). Therefore, the result obtained in this study agrees with the result obtained by several studies in this field.

Therefore, in this study, we tried to use the Chelex method for extracting DNA from different parts of *C. limon*. Generally, in other parts of *C. limon* the quality of DNA extraction was different. In the present study, extracting DNA from fruit failed, but we had suitable quality DNA in the leaves and stem. Furthermore, the PCR analyses showed that the DNA samples in different parts of *C. limon* could successfully use PCR amplification with an rDNA primer except for the fruit part.

**Conclusion**

Most plant DNA extraction has been done based on the CTAB method. Hence in this study, we used the Chelex method, which was simple, efficient, non-toxic, inexpensive reagents and required only a few steps. Therefore, in this study, we tried to use the Chelex method for extracting DNA from different parts of *C. limon*. Generally, in different parts of *C. limon* the quality of DNA extraction was different. We had DNA from all parts of the lemon tree, but the stem and leaves gave us better results regarding high-quality DNA. Therefore, the Chelex method could be used for genetic diversity and phylogenetic purposes and is highly recommended for DNA extraction from *C. limon,* especially in the stem part.

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**Author Contributions**

Mehrnoush Aminisarteshnizi designed, carried out the experiments, analyzed data, and wrote the manuscript.

**Conflict of Interest**

The author declares that there is no conflict of interest.

**Data Availability**

The author confirms that the data will be provided with a fair request to the corresponding author.

**Ethics Approval**

Not applicable to this paper.

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**Table 1:** Mean DNA extraction obtained for all samples from Leaf, Stem and Fruit (Mean±SE) in lemon.

|  |  |  |  |
| --- | --- | --- | --- |
| Sample Name | Nucleic Acid(ng/uL) | 260/280 | 260/230 |
| Leaf1 | 31.59±2 | 1.59±0.01 | 0.54±0.01 |
| Leaf2 | 24.99±3 | 1.57±0.02 | 0.58±0.01 |
| Leaf3 | 28.8±4 | 1.62±0.01 | 0.55±0.02 |
| Stem1 | 60.00±2 | 1.81±0.02 | 0.71±0.01 |
| Stem2 | 66.84±3 | 1.79±0.03 | 0.73±0.01 |
| Stem3 | 62.7±1 | 1.82±0.01 | 0.74±0.02 |
| Fruit1 | 19.13±4 | 1.98±0.02 | 0.69±0.01 |
| Fruit2 | 21.38±5 | 2.01±0.01 | 0.54±0.03 |
| Fruit3 | 18.82±2 | 1.99±0.03 | 0.48±0.02 |



**Fig. 1:** Total protein (mg/ml) obtained for all samples extracts in Lemon



**Fig. 2:** Agarose gel electrophoresis of rDNA-26S primers in Leaf (1,2,3), Stem (4,5,6) and Fruit (7,8,9) for Lemon.