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# Determination of efficient DNA recovery method in *Pyracantha coccinea* for use in forensic botany

**Tuğba ÜNSAL SAPAN[1*], Elvan ACARKAN[2], Nurdan SANDIKÇI[3]**

[1,3]*Üsküdar University, Institute of Addiction and Forensic Sciences, 34662, İstanbul, Türkiye*
[1,2,3]*Üsküdar University, Faculty of Engineering and Natural Sciences, Department of Forensic Sciences 34662, İstanbul, Türkiye*
**tugba.unsalsapan@uskudar.edu.tr, [2]elvanacarkan15@gmail.com, [3]nurdan.sandikci@uskudar.edu.tr*

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# *Pyracantha coccinea*'da etkili DNA geri kazanım yönteminin belirlenmesi ve adli botanikte kullanımı

**Abstract:** Forensic Botany aims to uncover the connection between crime scenes, perpetrators, or victims using plant materials. Since the presence of metabolites in plants varies among different species, DNA isolation methods should be adjusted specifically for each plant species and even each plant tissue. In this study, the objective was to determine the most suitable DNA isolation method for the fruits of firethorn (*Pyracantha coccinea* M.Roem.) growing in Türkiye without using liquid nitrogen. In the study, the fruits of Firethorn were divided into two groups as fresh (12 pieces) and dried (12 pieces), which each fruit was subjected to different homogenization methods using a single sample for each analysis. After completing the DNA isolation of the samples, the DNA quantities were measured to determine which method yielded optimal results. The highest DNA quantity of 4.80 ng/µl was obtained from dried fruits homogenized using a mortar and tissue lyser with beads, while the lowest DNA quantity of 0.13 ng/µl was obtained from fresh fruits homogenized only with a kitchen blender. The findings of the study indicate that for both fresh and dried fruits, homogenization with a mortar and tissue lyser with beads would result in a high recovery of DNA from plant materials. This study provides an optimization for DNA isolation in forensic cases where the Firethorn plant may be involved.

**Key words:** Forensic sciences, forensic botany, plant DNA extraction, homogenization methods, *Pyracantha coccinea* (Firethorn)

**Özet:** Adli Botanik disiplini bitki materyallerini kullanarak olay yeri, fail veya kurban arasındaki bağlantıyı ortaya çıkarmayı hedeflemektedir. Bitkilerde bulunan ikincil metabolitlerin varlığı farklı bitki türlerinde değişiklik gösterdiğinden DNA izolasyon yöntemleri her bitki türüne hatta her bitki dokusuna özel olarak ayarlanmalıdır. Çalışmamızda, Türkiye'de yetişen Ateş Dikeni (*Pyracantha coccinea* M.Roem.) bitkisinin meyveleri için sıvı nitrojen kullanılmadan en uygun DNA izolasyon metodunun belirlenmesi amaçlanmıştır. Çalışmamızda, ateş dikeni bitkisinin meyveleri taze (12 adet) ve kurutulmuş (12 adet) olarak iki gruba ayrılmış ve her analizde tek meyve kullanılarak farklı homojenizasyon yöntemlerine tabi tutulmuştur. DNA izolasyonu tamamlanan örneklerin DNA miktarları ölçülerek hangi metodun optimum sonuç verdiği belirlenmiştir. En yüksek DNA miktarı 4,80 ng/µl olarak havan ve boncuklu doku öğütücü ile homojenize edilen kurutulmuş meyvelerden elde edilirken, en düşük DNA miktarı ise 0,13 ng/µl olarak yalnızca mutfak robotu ile homojenize edilen taze meyvelerden elde edilmiştir. Çalışmamızın bulguları hem taze hem de kurutulmuş meyveler için, homojenizasyonun havan ve boncuklu doku öğütücü ile gerçekleştirilmesi halinde bitki materyallerinden yüksek miktarda DNA geri kazanımı olacağı yönündedir. Yaptığımız çalışma, Ateş Dikeni bitkisinin dahil olabileceği adli vakalarda DNA izolasyonu için optimizasyon sunmaktadır.

**Anahtar Kelimeler:** Adli bilimler, adli botanik, bitki DNA izolasyonu, homojenizasyon metotları, *Pyracantha coccinea* (ateş dikeni)

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## 1. Introduction

Forensic botany is one of the sub-disciplines of forensic sciences that examines various aspects of plants, such as their morphological, anatomical, chemical, and genetic characteristics. DNA can be obtained from the leaves, stems, roots, seeds, flowers, and fruits of plants. Just as identification can be performed for human DNA, it can also be done for animal and plant DNA. The identification of plant materials found at a crime scene can directly link a suspect to the crime scene or the victim. After identifying plant species by examining the morphological and histological features of plant materials, DNA analysis is conducted to perform identification.

In addition to identifying plant specimens, determining which region they belong to, and whether a crime scene is a primary or secondary location, are also within the scope of forensic botany (Aquila et al., 2014). In this context, forensic botany has long held its place within forensic sciences (Craft et al., 2007; Wickneswari and Rajanaidu, 2011).

The first step in examining plant samples is to identify the species. However, since the morphology and anatomy of plant samples obtained from a crime scene are often fragmented or deteriorated, species identification is not always possible. In such cases, the examination should be supported by molecular biology analysis methods (Aquila et al., 2014). In the analysis of plant samples, it is common to encounter the issue of the samples being present in limited quantities as well as the tissue integrity being

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compromised. Therefore, the analysis must be conducted with great care. Additionally, since there is no specific identification procedure for each plant species, researchers apply the general protocols provided in the isolation kits or the methods they use in their laboratories. Consequently, using protocols specifically developed for plant species will ensure optimal results in DNA analyses. In the study, which aims to address this deficiency, it was focused on the initial steps of identification (DNA isolation and quantification) using a selected plant with specific parameters.

In this study, the fruits of the Firethorn (*Pyracantha coccinea*) shrub, an evergreen plant with red-orange berries and thorny stems, commonly used for ornamental and security purposes in Atatürk Arboretum Garden borders were used. The plant's perennial and shrubby-thorny structure, which makes it easy to transfer and spread widely, has been an important factor that increases the probability of its presence at the crime scene, and for this reason it was chosen as material in this study (Akguc et al., 2010). The fruit samples of each plant were divided into two groups, fresh and dried, and different methods were used in DNA isolation to investigate which method provided the highest DNA yield. The quantities of the isolated DNA samples were measured, and the results were compared to gain perspective for method optimization. This way, the Firethorn shrub, which can easily grow, will illuminate the path for cases involving theft, murder, sexual assault, etc., with its thorny stem and small fruits.

After analysing the plant species, homogenization, which involves physically breaking down the cell walls found in plants, is required for DNA isolation. Among the commonly used methods in the homogenization step is grinding with liquid nitrogen. However, in this study, an alternative method using a bead tissue grinder and a kitchen blender without liquid nitrogen was used, and the results were examined.

## 2. Materials and Method

In this study, fruits of the Firethorn (*Pyracantha coccinea*) plant, an evergreen plant with thorny stems and striking orange-red berries that can easily grow in forested areas, found in various regions of Türkiye, were used.

In this context, Firethorn fruits were collected from 12 different shrubs in the province of İstanbul. Of the 12 plants that formed the sample of this study, five were collected from the Atatürk Arboretum in Sarıyer, İstanbul, which provides a reliable resource for educational and scientific research, and the remaining seven were collected from the surroundings of Üsküdar, İstanbul, where the species were identified according to morphological parameters. The fruit samples, carefully collected to prevent contamination, were placed in different paper envelopes, sealed, and transported to the laboratory with ice packs. The fruit samples collected from each shrub were divided into two groups for analysis as fresh and dried in room conditions (24 ºC, %60 humidity) and subjected to different storage conditions. The samples to be studied fresh were stored at +4ºC and analysed within 48 hours of collection, while the dried samples were analysed 12 days after collection, allowing for air circulation and protection from sunlight, with minimal contact.

For each sample to be isolated, the starting material was adjusted to a single fruit (~100 mg). The first stage of the isolation step, homogenization, was performed using a mortar, kitchen blender/hand blender (Sinbo, İstanbul-Türkiye), and bead tissue grinder (TissueLyser LT Qiagen, Hilden-Germany) without liquid nitrogen. Half of the fresh fruits were ground in a mortar and pulverized in a bead tissue grinder according to the "Purification of DNA from Plant Tissues" protocol found in the device manual, while the other half was homogenized using only the kitchen blender (QIAGEN® Technologies, 2009). For dried fruits, the samples that were ground in the blender were also ground in a mortar. The DNA of the samples homogenized with different combinations was isolated using the "Fruit" protocol contained in the i-genomic Plant DNA Extraction Mini Kit (iNtRON Biotechnology, 2012). In order to avoid contamination, the blender and mortar were sterilized by keeping them under bleach and UV light after each sample process.

The DNA concentration of the isolated samples was determined using the Qubit 4 Fluorometer (Invitrogen, Massachusetts-United States) device according to the protocol included in the Qubit™ dsDNA HS Assay Kit (Thermo Fisher Scientific Inc, Massachusetts-United States) (Thermo Fisher Scientific Inc., 2022) (Figure 1).

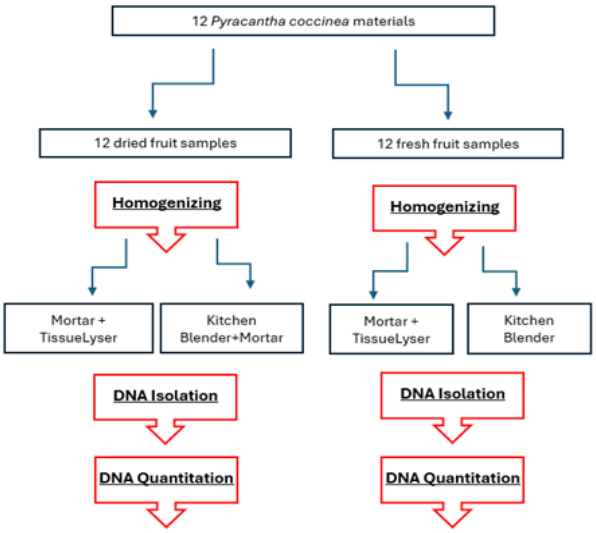


**Figure 1.** Flow chart of the study.

For statistical analysis Wilcoxon Signed-Rank Test in SPSS Statistics for Windows, version 20.0 (SPSS Inc., Chicago, Ill., USA) program was used to compare sample results.

## 3. Results

To find the most suitable DNA isolation method in this study, fruits taken from each plant were divided into two groups: fresh and dried (12 days) and subjected to different homogenization steps (mortar grinding, bead tissue grinder grinding, and blending). A single fruit was used for each analysis, and DNA isolation was performed on a total of 24 fruits. After the DNA isolation of the fruits was completed, DNA quantification was performed using the Qubit 4 Fluorometer (Invitrogen) device according to the Qubit™ dsDNA HS Assay Kit (Invitrogen) manual, and the results were compared.

Table 1 presents the DNA quantification results of the fruit samples, for which DNA isolation was performed on fresh material.

The DNA quantification results of the fruit samples, which were dried and had DNA isolation performed 12 days after collection, are shown in Table 2.

**Table 1.** Summary of DNA Quantities Obtained from Fresh Firethorn Fruits.

| | | Sample | DNA amount (ng/µl) | Mean value | Standart deviation |
|---|---|---|---|---|---|
| Fresh fruit samples | Mortar + Tissue lyser | 1 | 0.28[b] | 0.63 | 0.40 |
| | | 2 | 0.45 | | |
| | | 3 | 0.70 | | |
| | | 4 | 0.58 | | |
| | | 5 | 0.36 | | |
| | | 6 | 1.39[a] | | |
| | Kitchen blender | 7 | 0.26 | 0.35 | 0.20 |
| | | 8 | 0.64[a] | | |
| | | 9 | 0.19 | | |
| | | 10 | 0.50 | | |
| | | 11 | 0.13[b] | | |
| | | 12 | 0.38 | | |

*[a]= maximum value, [b]= minimum value*

**Table 2.** DNA Recovery from dried firethorn fruits.

| | | Sample | DNA amount (ng/µl) | Mean value |
|---|---|---|---|---|
| Dried fruit samples | Mortar + Tissue lyser | 1 | 2.32 | 3.23 |
| | | 2 | 4.80[a] | |
| | | 3 | 4.43 | |
| | | 4 | 1.55[b] | |
| | | 5 | 3.49 | |
| | | 6 | 2.77 | |
| | Kitchen blender + Mortar | 7 | 1.59 | 1.25 |
| | | 8 | 1.35 | |
| | | 9 | 1.07 | |
| | | 10 | 2.08[a] | |
| | | 11 | 1.02 | |
| | | 12 | 0.363[b] | |

*[a]= maximum value, [b]= minimum value*

The DNA amounts obtained as a result of DNA isolation using different methods on fresh and dried fruits of firethorn plant are shown comparatively in Figure 2.

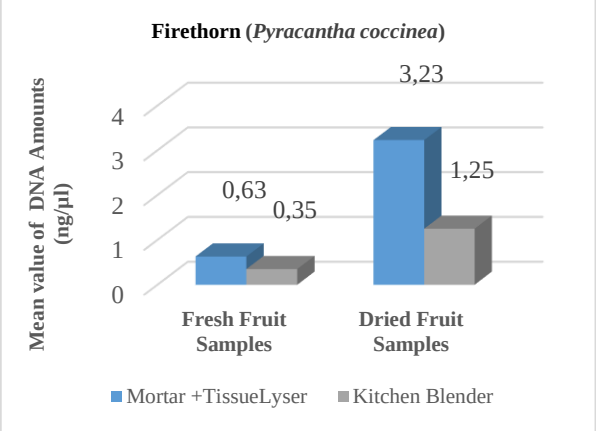


**Figure 2.** Comparison chart of DNA quantities from fruit samples.

The methods used in the preparation of dried and fresh fruit samples were evaluated among themselves using the Wilcoxon Signed-Rank Test in SPSS Statistics for Windows, version 20.0 (SPSS Inc., Chicago, Ill., USA). As a result of the test, insignificant results were obtained with a value of 0.1116 (>0.05) in fresh samples and significant results were obtained with a value of 0.046.

**Table 3.** Wilcoxon Signed-Rank Test results

| Test statistics | Method comparison between fresh samples | Method comparison between dried samples |
|---|---|---|
| Z | -1,572a | -1,992a |
| Asymp. Sig. (2-tailed) | 0,116 | 0,046 |

## 4. Discussions

Forensic botany, one of the subfields of forensic sciences, is not yet the primary discipline used in solving forensic cases, but new developments and approaches in this field are emerging daily.

Plant materials (e.g., leaves, fruits, seeds) frequently play a role in criminal cases such as murder, theft, and sexual assault due to their unnoticed presence. This places a responsibility on investigators to first detect and identify the plant species, and then conduct genetic analysis to obtain definitive results. Like human DNA, plant DNA can be analyzed, but the process begins with DNA isolation. Isolating pure, high-quality DNA from plants is critical, though often difficult due to contamination from secondary metabolites. Therefore, DNA isolation methods must be tailored to each plant species and tissue (Sahu et al., 2012).

Unlike humans, plant cells have a cell wall composed of cellulose. Therefore, the cell wall must be mechanically broken down to isolate DNA from plant samples. This step, known as homogenization, is typically performed using liquid nitrogen according to protocols in DNA isolation kits. However, liquid nitrogen can be problematic due to its difficulty of use, cost, and it's not being available in every laboratory. Additionally, the protocol in the DNA isolation kit manual suggests using 100 mg of fruit samples that have been lyophilized with liquid nitrogen (iNtRON Biotechnology, 2012). However, fruits collected from crime scenes or victims may not be fresh or available in the desired quantity. Therefore, alternatives to address potential issues should be developed, and method optimization for DNA isolation from the easily cultivated Firethorn (*Pyracantha coccinea*) plant was aimed in this study. This study is one of the pioneering works in DNA recovery for *Pyracantha coccinea* species.

The main factors affecting DNA quantities in this study are the homogenization methods used and the amount of starting material.

For fresh fruits, the highest DNA concentration obtained from samples homogenized with a mortar and bead mill was 1.39 ng/µl, while the highest concentration from samples homogenized with a household blender was 0.643 ng/µl. This difference is attributed to incomplete homogenization of samples processed with the blender and the bead mill's superior homogenization compared to the blender. For dried fruits, samples processed with the blender were additionally homogenized with a mortar, closing the gap in this stage. In order to avoid

contamination, the blender and mortar were sterilized by keeping them under bleach and UV light after each sample process.

For dried fruits, the highest DNA concentration obtained from samples homogenized with a mortar and bead mill was 4.8 ng/µl, while the highest concentration from samples using a household blender and mortar was 2.08 ng/µl. Using the mortar in both homogenization methods provided an opportunity to compare the bead mill and blender. The contribution of grinding larger pieces from blender-processed samples in the mortar supported homogenization, though it also resulted in minimal tissue loss, which likely had a slight effect on DNA quantities. It was observed that the DNA amounts obtained were suitable for the next step, PCR, for identification (QIAGEN®, 2010).

As an alternative method, there are studies showing that homogenization with the help of a blender can be performed for a wide range of plants and that these methods lead to good performance (Guillemaut and Maréchal-Drouard, 1992). However, as observed, this is not the case for Firethorn berries. It was not found to be an efficient method for plant samples with softer tissues, such as firethorn berries. Supporting our conclusion, another study reported that leaf tissues can be mechanically disintegrated, typically by grinding or beading for homogenization. However, it has been reported that too much mechanical force is applied in these methods, cutting the DNA and reducing its yield and quality. It has also been reported that mechanical force applied to plant samples causes DNA molecules to break into smaller fragments (Couch and Fritz, 1990).

For each DNA isolation, a single fruit of the Firethorn plant was used without measuring the quantities. The manual of the isolation kit suggests that after lyophilizing and grinding the fruit samples in a mortar, a starting amount of 100 mg dry weight is used. However, in this study, although the weight of a single fresh fruit was approximately 100 mg, it contained a high amount of water, resulting in a lower dry weight of the starting material. Additionally, although dried samples were worked with smaller starting material amounts, the DNA quantities yielded better results. These findings suggest that dried samples have considerable potential for DNA isolation.

### 4.1. Sufficiency of obtained DNA quantities for amplification

The minimum DNA amount required for plant amplification varies depending on the specific application and the quality of the DNA. A balance must be established between the amount of template DNA and the required degree of amplification. A very low amount of template DNA will require more amplification cycles, increasing the chance of errors. On the other hand, a high amount of template DNA can result in low yield and "dirty" PCR with many non-specific amplifications.

Uchiyama et al. (2013) used 5 ng DNA in their study to develope 32 EST-SSR markes for *Abies firma.*

Another study involving maize, tomato, and soybean used GenomePlex® Whole Genome Amplification (WGA) Kit with 1 ng/µl DNA concentrations for whole genome amplification, resulting in PCR products with an average length of 400 bp (Sigma-Aldrich, 2024).

According to QIAGEN® Multiplex PCR Handbook; the kit optimized at DNA concentrations between 1 ug and 1 ng (QIAGEN®, 2010) .

Based on the information provided and the referenced studies, there are no established standards for DNA amplification in plant samples. It is suggested that an amplification method should be used depending on each plant species, quantity, quality of the DNA obtained, and the intended analysis. With the DNA concentrations obtained from this study, it was concluded that a partial profile, which contains only a subset of genetic markers, could be obtained from some of the fresh samples and a full profile, which refers to the complete set of genetic markers used to uniquely identify an individual plant, from all dried samples using the GenomePlex® Whole Genome Amplification (WGA) Kit.

The increasing research in forensic botany and the significant role of plant materials in solving forensic cases are well-recognized. Techniques and methods are continuously evolving to enhance the usability of this field. Examples of these developments include genetic maps for plant origins, protocols specifically developed for plant species, and C-value databases created for plant DNA (Leitch et al., 2019).

Additionally, the chloroplast genome of *Pyracantha fortuneana* was analyzed using high-throughput sequencing technology and high genome similarity was found between the red and white flower phenotypes. Four mutational hotspots were identified and proposed as potential molecular markers for *Pyracantha* species. This information is important for the molecular taxonomy of *Pyracantha* species and for forensic botanical applications (Ding et al., 2022).

In addition, in the study by Huang et al., *Hamaecyparis taiwanensis* individuals were identified with 99.99% confidence level using 30 SSR markers to detect illegal logging. This success of genetic marker systems emphasizes the importance of DNA profiling in forensic botany. (Huang et al., 2020).

In forensic botany, it is crucial not only to define the plant species morphologically and anatomically but also to utilize molecular biology and genetic analyses when presenting evidence to courts and prosecutors. Genetic analyses can be used to prove that plants are endangered or endemic species, determine the origin of plant materials, or confirm if plant samples match. These analyses are carried out by targeting specific regions of plant DNA, DNA sequences, genetic polymorphisms, or profile matches.

This study aimed to optimize the homogenization methods used in DNA isolation from Firethorn (*Pyracantha coccinea*) fruits.

When working with firethorn fruits, drying and then homogenizing in a mortar followed by bead mill homogenization and using silica-based spin column-based plant DNA isolation kits yields the highest concentrations of DNA. However, in cases with time constraints, working with fresh fruit samples is also feasible. By homogenizing fresh fruit samples in a mortar and then in a bead mill and using spin column-based plant DNA isolation kits, it is possible to obtain high concentrations of DNA.

This study provides an important perspective on method

optimization for DNA isolation of Fire Thorn species. In future studies, it is important to increase the number of samples and to examine the discrimination power between individuals by identification. In future studies, the study will be improved by increasing the number of samples and performing other identification steps.

**Conflict of Interest**

Authors have declared no conflict of interest.

**Authors' Contribution**

TUS designed the experiment, supervised and edited. EA conducted the experiment, prepared the article. NS revised and edited the article.

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