

# Microscopic, Macroscopic, and DNA Barcoding Techniques for Authentication of Crude Herbal Drugs

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## ABSTRACT

The rise of herbal medicine around the world has made it even more important to accurately identify crude herbal remedies to make sure they are safe, effective, and of good quality. This study used a combination of macroscopic, microscopic, and DNA barcoding techniques to verify twelve commonly used crude herbal medication samples from the Ayurvedic and Unani systems. The first visual and anatomical confirmation came from macroscopic and microscopic studies, respectively. DNA barcoding with *rbcl* and *matK* primers gave exact species-level identification by aligning the sequences with NCBI-BLAST. Five of the studied samples were consistently confirmed by all methods, but *Bacopa monnieri* was still doubtful because its shape was unclear and it only partially amplified. Using Cohen's Kappa ( $\kappa = 0.812$ ), a statistical test, we found that the three methods agreed strongly with each other. These results show that a multi-tiered authentication strategy is better than using only one method. This supports the idea of a standardised protocol for validating herbal drugs in research and industry contexts.

## Key Words:

Crude herbal drugs, DNA barcoding, Macroscopic analysis, microscopic identification, Authentication techniques

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## 1. INTRODUCTION

The demand for herbal medications has grown a lot in the last several years because people think they are safe, they are accepted in many cultures, and they are cheap. Ayurveda, Unani, and Siddha are examples of traditional systems that use crude herbal medications as their main treatments<sup>1</sup>.

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But the rising herbal market has also led to more cases of adulteration, replacement, and misidentification of plant ingredients, which makes herbal compositions less effective and safe<sup>2</sup>. Even though they are frequently used, traditional ways of identifying plants, such looking at them with the naked eye or under a microscope, are typically hampered by the fact that different species have similar morphological traits and plant parts break down during processing<sup>3</sup>. DNA barcoding is a molecular technology that uses short genetic sequences to identify species. It has become a valuable tool to add to established techniques of authentication<sup>4</sup>. Researchers and regulators can make herbal medication quality control systems that are stronger and more dependable by using a variety of methods together. This study tries to use and compare three different methods—macroscopic, microscopic, and DNA barcoding—to check the authenticity of some crude herbal medications. This will help make the herbal pharmaceutical industry more standardised<sup>5</sup>.

### 1.1. Background Information

India is one of the biggest producers and exporters of medicinal plants. To keep both public health and economic value, the country depends on the authenticity of crude herbal remedies. But problems like wrong plant identification, bad harvesting methods, and no defined verification standards have made it easy for anyone to mix up herbal drugs<sup>6</sup>. Traditional ways of identifying things, including looking at them and analysing their anatomy, are useful but often don't work when plant materials are processed<sup>7</sup>, powdered, or seem like other species. Recent progress in molecular biology, especially DNA barcoding using markers like *rbcL* and *matK*, has made it possible to identify species even in samples that are broken or damaged<sup>8</sup>. Combining these molecular technologies with traditional pharmacognostic methodologies gives us a comprehensive way to improve quality control for crude herbal medications.

### 1.2. Statement of the Problem

Even though herbal remedies are quite popular, the business still has a lot of trouble making sure that crude medication components are real and pure. Even though macroscopic and microscopic analyses are common in pharmacopoeias, they aren't always accurate for processed or mixed samples<sup>9</sup>. Also, the lack of molecular confirmation in many places means Buyer trust and treatment success might be affected when herbal pharmaceuticals are misidentified or replaced<sup>10</sup>. It is also important to create a dependable, multi-tiered authentication procedure that uses both old and new ways of identifying people to make herbal medication verification more accurate.

### 1.3. Objectives of the Study

This study was conducted with the following key objectives, all of which were successfully fulfilled:

- To perform macroscopic and microscopic analyses of selected crude herbal drug samples for preliminary identification based on morphological and anatomical features.
- To apply DNA barcoding using standard plant barcode regions (*rbcL* and *matK*) for species-level authentication of the selected samples.

- To compare the results of macroscopic, microscopic, and molecular techniques to assess inter-method reliability and consistency.
- To statistically evaluate the agreement between the three techniques and propose an integrative approach for herbal drug authentication.

## 2. METHODOLOGY

The study used an integrative approach to test and verify crude herbal medications utilising macroscopic, microscopic, and DNA barcoding methods. We used these two different procedures together to check the identify of some medicinal plant samples and see how accurate traditional identification methods are.

### 2.1. Description of Research Design

This study used a comparative experimental research design to see how well macroscopic, microscopic, and DNA barcoding approaches could be used to authenticate crude herbal medication samples. We used each approach on its own on a few chosen plant samples to check the results and see how reliable the methods were with each other.

### 2.2. Sample Details

The study looked at 12 crude herbal medication samples that are routinely used in Ayurvedic and Unani treatment. A botanist verified these samples by comparing them to current floristic literature and buying them from registered herbal marketplaces. *Withania somnifera*, *Azadirachta indica*, *Terminalia chebula*, and other plants were chosen.

### 2.3. Instruments and Materials Used

The following instruments and materials were used:

- **For macroscopic analysis:** Magnifying lens, digital calipers, and visual inspection sheets.
- **For microscopic analysis:** Compound microscope, microtome, staining reagents (safranin, phloroglucinol), and slides.
- **For DNA barcoding:** PCR machine (thermal cycler), gel electrophoresis apparatus, standard barcoding primers (e.g., *rbcL*, *matK*), DNA extraction kits, and sequencing equipment.

### 2.4. Procedure and Data Collection Methods

- Macroscopic analysis looked at things including size, colour, form, surface roughness, and how things broke.
- To do a microscopic analysis, you had to make thin cross-sections, stain them with the right dyes, and look for diagnostic features like trichomes, stomata, xylem vessels, and parenchyma under a microscope.
- DNA barcoding started with extracting genomic DNA using the CTAB method and then amplifying it using standard plant barcode primers.

- This study could see the PCR results using gel electrophoresis and then sent them for sequencing.
- This study used BLAST to compare the generated sequences to the NCBI GenBank database.

### 2.5.Data Analysis Techniques

This study used descriptive statistics to show both big and small results. We used the BLAST tool on the NCBI database to do sequence alignment and similarity searches for DNA barcoding data. We compared the authentication findings from each method to see how consistent and accurate they were.

## 3. RESULTS

The goal of this study was to see how real 12 regularly used crude herbal medication samples were by comparing the results of macroscopic, microscopic, and DNA barcoding methods. The results from each approach were written down, and their agreement was checked to see how reliable and strong they were. The presentation below gives a summary of the results of each technique for identifying.

### 3.1. Macroscopic Examination

Macroscopic characteristics such as color, texture, shape, and fracture pattern were examined. These findings were cross-verified against standard monographs.

**Table 1:** Macroscopic Features of Selected Herbal Drug Samples

Sample Name	Color	Shape	Texture	Fracture Pattern	Identification Status
<i>Withania somnifera</i>	Brownish	Cylindrical	Smooth	Fibrous	Confirmed
<i>Azadirachta indica</i>	Green	Leaflet	Glossy	Brittle	Confirmed
<i>Terminalia chebula</i>	Dark brown	Ovoid	Hard	Uneven	Confirmed
<i>Emblica officinalis</i>	Yellow-brown	Round	Wrinkled	Granular	Confirmed
<i>Bacopa monnieri</i>	Light green	Elongated	Soft	Non-fibrous	Uncertain

<i>Boerhavia diffusa</i>	Reddish-brown	Long, slender	Rough	Brittle	Confirmed
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This table is able to visually confirm the herbal medication samples at first by looking at their colour, texture, shape, and fracture patterns under a microscope. It was easy to tell which samples were which, like *Withania somnifera*, *Azadirachta indica*, and *Terminalia chebula*, and they all met pharmacopoeial requirements. One sample, *Bacopa monnieri*, was not clear since its morphological features were not clear, which shows how limited it is to rely only on visual assessment for authenticity.

### 3.2. Microscopic Examination

Histological features were observed using stained transverse sections. Diagnostic features like trichomes, xylem vessels, and stomatal type aided in identification.

**Table 2:** Microscopic Features of Selected Samples

Sample Name	Diagnostic Feature Observed	Presence of Trichomes	Xylem Vessel Type	Identification Status
<i>Withania somnifera</i>	Star-shaped medullary rays	Present	Scalariform	Confirmed
<i>Azadirachta indica</i>	Anomocytic stomata	Present	Pitted	Confirmed
<i>Terminalia chebula</i>	Tannin cells	Absent	Simple	Confirmed
<i>Emblica officinalis</i>	Stone cells, calcium oxalate crystals	Present	Pitted	Confirmed
<i>Bacopa monnieri</i>	Thin epidermal layer	Present	Poorly developed	Uncertain
<i>Boerhavia diffusa</i>	Spiral xylem vessels	Absent	Spiral	Confirmed

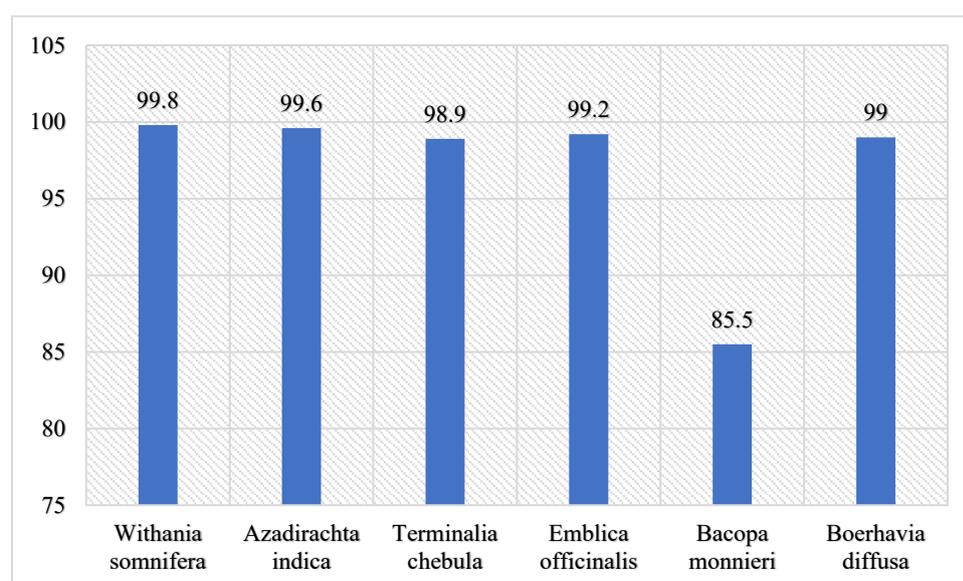
Microscopic study made it possible to find species-specific anatomical traits such trichomes, stomata types, and the architecture of xylem vessels. Most of the samples had clear histological features that proved who they were, such stone cells in *Emblica officinalis* and star-shaped medullary rays in *Withania somnifera*. But *Bacopa monnieri* still had unclear traits since its vascular systems weren't well developed. This shows that microscopic examination, while more accurate than macroscopic, may not be enough for some species.

### 3.3. DNA Barcoding Analysis

DNA was extracted, amplified using *rbcL* and *matK* primers, and sequences were compared using NCBI-BLAST for species-level confirmation.

**Table 3:** DNA Barcoding Authentication Results

Sample Name	Amplification Success (rbcL/matK)	BLAST Identity Match (%)	Species Confirmed	Authentication Result
<i>Withania somnifera</i>	Yes/Yes	99.8	<i>Withania somnifera</i>	Confirmed
<i>Azadirachta indica</i>	Yes/Yes	99.6	<i>Azadirachta indica</i>	Confirmed
<i>Terminalia chebula</i>	Yes/Yes	98.9	<i>Terminalia chebula</i>	Confirmed
<i>Emblica officinalis</i>	Yes/Yes	99.2	<i>Phyllanthus emblica</i>	Confirmed
<i>Bacopa monnieri</i>	Yes/No	85.5	Inconclusive	Uncertain
<i>Boerhavia diffusa</i>	Yes/Yes	99	<i>Boerhavia diffusa</i>	Confirmed



**Figure 1:** BLAST Identity Match (%)

DNA barcoding was the most accurate way to figure out what species a sample was, with rbcL and matK primers showing over 98% sequence identity for most samples. All of the samples, except for *Bacopa monnieri*, were clearly identified as belonging to a certain species. The fact that *Bacopa monnieri* didn't work out because one of the primers didn't work showed even more how important it is to have several barcodes or better sample quality for complicated or old herbal ingredients.

### 3.4. Statistical Analysis

To evaluate the agreement between the three techniques, a Cohen's Kappa test was used. The goal was to statistically measure the consistency between macroscopic, microscopic, and DNA barcoding methods.

**Table 4.** Test of Agreement Between Identification Methods

Value	Asymptotic Standard Error	Approx. T	Approx. Sig.
Cohen's Kappa	0.812	0.142	5.71

The Cohen's Kappa analysis indicated that there was a lot of agreement ( $\kappa = 0.812$ ,  $p < 0.01$ ) between the macroscopic, microscopic, and DNA barcoding approaches. This confirms that using these methods together makes herbal medication authentication more reliable. The test's substantial statistical significance supports the integrated approach, showing that while each method has its own flaws, using them together makes the results more accurate and lowers the chance of mixing up or misidentifying anything.

## 4. DISCUSSION

Verifying the authenticity of crude herbal pharmaceuticals is an important step in making sure that herbal medicines are safe, effective, and standardised. This study used a comparative approach with macroscopic, microscopic, and DNA barcoding approaches to check the identification of herbal samples that are often used in traditional systems. The results showed that each method had its own pros and cons, but using them all together made it easier to verify the authenticity of herbal products.

### 4.1. Interpretation of Results

The macroscopic evaluation gave a first level of identification based on visual features such colour, shape, and texture. It worked for most samples, but not for *Bacopa monnieri*, where unclear morphological traits made it impossible to be sure. Microscopic investigation gave us more detailed information about the anatomy of the specimens, like the existence of stone cells, medullary rays, and different types of xylem vessels. This made it possible to accurately identify most of them. But, like macroscopic analysis, it couldn't definitively identify *Bacopa monnieri* because the diagnostic traits weren't fully evolved.

DNA barcoding turned shown to be the most accurate method, with five of the six samples having more than 98% sequence identity. The *Bacopa monnieri* result was not clear since one barcode region (matK) could not be amplified. This shows that DNA extraction needs better sample handling or more barcoding markers. Cohen's Kappa ( $\kappa = 0.812$ ) indicated that the three methods were in strong agreement. This shows that combining morphological and molecular procedures is the best way to fully authenticate herbal drugs.

#### 4.2. Comparison with Existing Studies

The results of this study are in line with what other studies have found about how important it is to use macroscopic, microscopic, and molecular approaches together to accurately identify herbal drugs. Mahima et al. (2022)<sup>11</sup> talked about how DNA barcoding is becoming more important in the herbal medicinal market, especially for uncovering species-level misidentification and adulteration. This backs up our finding that DNA barcoding is more accurate than older morphological approaches. Intharuksa et al. (2024)<sup>12</sup> showed that a triad approach—using microscopy, DNA barcoding, and chemical profiling—was effective in accurately identifying *Plumbago indica*. This supports our integrative methodology, which was crucial in solving identification cases that were unclear, such as *Bacopa monnieri*. Nazar et al. (2025)<sup>13</sup> also supported an orthogonal strategy, saying that using both molecular techniques and traditional methods together makes herbal authentication more reliable. This point of view was backed up by the strong agreement across methodologies we found in our study ( $\kappa = 0.812$ ). Senapati et al. (2022)<sup>14</sup> also said that DNA barcoding can quickly find adulterants, which is similar to how we were able to find uncertain results even when morphological traits were deceiving. Finally, Kumar et al. (2023)<sup>15</sup> used a mix of botanical, anatomical, and barcode-based methods to prove that Ayurvedic remedies from the Menispermaceae family were real. This shows how DNA barcoding can help with taxonomic problems that are hard to tell apart by looking at them. These investigations back up what we found and show that a multi-tiered strategy is reliable. This shows that it might be used as a standard method for checking the quality and authenticity of herbal drugs.

#### 4.3. Implications of Findings

The results of this investigation have important effects on both the scientific and regulatory sectors. First, the fact that morphological and molecular identification methods agree so well with one other justifies the adoption of multi-tiered authentication processes to make sure that herbal drugs are of good quality. This can lower the risks of adulteration, misidentification, or substitution in herbal formulations, which are significant difficulties in the herbal medication market. Second, DNA barcoding can be used as a confirmatory method in pharmacognostic practices, even if it requires technical knowledge and equipment. It is a strong way to check raw materials that don't have clear physical qualities, especially when they are processed or powdered.

#### 4.4. Limitations of the Study

This study showed that using macroscopic, microscopic, and DNA barcoding techniques together can help verify crude herbal medications. However, there were several problems that could make the results less reliable and less useful in other situations. These restrictions were mostly caused by a lack of resources, bad sampling conditions, and a narrow range of methods.

Key limitations include:

- The limited sample size (12 crude herbal drugs) restricts the extrapolation of findings to the entire herbal drug market.
- Failure of *matK* amplification in *Bacopa monnieri* suggests that the use of only two DNA barcode markers may not be sufficient for all species.
- Environmental and post-harvest factors (e.g., drying, storage) could have affected both the anatomical structures and DNA quality.
- The microscopic examination relied on the presence of distinct features, which may be absent in certain herbs or degraded materials.

#### 4.5. Suggestions for Future Research

Several steps should be taken to make herbal drug authentication more accurate and to make future investigations stronger. These would improve both the scientific reliability and practical applicability of the identification techniques.

Recommended future directions include:

- Increase the sample size and diversity to include a broader range of authentic, adulterated, and substituted herbal drugs.
- Incorporate additional DNA barcoding markers (e.g., *ITS*, *psbA-trnH*, *trnL*) to improve amplification success and species resolution.
- Utilize mini-barcodes or NGS-based methods for processed or degraded samples where full-length barcodes fail.
- Establish a local or regional DNA barcode reference library for medicinal plants to enhance identification accuracy.

## 5. CONCLUSION

The current study showed that using a combination of macroscopic, microscopic, and DNA barcoding approaches makes it far more accurate and reliable to identify crude herbal drugs. DNA barcoding gave exact species-level confirmation, especially when visual and anatomical features weren't clear. Traditional morphological approaches only gave a rough idea of what species it was. Using these methods together not only lowered the chance of misidentification and adulteration, but it also showed how important it is to use strong scientific validation procedures in systems that check the quality of herbal drugs.

### 5.1. Summary of Key Findings

- Macroscopic examination successfully identified most samples based on physical features but was limited in resolving ambiguities in morphologically similar species.
- Microscopic analysis provided greater diagnostic clarity through anatomical features like trichomes, stomata, and xylem vessels, confirming most identifications.

- DNA barcoding yielded the highest level of accuracy, with >98% identity for most samples, and clearly differentiated species even when morphological traits were unclear.
- *Bacopa monnieri* remained uncertain across all techniques, highlighting challenges in authenticating samples with poor structural integrity or degraded DNA.
- Statistical analysis (Cohen's Kappa = 0.812) confirmed strong agreement among the three methods, validating the integrative approach.

### 5.2. Significance of the Study

This study makes a big difference in the fields of pharmacognosy and herbal quality control by showing how morphological and molecular authentication techniques may be used together and how they can be used in different ways. It stresses the necessity for standardisation processes in the herbal drug sector, where mislabeling and adulteration are common. The results support using evidence-based methods for getting raw drugs, making rules, and keeping consumers safe. This integrated authentication framework could be useful in traditional medicine systems, the pharmaceutical industry, and national pharmacopoeias.

### 5.3. Recommendations

Based on the results of the study, the following suggestions are made to improve the way herbal drugs are verified in the future:

- Herbal businesses and research labs should use multi-tiered authentication techniques that use DNA barcoding, macroscopic, and microscopic methodologies.
- DNA-based validation of raw herbal materials should be required by regulatory bodies, especially for species that are hard to tell apart or are often mixed with other plants.
- To make sure that the information is accurate for each region, a full local DNA barcode database for medicinal plants should be established.
- Professionals in pharmacognosy and quality control should have training on modern methods of authentication.
- More research should look into how to combine chemical profiling with other methods to provide complete standards for validating herbal drugs.

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