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Original Article

Determining Genetic Variability and Taxonomy of *Hibiscus Rosa-sinensis* Through Rbcl Molecular Marker

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ABSTRACT

Medicinal plants have been used in traditional medicine for a long time. These plants contain phytochemicals that have a variety of medicinal properties. However, accurate identification and authentication of medicinal plant species ensured their safety and efficacy. DNA barcoding using molecular markers has proven to be a useful method for plant species identification. The rbcL molecular marker was used for detailed characterization, amplification, and phylogenetic studies of *Hibiscus rosa-sinensis*. **Objective:** To evaluate the therapeutic properties and potential applications of *Hibiscus rosa-sinensis*. **Methods:** Samples of *H. rosa-sinensis* were collected, and DNA was isolated by the Doyle and Doyle method. The presence of DNA was confirmed by gel electrophoresis, and specific primers were used for PCR amplification. The PCR results were sequenced using next-generation sequencing techniques. After that, a neighbor-joining technique was used for phylogenetic analysis and to obtain pairwise nucleotide distances. Gel electrophoresis confirmed the presence of DNA in plant samples, and PCR amplification using rbcL primers generates successful amplification results. **Results:** The obtained sequence was 99.7% identical to the previously reported rbcL gene sequence from *H. rosa-sinensis*. Based on phylogenetic research, *H. rosa-sinensis* was discovered as a closely related species. **Conclusions:** The rbcL gene has been found as a viable molecular marker for *H. rosa-sinensis* identification and phylogenetic analysis. The results of this study demonstrated the therapeutic potential of *H. rosa-sinensis* and the importance of species identification in herbal medicine. DNA barcoding proved a reliable authentication and quality control technology in the herbal medicine business.

INTRODUCTION

Medicinal plants, known as medicinal herbs, have been used in traditional medicine since ancient times. These plants produce a variety of chemical compounds for various purposes, such as protection against insects, fungi, diseases, and herbivorous mammals [1]. Various potentially effective phytochemicals have been discovered. However, due to the wide range of phytochemicals in a single plant, using whole plants for medicinal purposes requires further investigation. Furthermore, thorough scientific research is required to evaluate the phytochemical content and pharmacological properties of many plants with medicinal potential to determine their efficacy and safety [2]. Medicinal plants are widely used in modern and traditional medicine to maintain health and treat specific conditions. According to Food and Agriculture Organization estimates, in 2002, the global use of medicinal plants exceeded 50,000 [3]. The use of medicinal plants has threefold benefits. First, they provide health benefits to those who use them medicinally. Second, they offer financial benefits to individuals who harvest, process and distribute these plants commercially. Finally, society benefits from medicinal plants through job creation, tax revenue and a healthy workforce [4]. The Malvaceae family is found in both tropical and temperate regions. About 125 Malvaceae species have been reported in Asia, belonging to 22 genera [5]. *Hibiscus rosa-sinensis*, commonly called China rose and belonging to the Malvaceae family, is a plant of crucial medicinal value. It is

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an effective remedy for wounds, inflammation, fever, cough, diabetes, bacterial and fungal infections, hair loss, and stomach ulcers in tropical regions [6]. Studies have shown that *Hibiscus* flowers have anti-tumor properties and can be used as analgesic, antipyretic, anti-asthmatic, and anti-inflammatory agents. Additionally, these flowers are rich in antioxidants and exhibit potent antimicrobial and antifungal activity [7]. Studies on various parts of *Hibiscus*, including extracts of its stems, roots, leaves and flowers, have revealed the health benefits of its phytochemical constituents. These ingredients exhibit antioxidant activity, effectively scavenging free radicals in the body that can potentially damage DNA [8]. Figure 1 shows the flower and plant perspective of *H. rosa-sinensis*, which is highly valued in the economy for its herbal and medicinal uses [9]

Figure 1: (a) Plant view (b) Flower view

Table 1: Morphological characteristics of *Hibiscus rosa-sinensis L.*

Common names	China rose, Shoe flower, Gudhal, Jasum, Gul gurhal taza [10]
Family	Malvaceae
Habitat	The world's tropics and sub-tropics regions range from 30 degrees north latitude to 30 degrees south latitude.
Distribution in Pakistan	It is extensively cultivated as an ornamental plant in tropical areas of Pakistan.
Worldwide Distribution	China, India, Nepal, Africa
Habit	Shrubs
Life form	Perennial
Flowering Period	Throughout the year
Morphological characters	The plant can grow to a height of 1-5m and has a woody stem with a globe shape. The **stem** has branches and is sparsely pubescent with simple dark hairs. The plant **leaves** are broadly lanceolate, serrate, lobed or ovate, with linear stipules 5-10 mm long and lanceolate. The **petiole** measures 0.5-2 cm. The plant's flower is axillary and solitary, with an erect or sub-pendulous side. The single or double pedicel is 1–8 cm long and conspicuous near the top. The flower centre can be yellow to purple, and the peduncle is 1–4 cm long, with a firm and dense fruit. The **calyx** of the plant is 5-lobed, tubular-campanulate, and 1.5-2 cm long. The shape can range from triangular to lanceolate. The **corolla** is 5-lobed and measures 4-9 cm. It can appear pink to red, with or without dark central petals. Petals are ovate or oblong, 5-9 cm long and 3-7 cm wide. The **stamen** is short or almost as long as the petals and has a broad base. The apex of the filaments is either truncate or 5-dentate, and the anthers are basally fixed, entire or at the apex. The **carpel** of the plant is superior, 5-locular to rarely 10-locular. It has axillary placentation with ovules that are 3 or more per locule. The **style** measures 1 or 5 branches apart, with a discoid-capitate or indehiscent stigma. The fruit of the plant is a capsule of globose-cylindrical shape with an apex that is apiculate, acute or acuminate. It fades locally and may be glabrous or hairy. The plant produces many reniform or subglobose seeds that may be smooth or hairy [11].
Ethnobotanical uses	This herb exhibits astringent properties and has a cooling effect. It has the ability to control bleeding, soothe irritated tissues and relax muscle spasms. The herb's flowers have astringent, aphrodisiac, and refrigerant properties. They are used internally to treat diseases such as cystitis, bronchial catarrh, excessive and painful menstruation, febrile illness, Intestinal diseases and cough. The flowers are also used for hair growth and as a cooling drink for sick people. Additionally, they work to treat mumps, fever, and wounds. The herb leaves have anodic, emollient, and laxative properties and are used externally as a lotion in treating fever [10].

The unscrupulous practice of compounding herbal medicines has eroded trust in recent decades. It involves mixing or replacing a specific medicinal plant with a morphologically similar plant that lacks therapeutic properties. Such unethical practices have led to declining the efficacy of herbal remedies and, consequently, losing faith in treatment methods [12]. Adulteration is a common problem in the herbal medicine industry that poses significant health risks to consumers and severely affects the industry. Medicinal plants are commonly purchased in markets and traditional herbal shops in various forms, such as dried leaves, roots and bark, and processed mixtures or extracts. To ensure accurate identification by retailers and customers, plant material must have appropriate morphological characteristics [13]. In the current literature, the term 'DNA barcoding' has emerged as a new name for a concept that has been used for a long time [14]. A quick and accurate species identification method involves using a standard DNA segment as a marker [15]. DNA barcoding is a globally recognised technique for species identification, which involves using specific regions of DNA and established protocols to generate a comprehensive database of organisms [16]. The importance of plant DNA barcoding is rooted in the need for accurate species identification, which is vital for plant conservation and utilisation. However, this process may require extensive taxonomic knowledge in multiple regions worldwide [17]. DNA barcoding can provide an accurate and reliable alternative to morphological identification for biological material and is often used when identification by macroscopic or microscopic methods is difficult [18]. This technology can differentiate and classify species at any stage of their development or processing as long as DNA extraction is possible [19]. DNA barcoding in plants is currently being applied in many applications. Different methods have been used to authenticate plant products, such as medicinal plants [18], kitchen spices [20], berries

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[21], olive oil [22], and tea [23]. Phylogenetic trees for community ecology are constructed using genetic sequences obtained by DNA barcoding [24]. Identification of different species depends on the use of specific regions of DNA. It is crucial to accurately document the laboratory procedures used during sample processing along with the primers used. End users can access DNA sequencing trace files and quality statistics [25]. GenBank and the Barcode of Life Data System (BOLD) are viable options for collecting DNA sequences because all data are publicly available. However, it is recommended to use bold for this purpose [17]. BOLD offers a comprehensive solution for project management by enabling the storage of DNA sequences and trace files [17]. The present study aims to create awareness about the importance of using natural medicinal plants, the prevalence of adulteration and the impact of the quality of certain medicinal plants on human health. Promoting the treatment and export of herbal plants can improve the economy and maintain the quality of a particular herbal medicine. This study provides resources for information on techniques used to detect the presence of adulterants in plants, such as DNA barcoding.

METHODS

Hibiscus rosa-sinensis plant was obtained from Minchin Abad Tehsil of Bahawalnagar district located at 30°05'53.1"N 73°37'28.4" E. Taxonomic expert Prof. Dr Muhammad Zia-ur-Rehman carried out the initial identification procedure using the available flora [26]. Collected plants were stored in zip lock bags carefully labelled with the location and date of collection, common name and identifier. The plants were then transported to the Plant Molecular Lab of the Department of Botany, Government Graduate College of Science, Lahore, where the leaves were stored in a -20°C freezer for further processing. DNA analysis was performed to detect any possible adulteration in the plant sample, which is the most accurate and sensitive method. Plant samples were processed for DNA analysis, and a protocol for admixture detection was developed based on the results. The Doyle and Doyle method was used for DNA extraction [27]. A sterile ice-cold pestle and mortar were used to crush 1 g of sample with liquid nitrogen to extract genomic DNA from plant samples. The resulting material was transferred to an Eppendorf, and 750 µl of Cetyltrimethylammonium Bromide (CTAB) was added. The mixture was then incubated in a water bath at 65 °C for 30–45 min. Then, a solution of equal volume of chloroform and isoamyl alcohol (24:1) was added to Eppendorf and centrifuged at 10,000 rpm for 15 min. The supernatant was then transferred to a new Eppendorf tube, and 750 µl of chloroform and isoamyl alcohol solution was added. The mixture was then centrifuged at 10,000 rpm for 15 min. This process was repeated three times, and the clear supernatant was collected in another Eppendorf. 2/3 volume of ice-cold isopropanol was added to each Eppendorf-containing supernatant to precipitate the DNA strands. The DNA strands were visualised as minute threads and left to Eppendorf overnight in a freezer at -20°C. The following day, the Eppendorf tubes were centrifugated at 10,000 rpm to allow the DNA strand to settle to the bottom as a pellet. The supernatant was discarded, ensuring the safety of the pellet. The pellet was washed three times with 200 µl washing buffer and then allowed to dry. Next, the DNA was dissolved in 50–70 µl of distilled water. Two methods were used to confirm the presence of plant DNA samples. DNA samples' concentration, purity and quantity were confirmed by measuring the absorbance at 260 nm using a nanodrop spectrophotometer (Thermo Scientific, Wilmington, USA). Finally, DNA samples were typically diluted 20 times (dilution factor) but varied based on DNA concentration. A Halo DB20 double-beam spectrophotometer model was used for DNA quantification, with TE buffer as the reference buffer. Additionally, optimizations were made according to the respective protocols [28]. Gel electrophoresis is a valuable technique for the identification of plant DNA. A 1% agarose gel was formed using 0.25 g agarose powder and 25 ml of 1X TAE buffer. The mixture was heated in a microwave oven for one minute until it became transparent and then cooled to 60 °C. Ethidium bromide was added to the liquid gel at a 0.5 µg/mL concentration. The liquid gel was then poured into a tray with two appropriately sized combs and allowed to solidify. The gel tray was then immersed in a gel electrophoresis tank containing 1X TAE buffer. The combs were carefully removed, and 10 µl DNA sample and 4 µl loading dye were mixed and loaded onto the gel with a 100 bp ladder (Thermo Fisher Scientific, USA). The gel was then run at 100 volts for 30 min and observed under UV illumination in a gel documentation system (Bio-Rad, USA), which captured images. The bands in the gel indicate the presence of DNA in the samples [29]. After DNA confirmation, PCR was used to amplify all DNA samples. Extracted genomic DNA extracts were prepared using the equation $M_1V_1=M_2V_2$ and double-distilled water. Highly efficient PCR techniques were used to amplify specific gene regions of plant DNA, including matK, rbcL, nrITS, and trnH-psbA, using a master mix and appropriately selected primers. PCR of the plant DNA sample was performed with appropriate primers [30]. Table 2 lists the primer sequences that were selected based on a thorough review of the literature.

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Table 2: Short Oligonucleotide Primers

Barcode	Primer	Primer sequence	Reference
matK	matK F	5′-TAATTTACGATCAATTCATTC-3′	[31]
	matK R	5′-CTTCCTCTGTAAAGAATTC-3′	
rbcL	rbcL F	5′-ATGTCACCACAAACAGAAAC-3′	[32]
	rbcL R	5′-TCG CAT GTA CCY GCA GTT GC-3′	
nrITS	nrITS F	5′-CCTTATCATTTAGAGGAAGGAG-3′	[33]
	nrITS R	5′-GGAAGTAAAAGTCGTAACAAG-3′	
trnH-psbA	trnH-psbA F	5′-GTTATGCATGAACGTAATGCTC-3′	[34]
	trnH-psbA R	5′-CGCGCATGGTGGATTCACAAATC-3′	

25 µl of the reaction mixture was aliquoted in PCR tubes. The PCR profile was set with an initial temperature of 94 °C for 3 min, an annealing temperature of 48 °C for 1 min, and an extension temperature of 72 °C for 10 min. All temperature settings were maintained to run for 40 cycles. The PCR product (5 µl) was mixed with 3 µl loading dye (5X) and run on a 1% agarose gel to confirm DNA amplification (Figure 2).

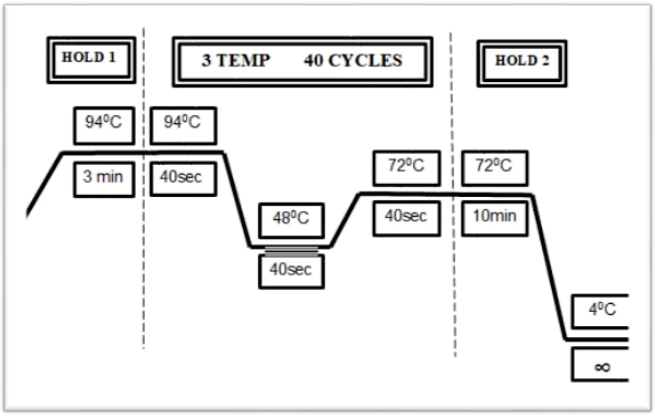

Figure 2: Steps of PCR with conditions

After DNA amplification, the next step involved purifying the plant DNA sample. To achieve this purity, distilled water was taken into the Eppendorf containing the DNA, resulting in a total volume of 100 µl. Equal volumes (100 µl) of the phenol and chloroform mixture were added to the same Eppendorf, and the entire mixture was inverted. Eppendorf tubes were centrifuged at 14000 rpm for 6 min to obtain the top aqueous phase of the solution, which was then transferred to a new tube. To further purify the DNA, 9 µl of a 3 molar sodium acetate solution (1/10th volume, pH 5.2) and 250 µl of absolute ethanol (2.5% volume) were added to Eppendorf and stored at -20 °C. But was left for 30 minutes. The tubes were centrifuged at 14000 rpm for 10 minutes to precipitate the DNA and extract ethanol. The DNA pellet was washed with 100 µl of 70% ethanol, centrifuged at 14000 rpm for 2 min, and the ethanol was removed. After isolation of the DNA pellet, it was essential to keep it dry and dissolve it in 20 µl of sterile distilled water (SDW). This method has proven to be highly effective in purifying plant DNA samples. To confirm DNA purification, 4 µl of DNA solution was loaded onto a 1% agarose gel. Purified PCR products were then sent to "CELEMICS BTSeq™, Seoul, Korea" for sequencing. The results were analyzed through a comparative study. Next-generation sequencing (NGS) was used instead of Sanger sequencing because it is more accurate and reliable. NGS is a massively parallel sequencing technique that offers extremely high throughput, scalability, and speed. Therefore, this technology was used to determine the sequence of nucleotides in the entire genome or targeted regions of DNA or RNA [35]. To identify closely related sequences, we performed a blast search on NCBI. For DNA analysis, we downloaded twelve adjacent sequences from plant samples. To perform phylogenetic analysis, we used the neighbor-joining method [36] with MEGAX software [37]. As part of the phylogenetic tree construction process, a bootstrap test was run with 1000 replicates.

RESULTS

DNA samples were analyzed by agarose gel electrophoresis, allowing the gel to run at 100 volts for 30 min. A gel documentation system (Bio-Rad, USA) was used to observe the gel under UV illumination, and the presence of bands in the gel confirmed DNA in the samples (Figure 3).

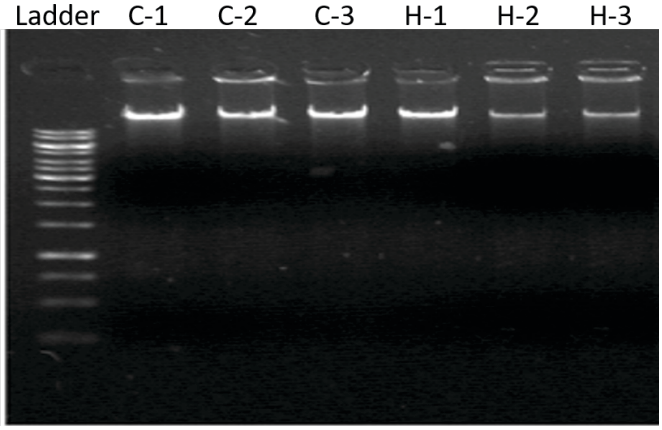

Figure 3: 1% Gel electrophoresis showing DNA profile of Plant samples

The DNA concentration of the plant samples was measured using a spectrophotometer at a wavelength of 260 nm, with distilled water as a reference. The concentration was calculated using the formula provided [28]. DNA concentration (µg/ml) = E × OD260 × dilution factor. (E is extinction coefficient = 50 for dsDNA) (Table 3).

Table 3: Concentration of DNA at 260 nm

Serial. No	Sample ID	OD at 260 nm	DNA in µg/mL
1	C-1	0.7276	1491.58
2	C-2	0.658	1348.9
3	C-3	0.655	1342.75
4	H-1	0.587	1203.35
5	H-2	0.606	1242.3
6	H-3	0.62	1271

After analysing the OD values at 260 and 280 nm wavelength using the formula Quality of DNA = OD_{260}/OD_{280},

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we can confirm the quality of the DNA. Usually the net ratio of DNA is about 1.8 [38]. However, if the ratio falls below 1.6, it may indicate the presence of contaminants, such as proteins or phenols, which absorb at or near 280 nm. Based on this, C-1 and H-1 were selected for further work [39], as shown in Table 4.

Table 4: Quality of DNA at OD 260/280

Sample	OD at 260	OD at 280	Ratio
C-1	0.7276	0.404	1.80
C-2	0.658	0.369	1.78
C-3	0.655	0.370	1.77
H-1	0.587	0.326	1.80
H-2	0.606	0.344	1.76
H-3	0.62	0.349	1.79

DNA barcoding primer pairs (matK, rbcL, nrITS, and trnH-psbA) were used to PCR the plant DNA sample on a Bio-Rad thermocycler [30]. In a 1% agarose gel electrophoresis procedure, 10 µl of the PCR product was used, and a band of the desired size was successfully obtained. Notably, it was observed that only the rbcL primer pair yielded positive results for the plant sample, as shown in Figure 4.

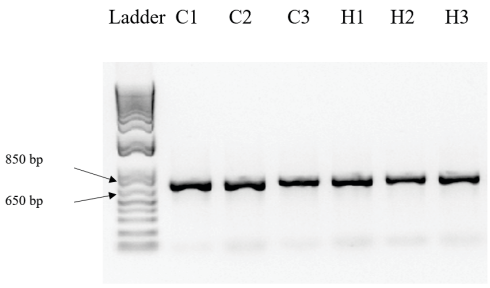

Figure 4: 1% Agarose Gel electrophoresis of PCR product representing the DNA bands of *Hibiscus rosa-sinensis*

The DNA sequences from the rbcL primer were edited and assembled through EditSeq software from DNA Star. Afterwards, these sequences were utilised to perform a blast on the NCBI database (https://www.ncbi.nlm.nih.gov/) to download related sequences. The evolutionary history of the sequenced sample was determined using the neighbor-joining method [36]. The muscle method in MEGA11 software was used to align multiple sequences for homology analysis. Pairwise nucleotide identities were calculated for phylogenetic analysis using MEGALIGN (DNA Star) software. The rbcL gene sequence from *Hibiscus rosa-sinensis* was found to have 99.7% pairwise nucleotide sequence identity with the previously reported rbcL gene sequence from *Hibiscus rosa-sinensis* [NC_042239], *Hibiscus rosa-sinensis* [MK382984], and 98.8 % *Hibiscus mutabilis* [MK820657] that was reported from Islamabad. Figure 5 illustrates these results.

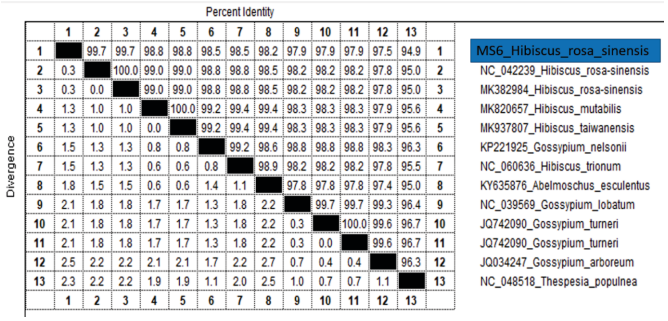

Figure 5: Sequence distancing of *Hibiscus rosa-sinensis*

DISCUSSION

Specimens of *H. rosa-sinensis* were systematically collected from different Punjab, Pakistan regions and were appropriately labelled with name, locality, and collection date. The samples were then stored in zip lock bags and sent in an ice box to the Molecular Biology Laboratory of the Government Graduate College of Science at Wahdat Road Lahore for morphological characteristics, including stems, leaves, flowers, seeds, and families. A standard protocol was used for DNA extraction, and confirmation was performed by 1% gel electrophoresis. The extracted DNA was further amplified by polymerase chain reaction (PCR) using primers including matK, rbcL, psbA-trnH, and ITS. In 2009, CBOL (The Consortium for the Barcode of Life) proposed that rbcL and matK be used as the standard plant DNA barcode consisting of two loci. Moreover, the psbA-trnH spacer and the nuclear internal transcribed spacer 2 (ITS2) are primarily functional [21, 40, 41]. The amplification of selected plants using the rbcL primer (750 bp) was successful, and the obtained amplifications were used for further processing. PCR products were purified, and nucleotides from the rbcL gene were sent to "CELEMICS BTSeq™, Seoul, Korea" for sequencing. Nucleotide sequencing was performed by next-generation sequencing. The rbcL gene has been identified as an efficient DNA barcoding tool for plants of the Malvaceae family, including *H. rosa-sinensis* [39]. The molecular marker rbcL gene has effectively identified processed plant products used in various fields such as food, medicine and cosmetics [42]. This information can significantly benefit experts who rely on accurately identifying plant products. The results agree with previously documented rbcL gene sequences, indicating a high level of consistency [39, 42]. Through an NCBI BLAST search on each sample sequence in GenBank, it was found that the closest matches to the same species were at 99% similarity. The rbcL gene sequence obtained from *Hibiscus rosa-sinensis* showed high similarity with previously reported sequences of the same species, with a percentage nucleotide sequence identity of 99.7%. Furthermore, it showed 98.8% similarity with the rbcL gene of *Hibiscus mutabilis*. The

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studied plants, particularly *H. rosa-sinensis*, were confirmed by NCBI BLAST and by examining different plants for their morphology. In addition, cross-matching was done by investigating different subjects. We analyzed pairwise nucleotide distances between selected plant species within and between species. The results showed that the homology percentage of nucleotides between different species was higher than within the same species. This allowed us to infer that a species exhibits high homogeneity with its closely related species. To develop a successful DNA barcode, it is essential to distinguish between intraspecific and intraspecific divergence [43]. Mitochondrial COI in animals shows more significant variation within species than between species, resulting in a 'barcode gap' that allows reliable species differentiation. However, the plastid DNA of land plant regions mark and rbcL do not provide the same 'barcode gap', especially in closely related species [44, 40]. A recent study successfully extracted DNA from seven infected samples and confirmed their presence by gel electrophoresis. Notably, three of these samples exhibited amplification by PCR reactions, specifically containing a beta-satellite component at 1.4 Kbps (S3, S4, and S6). Each of the three amplified samples was subjected to restriction enzyme assays with Bam H1, ECOR1, and Pst1, yielding different bp length results [45]. During our research, we analyzed intraspecific and interspecific sequence divergence. We observed a significant overlap of sequences, which did not require further analysis to identify a barcode gap. Although the barcode gap and distance method have been used to differentiate plant groups beyond the species or genus level, it is well documented that matK and rbcL barcodes differ among plants at the species level however, specific barcodes don't show the difference [46]. Cluster analysis was performed, and NJ trees were constructed with bootstrap analysis to assess the effectiveness of barcodes in distinguishing between species [47, 48]. The DNA sequence of the rbcL gene was used to construct a phylogenetic tree, which showed that *Hibiscus rosa-sinensis* (MS06) shared a clade with *Hibiscus rosa-sinensis* and *Hibiscus mutabilis,* whose bootstrap values are 99.7% and 98.8%. Our study demonstrated that the rbcL gene can correctly identify *H. rosa-sinensis*. The high discrimination efficiency of the gene for this species makes it a valuable barcode for identification purposes. Its short sequence length makes it an informative and powerful molecular tag for distinguishing weed and cultivated plant species.

CONCLUSIONS

The use of herbal products is widespread in various spheres of life. However, it is imperative to exercise caution and confirm their identity, as they can adversely affect human health. It is important to ensure that the herbal products are authentic and not fake, which can be used to fulfil personal goals. Molecular identification, especially DNA barcoding, can be a valuable technique to distinguish between plant species or plant-derived products, thus ensuring their efficient use in diverse fields.

Authors Contribution

Conceptualization: AI
Methodology: MZUR
Formal analysis: AI
Writing-review and editing: AI, MZUR

All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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