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Original Article

Characterization, Amplification, and Phylogenetic Analysis of *Gossypium herbaceum* using rbcL Molecular Marker

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ABSTRACT

People have used medicinal plants for centuries to produce traditional remedies that greatly interest modern health care. One of these plants, *Gossypium herbaceum* or commonly called Arabian cotton, has been used in various medicinal applications. Scientists are turning to DNA barcoding, a molecular technique that identifies species using standardized DNA regions. **Objective:** To evaluate samples of *Gossypium herbaceum* and their physical properties. **Methods:** DNA was extracted from the plant material, and its quality and quantity were checked. Using PCR and gel electrophoresis, amplification of the RBCL gene was done. Purification of the PCR products was done for DNA sequencing. After that, all the DNA sequences were compared with the available DNA sequences in public databases. The relationship between *Gossypium herbaceum* and other related species was evaluated using the neighbour-joining method for phylogenetic analysis. **Results:** The results showed a high percentage of pairwise nucleotide sequence identity with *Gossypium richmond* and *Gossypium hirsutum*. The study demonstrated the potential of DNA barcoding using the rbcL gene as a reliable method for identifying and confirming *Gossypium herbaceum*. Also, this study provides valuable insight into the phylogenetic relationships of this medicinal plant species. **Conclusions:** The findings support the conservation and appropriate use of medicinal plants and highlight the importance of ensuring the authenticity and quality of herbal products.

INTRODUCTION

For centuries, traditional medicine has used medicinal plants, or herbs, discovered long ago. Plants can produce a multitude of chemical compounds that serve various purposes, such as protection against insects, fungi, diseases, and herbivorous mammals [1]. Scientists have identified many phytochemicals that may or have already been shown to have biological activity. However, the effects of using an entire plant as a remedy are still being studied because a single plant contains a wide range of phytochemicals. Further, rigorous scientific research must assess many plants' phytochemical content and pharmacological actions with the medicinal potential to define efficacy and safety [2]. Maintaining health and treating certain conditions can be achieved through medicinal plants in modern and traditional medicine. According to the Food and Agriculture Organization, as of 2002, there are more than 50,000 documented uses of medicinal plants worldwide [3]. Medicinal plants provide three main benefits: health benefits to those who consume them as medicines, financial benefits to people who yield and distribute them for sale; and society-wide benefits, such as taxation income, and a better labour force [4].

Gossypium herbaceum is a cotton plant introduced in the native schemes of medicine. Flower and plant view of *G. herbaceum* is given in Figure 1.

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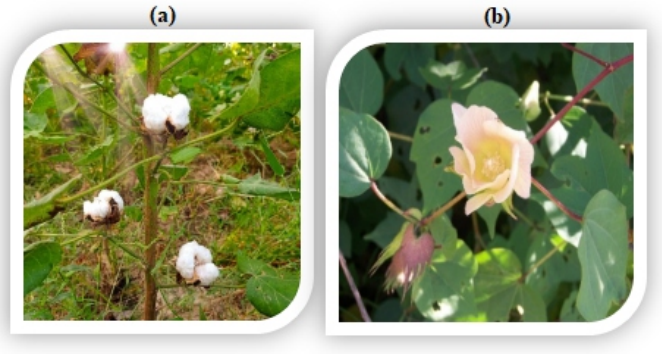

Figure 1: (a) Plant view (b) Flower view

Historically, this particular plant has been included in medicine and food preparation. Vitamin E can be found in cottonseed, which is used as a pain reliever, antioxidant and laxative. The root bark of the plant acts as an aphrodisiac, and a decoction made from it is used to treat amenorrhea [5]. In Unani medicine, leaves of *G. herbaceum* are useful in ishal al-atfal (childhood diarrhoea), and seeds are used to cure in qillatul laban [6]. The morphological characteristics of *Gossypium herbaceum* are given in Table 1.

Table 1: Morphological characteristics of *Gossypium herbaceum*

Common names	Cotton, Arabian cotton, Levant cotton [6]
Family	Malvaceae
Habitat	In tropical and subtropical regions
Distribution in Pakistan	Gossypium herbaceum is not a preferred choice for cotton production among cotton farmers in Pakistan, but it is occasionally cultivated in arid areas of Balochistan.
Worldwide Distribution	It is considered as native from Soviet Central Asia, and of adjacent Iran and Afghanistan.
Habit	Shrubby or herbaceous
Life form	Perennial
Flowering Period	Early July and blooms through August
Morphological characters	The plant can be a shrub or herb or annual, or perennial. Branches, petioles and pedicels may have stellate tomentose with or without common spreading hairs. The leaves are rounded in outline, measure 2-5 cm in length and width, and are glabrous above with a hairy margin. The lower nerves have stellate pubescence, which can sometimes be mixed with simple hairs. The Leaves are mainly deeply cordate and palmately (3-)5(-7)-lobed, with lobes that are oblong, elliptic, ovate, or broadly ovate. The lobes are entire and obtuse or obtuse at the apex. Stipules linear-lanceolate or obliquely ovate, 6-12 mm long and 1.5-5 mm wide, while the petiole is 2-3.5 cm long. Pedicel 7-15 mm long. Epicalx segments are deeply cordate at the base, measuring 1–2 cm long and wide, and 6–8 toothed or lobed at the apex. The central part is the largest, with lanceolate teeth. Calyx cup-shaped, densely black-dotted, 7-10 mm long and wide, and 5-lobed or shorter. The corolla is yellow, 4-6 cm long, and the petals are ovate, measuring 2.5-3.5 cm long and 3-4 cm wide. The staminal column is 1 cm long and contains anthrafurans at the top. The capsule is oblong or round, 2.5-3 cm long, comprehensive, and beaked at the apex. They are 3-5 celled, pitted and glabrous, each containing 5-7 seeds. The seeds are ovoid, measuring 6–9 mm long and 3–7 mm wide, covered with long white hairs (lints) covered with grey mist [6]
Ethnobotanical uses	Cotton has various uses, such as relieving symptoms of nausea, fever, headache, diarrhoea, dysentery, neuralgia and bleeding. Women have been known to use cotton to control menstrual disorders and menopausal symptoms. It has also been used to induce labour and childbirth and expel the afterbirth. For some women, cotton is used to increase breast milk production.

Unfortunately, for the last few decades, people's trust in the herbal mode of treatment is going decreased due to the unhygienic and unethical approach, i.e., adulteration (mixing or replacement of a particular medicinal plant with a morphologically similar plant that has no or least therapeutic values) [7]. Adulterating herbal medicines is becoming increasingly common, harming individuals and significantly negatively impacting the herbal industry. Medicinal plants are commonly traded in traditional herbal shops and markets as dried leaves, roots, bark, processed mixtures, extracts, and powdered parts. These plant materials must possess the necessary morphological characteristics to ensure accurate identification for retailers and consumers [8]. A more recent term used in the scientific literature [10] is "DNA barcoding", which involves using standardised DNA regions to accurately identify species [9]. The method follows globally recognised protocols and DNA regions to establish a comprehensive database of organisms [11]. The importance of DNA barcoding in plants lies in the need to identify specific species for their conservation and use, although in some regions, this may require more taxonomic expertise [12]. When macroscopic or microscopic identification methods present a challenge, DNA barcoding offers a reliable alternative for identifying biological materials [13]. It can detect and differentiate species at any stage of growth or processing, allowing DNA extraction [14]. DNA barcoding has already found practical applications in various fields, such as authenticating plant products, including medicinal plants [13], spices [15], olive oil [17], berries [16] and tea [18]. DNA sequences obtained from barcoding have also been used to construct phylogenetic trees for phylogenetic community ecology [19]. To accurately identify species, DNA regions must be used, and the lab procedures and primers used must be recorded. DNA sequence quality data and trace files are available to end users [20], and all data are publicly accessible. DNA sequences can be submitted to the Barcode of Life Data System (BOLD) [12], which manages projects and stores DNA sequences and trace files, scans

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and images of herbarium specimens. BOLD provides a means of managing projects and allows trace files, scans of herbarium specimens, and photographs to be stored alongside DNA sequences [12]. The purpose of this current study was to provide awareness among people about the importance of using authentic medicinal plants, adulteration in them, the effect of quality of particular medicinal plants and their harmful impacts on human health. We can improve our economy by promoting herbal plant treatment and their export. This is the only way to keep the quality of a particular herbal drug. The prime focus of this study is to provide facilities to learn more about the techniques used to indicate the adulteration persisting in plants, i.e., DNA Barcoding.

METHODS

Gossypium herbaceum herbal plant was obtained from Tehsil Munchanabad (30°05'53.1"N 73°37'28.4" E) of Bahawalnagar district and was identified by taxonomic expert Prof. Dr Muhammad Ziaur Rahman and available flora [21, 22]. The plant was placed in a zip-lock bag after collection and labelled with the location and date of collection, common name, and plant identifier. The plant was transferred to the Plant Molecular Lab, Department of Botany, Govt—Graduate College of Science, Wahdat Road Lahore, at Ice Box. The collected plant leaves were stored in a -20 °C freezer before further processing. DNA analysis was used to detect adulteration in plant samples, and a protocol was developed to detect possible adulteration. The Doyle and Doyle method [23] was used for DNA extraction. Genomic DNA extraction involved crushing a 1 g plant sample in a sterile ice-cold pestle and mortar with liquid nitrogen, and the ground material was transferred to an Eppendorf. 750 µl of Cetyltrimethylammonium Bromide (CTAB) was added and incubated in a water bath at 65 °C for 30–45 min. Eppendorf added equal volumes of chloroform and isoamyl alcohol (24:1) solution and centrifuged at 10,000 rpm for 15 min. The supernatant was removed to another Eppendorf, and 750 µl of chloroform and isoamyl alcohol solution was added. This was centrifuged at 10,000 rpm for 15 min, and this step was repeated three times. The clear supernatant was collected in another Eppendorf, and 2/3 volume of ice-cold isopropanol was added to each Eppendorf containing the supernatant. DNA strands appeared as minute threads, and these Eppendorf were placed in a -20°C freezer overnight to allow the DNA strands to thicken and set. The following day, these Eppendorf tubes were centrifuged at 10,000 rpm until the DNA strands resolved as a pellet, and the pellet was washed three times with 200 µl washing buffer. The pellet was allowed to dry, and the DNA was dissolved in 50–70 µl of distilled water. A nanodrop spectrophotometer (Thermo Scientific, Wilmington, USA) was used to confirm DNA samples' concentration, purity, and quantity by recording the absorbance at 260 nm. DNA samples were typically diluted 20-fold, and a double-beam spectrophotometer model Halo DB20 was used to quantify DNA [24]. The gel electrophoresis technique was used to confirm plant DNA. A 1% agarose gel was formed in 1X TAE buffer, and the gel was allowed to solidify. The gel tray was immersed in a gel electrophoresis tank containing TAE buffer (1X), and the comb was carefully removed. To load the samples into the gel, 10 µl DNA sample and 4 µl loading dye were mixed well and loaded with a 100 bp ladder (Thermo Fisher Scientific, USA). The gel was allowed to run at 100 volts for 30 minutes, and the appearance of bands in the gel confirmed DNA in the samples. After DNA confirmation, all DNA samples were amplified by PCR [25]. The extracted genomic DNA was eluted in double-distilled water according to the equation: M1V1=M2V2. PCR of plant DNA samples was planned in the presence of appropriate primers, namely, matK, rbcL, nrITS and trnH-psbA [26], which were selected through literature review, and their sequences were listed in Table 2.

Table 2: Short Oligonucleotide Primers

Barcode	Primer	Primer sequence	Reference
matK	matK F	5'-TAATTTACGATCAATTCATTC-3'	[27]
	matK R	5'-CTTCCTCTGTAAAGAATTC-3'	
rbcL	rbcL F	5'-ATGTCACCACAAACAGAAAC-3'	[28]
	rbcL R	5'-TCG CAT GTA CCY GCA GTT Gc-3'	
nrITS	nrITS F	5'-CCTTATCATTTAGAGGAAGGAG-3'	[29]
	nrITS R	5'-GGAAGTAAAAGTCGTAACAAG-3'	
trnH-psbA	trnH-psbA F	5'-GTTATGCATGAACGTAATGCTC-3'	[30]
	trnH-psbA R	5'-CGCGCATGGTGGATTCACAAATC-3'	

A volume of 25 µl was prepared in PCR tubes containing the reaction mixture. PCR profile was adjusted on the machine at the initial denaturation temperature of 94°C for 3 minutes, the annealing temperature of 48°C for 1 minute, and the extension temperature of 72°C for 10 minutes. All the temperature sequences were maintained to operate for 40 cycles. The PCR product (5 µl) was mixed with 3 µl of loading dye (5X) and allowed to run on 1% agarose gel to confirm DNA amplification (Figure 2).

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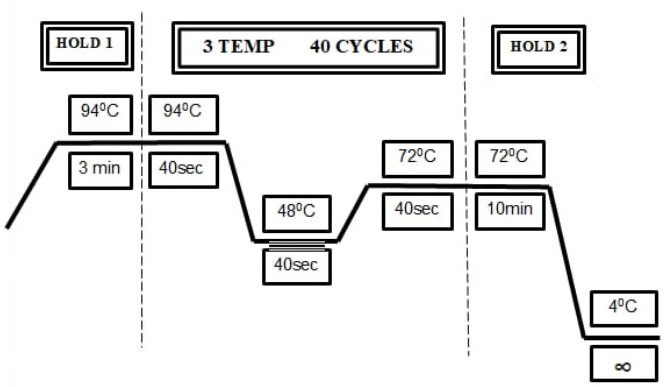

Figure 2: Steps of PCR with conditions

After DNA amplification, the next step involves purifying the plant DNA sample. To achieve this, a technique involved adding distilled water to Eppendorf containing DNA, resulting in a total volume of 100 µl. An equal volume of a mixture of phenol and chloroform (100 µl) was added to the same Eppendorf, and the entire mixture was mixed by inversion. The Eppendorf was then centrifuged at 14000 rpm for 6 min, and the top aqueous phase of the solution was transferred to a new tube. Then, 9 µl of a 3-molar solution of sodium acetate (1/10th volume, pH 5.2) and 250 µl of absolute ethanol (2.5% volume) were added to the Eppendorf and then incubated at -20 °C for 30 min. The tubes were centrifuged at 14000 rpm for 10 min to precipitate the DNA and remove the ethanol. The DNA pellet was washed with 100 µl 70% ethanol, centrifuged at 14000 rpm for 2 min, and then the ethanol was removed. The DNA pellet was kept dry, and 20 µl sterile distilled water (SDW) was added to dissolve it. To validate DNA purification, 4 µl of DNA solution was loaded onto a 1% agarose gel. Purified PCR products were sent to "CELEMICS BTSeq™, Seoul, Korea" for sequencing, and the results were analysed by comparative study. Next-generation sequencing (NGS) was used to perform sequencing instead of Sanger sequencing because it is a more reliable and accurate method. NGS is an impressive sequencing technique that provides extraordinary levels of throughput, scalability, and speed, determining the nucleotide sequence of entire genomes or specific regions of DNA or RNA. With NGS, the possibilities are endless, and the future looks bright for advances in genomics research [31]. The sequences were blasted on NCBI to find the most closely related sequences. Twelve closely related sequences for plant samples were downloaded and used for the DNA analysis. The neighbour-joining method [32] was conducted using MEGAX software [33] for the phylogenetic analysis. The Bootstrap test with 1000 replications was included during the phylogenetic tree construction.

RESULTS

Agarose gel electrophoresis was performed by allowing the gel to run for 30 minutes at a voltage of 100 volts. The observance was done under a UV illuminator in the Gel Documentation system (Bio-Rad, USA), and the appearance of bands in the gel confirmed the DNA in the samples (Figure 3).

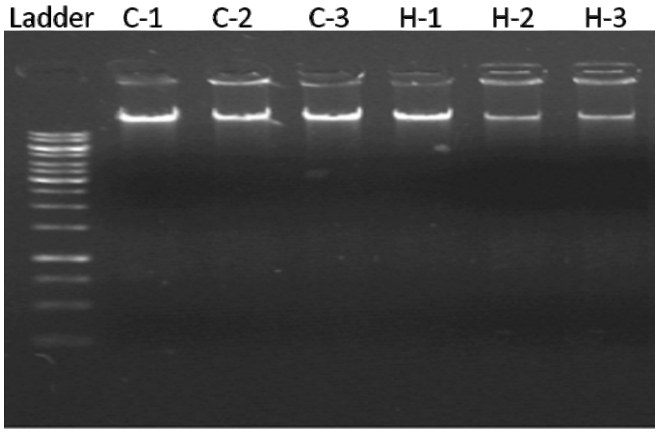

Figure 3: 1% Gel electrophoresis showing DNA profile of Plant samples

The concentration of ds DNAs of plants was determined using a spectrophotometer at 260 nm wavelength using distilled water as blank. The following formula calculated the DNA concentration [24]. 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

Quality of DNA was ensured after observing the OD values at 260 and 280 nm wavelengths using the formula: Quality of DNA= OD_{260}/OD_{280}. It is generally believed that the ratio of DNA to pure is about 1.8. However, if the ratio falls below 1.6, it may indicate the presence of contaminants such as proteins or phenols, which absorb strongly at or near 280 nm [34]. Therefore, the C-1 and H-1 used for further working [35] (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

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H-2	0.606	0.344	1.76
H-3	0.62	0.349	1.79

Polymerase Chain Reaction (PCR)

The PCR of the plant DNA sample was carried out using DNA barcoding primer pairs (matK, rbcL, nrITS and trnH-psbA) [26] in Bio Rad Thermocycler. 10 µl of PCR product was used in the 1% agarose gel electrophoresis. The required size band was obtained and captured with the help of available gel documentation. Only the rbcL primer pair gave positive results for the plant sample (Figure 4).

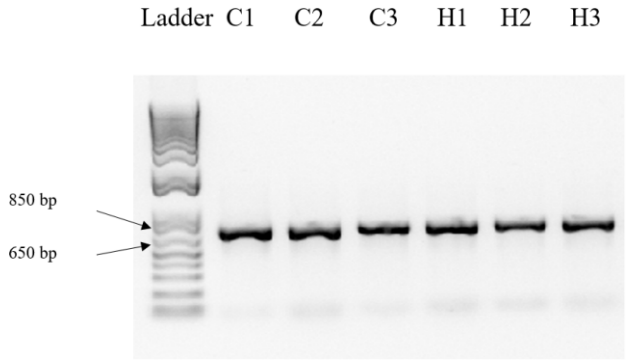

Figure 4: 1% Agarose Gel electrophoresis of PCR product representing the DNA bands of selected plants

DNA Sequences obtained from rbcL primer were edited and assembled using EditSeq. (DNA Star software). Then sequences were used to blast the NCBI database (https://www.ncbi.nlm.nih.gov/) and to download the related sequences. The Sequenced sample's evolutionary history was inferred using the neighbour-joining method [32]. These multiple sequences were aligned for homology analysis using the Muscle method in MEGA11 software. Pairwise nucleotide identity was calculated using the MEGALIGN (DNA Star) software for phylogenetic analysis. The DNA sequence of the rbcL gene amplified from *Gossypium herbaceum* showed a maximum percentage pairwise nucleotide sequence identity at 99.2% with the already reported rbcL gene sequence of *Gossypium richmondii* [MK792869], *Gossypium hirsutum* [MK792865] and 99.0% with *Gossypium barbadense* [HQ901198] reported from China [36], as shown in figure 3.10. The nucleotide sequence of *G. herbaceum* was not **f**ound in GenBank (Figure 5).

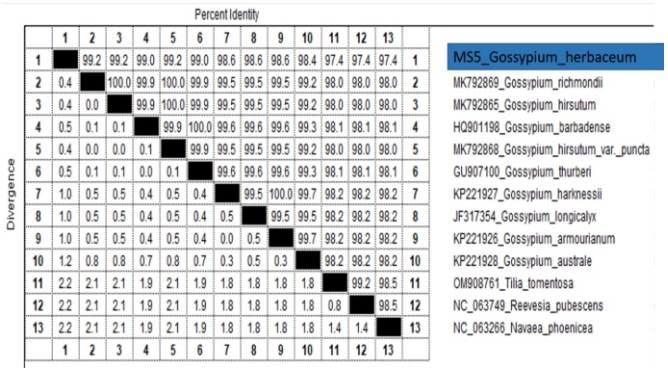

Figure 5: Sequence distancing of *G. herbaceum*

DISCUSSION

Gossypium herbaceum samples were collected from different province of Punjab, Pakistan, stored in labelled zip lock bags and transported to the Plant Molecular Biology Laboratory at Government Graduate College of Science, Wahdat Road, Lahore. The plant was characterised morphologically, including its common name, genus, stem, leaves, flowers and seeds. DNA was extracted using standard protocols and confirmed by 1% gel electrophoresis before amplification by polymerase chain reaction (PCR) with the various primers matK, rbcL, psbA-trnH, and ITS. was In 2009, CBOL (The Consortium for the Barcode of Life) recommended the use of the two loci rbcL and matK as a universal plant DNA barcode. The psbA-trnH spacer and nuclear internal transcribed spacer 2 (ITS2) can also be widely used [37]. Selected plants were successfully amplified with primer rbcL, and the resulting amplifications were purified. Nucleotides obtained from the rbcL gene were sequenced using next-generation sequencing technology at "CELEMICS BTSeq™, Seoul, Korea". The rbcL gene is an efficient DNA barcoding tool for *G. herbaceum* belonging to the *Malvaceae* family. It can identify processed plant products used in food, medicine or cosmetics. The results were consistent with previously reported rbcL gene sequences [38], and NCBI BLAST searches revealed the closest matches with the same species at 99%. The amplified rbcL gene sequence from *Gossypium herbaceum* showed a maximum percentage pairwise nucleotide sequence identity of 99.2% with rbcL gene sequences of *G. richmondii* and *G. hirsutum* and 99.0% with *Gossypium barbadense*. The identity of the studied plant (*G. herbaceum*) was confirmed by NCBI Blast and by studying many plants for their morphology and cross-referencing with different articles. We examined the nucleotide distances of different plants by comparing intraspecific and interspecific pairwise sequences. Interspecific homology was more significant than intraspecific homology, indicating species having a higher percentage of homology with its closest relative. A

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successful DNA barcode must show the difference between intraspecific and interspecific distances. Animals typically show a high degree of variation between species in mitochondrial COI, resulting in a "barcode gap" that allows species differentiation with reasonable certainty. However, this "barcode gap" in the plastid DNA regions mark, and rbcL is absent in closely related land plant species. In our study, when we performed intraspecific versus intraspecific sequence divergence [39] analysis, many sequences overlapped, requiring no further testing to determine the barcode gap. Some researchers have used the barcode gap and distance method to distinguish plant groups beyond the species or generic levels, many plant species' mathematics at the species level and the absence of barcode gaps [40] in rbcL have been used and well documented. To assess the effectiveness of barcodes in separating species, we performed cluster analysis and constructed NJ trees with bootstrap analysis [42]. *Gossypium herbaceum* (MS05) was found to be in the same clade as *Gossypium richmondii* and *Gossypium hirsutum* with a 99.2% bootstrap value based on the phylogenetic tree using the DNA sequence of the rbcL gene. Our results demonstrated that a barcoding gene (rbcL) can identify *G. herbaceum*. The rbcL gene sequence has a high discrimination efficiency for *G. herbaceum*, making it a valuable barcode for species identification. The short sequence of the single gene rbcL is an informative and potentially powerful molecular tag for identifying grass and cultivated plant species.

CONCLUSIONS

In this study, it has been found that herbal products are widely used in different aspects of life, but they need to be identified and checked consciously, and they may also lead to disastrous results for human health. Therefore, it is compulsory to confirm whether the herbal product is the same as required or whether it has been adopted to obtain their personal goals. In future, Molecular identification (DNA Barcoding) will be a beneficial tool to identify plant or plant products for their efficient use in various fields of life.

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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