Exploring the Medicinal Flora of District Musakhel, Pakistan: A DNA Barcoding and Ethnobotanical Investigation

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Research

Abstract

Background: District Musakhel in Pakistan is known for its unique geographical and climatic conditions, fostering diverse flora and fauna, including unexplored medicinal plants. This study aims to systematically categorize these medicinal plant species using traditional taxonomic methods and advanced DNA barcoding techniques.

Methods: The methodology included the ethnobotanical study and plant sampling, DNA extraction, PCR amplification of rbcL and ITS2 regions, sequencing, and subsequent taxonomic identification using BLAST and Phylogenetic analysis via the Neighbor-Joining method.

Results: The *rbcL* barcode exhibited efficacy in discerning taxonomic relationships at the familial and generic levels, while the *ITS2* barcode excelled in elucidating species-specific variations, albeit encountering challenges in clustering species within families. Nevertheless, the most precise taxonomic resolution was achieved by combining both barcodes. Furthermore, Phylogenetic tree analysis confirmed the effectiveness of the *rbcL* over *ITS2* and the combined barcoding approach. The *rbcL* tree displayed robust performance, with 76.9% of nodes surpassing the 50 bootstrap value threshold and successfully resolving 85% of the studied species. The *ITS2* tree, while weaker, still resolved 65% of the species. The combined analysis, integrating *rbcL* and *ITS2* data, achieved a 58% success rate in species resolution. This research explored the ethnobotanical features, highlighting that Plantago major had the highest utilization among the species surveyed. *Cirsium shansiense* showed the greatest use value (0.37), while *Ficus palmata* and *Solanum xanthocarpum* had the highest Relative Frequency of Citation (RFC) values.

Conclusions: In a nutshell, this study serves as a foundational contribution to thoroughly document, precisely identify, and conserve the priceless indigenous medicinal plant species in District Musakhel.

Keywords: DNA Barcoding, Ethnobotany, ITS2, Phylogenetic, *rbcL*, Species Resolution.

Background

Plants are pivotal to human survival, as they provide us with food and hold significant cultural, religious, and medicinal value. The co-evolutionary history between humans and plants is evident in the present era through significant domestication advancements aimed at fulfilling our nutritional, pharmaceutical, fiber, and energy requirements (Schaal 2018). Herbal plants have been utilized by local populations for years for treating various ailments and transferring indigenous knowledge and practices (Uzun and Koca 2020). Ethnobotanical studies have been vital in exploring and developing novel therapeutic drugs (Bulut et al. 2017). Currently, 25% of the herbal medicines included in new pharmacopoeias are plant-based, and numerous synthetic medicines are designed using chemical constituents extracted from plants. Approximately 50,000 flowering plants are used for therapeutic purposes the 422,000 documented species of angiosperms. The documentation of indigenous knowledge of traditional medicinal plants plays a crucial role in conservation efforts (Umair et al. 2017). The demand for plant-based compounds is on the rise due to numerous Western medicines' increasing use of herbal constituents in drug designing (Sucher and Carles 2008). In the rapidly growing demand for botanical products, there is an urgent need to ensure efficacy, quality, and safety. Medicinal plants are generally identified based on their morphological characteristics at the species level. However, morphological identification can be misleading, resulting in improper identification due to a lack of knowledge or clerical errors. Therefore, since 1990, an accurate identification method based on the gene level has been sought to authenticate the identification of medicinal plants (Sucher and Carles 2008). Such approaches utilize DNA sequence variations among species to identify organisms.

The year 2003, witnessed the introduction of DNA barcoding technology to provide authentic identification of species (Hebert et al. 2003). The technology uses the short, universal DNA sequence alias a DNA barcode as a marker for swift, authentic, and automatic species identification (Hebert and Gregory 2005). DNA barcoding has widely been viewed as a neo-taxonomic tool with various other applications, e.g., identification of herbal medicinal plants/products essential for human health (Yu et al. 2021). This method not only aids in the classification of organisms but also provides genetic information for ancestral inheritance and the identification of new species (El-Atroush et al. 2015). In 2009, during the third International Barcode of Life Conference, the combination of ribulose-1,5-bisphosphate carboxylase (*rbcL*) + maturase K (*matK*) loci was recommended as the core DNA sequence for plants, whereas Internal Transcribed Spacer (ITS) and trnH-psbA as complementary sequences (Kress et al. 2005; Group 2009).

Evidence suggests that the ITS2 sequence is a suitable primary barcode for identifying medicinal plants, along with some complementary regions, with applicability for phylogenetic analysis at both the genus and species levels (Schultz and Wolf 2009; Chen et al. 2010). Among the mentioned barcode markers, *rbcL* gene is the most extensively characterized plastid coding region in GenBank, with reasonable representation from all major groups. Thus, it serves as a useful baseline for comparison with other plastid genes (Newmaster et al. 2006). To date, no single DNA barcode has been found to be effective in identifying all plant species. A single barcode may lack the necessary variability to distinguish closely related species. Therefore, a combination of multiple loci may be a better choice in plant DNA barcoding, as it allows for more precise discrimination between species. However, the standard or core DNA barcodes in plants remain a topic of controversy (Hollingsworth 2011; Li et al. 2011). The study area chosen is located in the northeastern part of Balochistan, spanning the western slopes of the Sulaiman mountain ranges. It encompasses tribal territories from two distinct administrative districts: Musakhel in Balochistan and Dera Ghazi Khan in Punjab province (Buzdar 2008).

The objectives of this research paper are fourfold. Firstly, the study aims to identify and document the significant medicinal plants present in the region under investigation. This involves compiling a comprehensive list of these plants and their respective characteristics. Secondly, an ethnobotanical analysis were conducted to explore the therapeutic uses of these medicinal plants, examining traditional knowledge and practices associated with their medicinal properties. Thirdly, the research aims to assess the resolution power of DNA barcoding regions at different taxonomic levels (family, genus, and species) and determine the effectiveness of combining multiple barcodes. Finally, the study seeks to evaluate the authenticity and effectiveness of ITS2 and *rbcL* DNA barcoding regions in identifying medicinal plants, specifically in Musakhel, Pakistan. By addressing these objectives, the research paper aims to contribute valuable insights into the medicinal flora of the region and enhance our understanding of DNA barcoding techniques for plants identification.

Materials and Methods

Study area

The research was conducted in the Koh-e Sulaiman range, in the Musakhel District in the northeastern Baluchistan region and shares borders with Khyber Pakhtunkhwa and Punjab's Dera Ghazi Khan. Musakhel is located at coordinates 30.8478° north latitude and 69.9550° east longitude, covering an area of 5728 km2 (Figure 1). It is home to a variety of plant and animal species, some of which are exclusive to this region (Musakhel 2005).

Ethnobotanical data collection

In this study, 40 local inhabitants and ten traditional healers (Hakeem) were interviewed to gather information on their utilization of medicinal plants for treating various ailments. The Departmental Research Committee (DRC) of the Biotechnology Department and the Graduate Study Office (GSO) at Sardar Bahadur Khan Women's University thoroughly

assessed and reviewed all ethical aspects of this research, granting permission to conduct the study. Prior to the interviews, participants were fully informed about the research on medicinal plants. Subsequently, interviews were conducted through questionnaires after obtaining their informed consent. The interviews focused on capturing the indigenous knowledge and practices of the interviewees, with a particular emphasis on elderly women. Open-ended interviews and questionnaires were employed to investigate the medicinal properties of the collected plants. Considering that the local population primarily spoke Balochi, the interviews were conducted in Balochi and later translated into English for analysis. The documented data encompassed the vernacular and botanical names of the plants, their respective families, Barcode of Life Data Systems (BOLD) sample ID, ethnobotanical uses, and quantitative details.

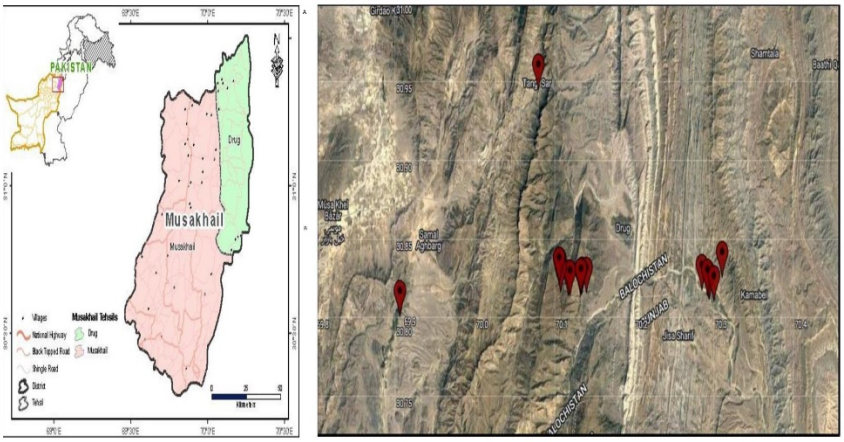

Figure 1. A) Map of District Musakhel (Source: MUSAKHEL DISTRICT EDUCATION PLAN FOR 2016-2017 TO 2020-2021) (B) GPS Coordinates based map of sampling sites.

Collection and identification of medicinal plants

To collect the plants, the researcher received assistance from the traditional healers and local inhabitants who possess a strong association and familiarity with the area and its flora. They guided the researcher to the natural habitats of the target species and, in some cases, helped in collecting voucher specimens. After collecting plant data, voucher specimens were pressed and dried in the field. Herbarium sheets were prepared, and the voucher samples were identified by a taxonomist and deposited at the Herbarium of Balochistan University of Information Technology Engineering and Management Sciences (BUITEMS) in Quetta, Pakistan. The proposed scientific names and synonyms were crosschecked with the Plant of the World Online (POWO) database (http://www.plantsoftheworldonline.org). Together with the voucher preparation, young plant material from all selected plants was collected, carefully placed in sealed plastic bags containing silica gel, and transported back to the laboratory. Relevant metadata about these plants as submitted in BOLD, specifically Specimen data file including detailed voucher information, botanical names of the taxa sampled, dates of collection, geographical coordinates, collectors, identifiers (Taxonomist) and high-quality specimen images from each plant.

Statistical analysis

The data collected from the questionnaires underwent statistical analysis using several quantitative indices, such as Use Reported (UR), Use Value (UV), Frequency of Citation (FC), and Relative Frequency Citation (RFC). The UR index quantifies the number of ailments treated by a specific plant, while the UV index measures the relative value of native plant species. To calculate the UV, the researchers employed the formula developed by Phillips et al. 1994, ensuring accuracy and consistency in the assessment.

$$UV = \Sigma U / n$$

In this formula, "U" represents the number of uses reported for a specific plant species by the informants, while "n" denotes the total number of informants who reported using that species. A higher UV score indicates a greater number of use reports for the plant, whereas a lower score indicates fewer use reports. Furthermore, two additional statistical analyses were performed: Frequency of Citation (FC) and Relative Frequency Citation (RFC). The Frequency of Citation (FC) refers to the number of informants who cited the use of a particular plant species. On the other hand, the Relative Frequency Citation (RFC) index was computed using the following formula:

RFC= FC/ N (0 < RFC < 1)

The RFC index is determined by considering the overall number of participants in the study, represented by "N." This index varies from "0," indicating that no informant mentioned the plant as being useful, to "1," indicating that all informants acknowledged the plant's usefulness (Vitalini et al. 2013).

DNA Extraction, PCR and sequencing

The genomic DNA was obtained from leaves dried on silica gel. Leaf samples were loaded into a 96-well box in accordance with established procedures. The DNA extraction followed the standard protocol devised by Ivanova et al. in 2008 and was conducted at the Canadian Centre for DNA Barcoding (CCDB) within the Biodiversity Institute of Ontario. Before processing, plant boxes were centrifuged at 1500 g for 2 min. Each tube containing dry tissue was equipped with one stainless steel bead and covered with fresh strip caps. The boxes, with lids removed, were inserted into TissueLyser (Qiagen) adapters and shaken at 28 Hz for 30 sec; the plates were rotated, and the process was repeated. Centrifugation at 1500 g for 2 min followed. Subsequently, 250-350 µl of 2×CTAB was added to each tube, covered with fresh strip caps, and gently inverted to mix. Centrifugation at 1500 g for 1 min ensued, followed by an incubation at 65ºC for 1.5 hours.

Afterward, 50 µl of lysate was transferred into a 96-well Eppendorf plate, and 100 µl of Plant Binding Buffer (PBB) was added to each sample. Incubation at room temperature for 5 min was performed, and the lysate was transferred (approximately 150 µl) from the microplate wells to the Glass Fiber (GF) plate wells, which were placed on top of a square-well block. The plate was sealed with self-adhering foil and centrifuged at 5000 g for 5 min to bind DNA to the GF membrane. For the first wash step, 180 µl of Protein Wash Buffer (PWB) was added to each well of the GF plate, which was then sealed and centrifuged at 5000 for 2 min. In the second wash step, 750 µl of Wash Buffer (WB) was added to each well of the GF plate, sealed with a new self-adhering foil, and centrifuged at 5000 for 5 min. The self-adhering foil was removed, and the GF plate was placed on the lid of a tip box. Incubation at 56 °C for 30 min followed to evaporate residual ethanol. A PALL collar was positioned on the collection microplate, and the GF plate was placed on top. Then, 50–60 µl of ddH20 (prewarmed to 56°C) was dispensed directly onto the membrane in each well of the GF plate, and incubation at room temperature for 1 min ensued. The plate was sealed and placed on a clean square-well block to prevent cracking of the collection plate, and centrifuged at 5000 g for 5 min to collect the DNA eluate. The GF plate was removed and discarded. Finally, the DNA plate was covered with cap strips, and the DNA could be stored at –20 °C for long-term storage. For PCR, 1-2 µl of the DNA was used. PCR amplification via ITS2 and *rbcL* (Table 1), and sequencing were performed at the Canadian Centre for DNA Barcoding (CCDB) within the Biodiversity Institute of Ontario.

Table 1. Primers used for DNA amplification of *ITS2* and *rbcL*

Gene Region	Name	Sequences
RbcL	rbcLa-F	ATGTCACCACAAACAGAGACTAAAGC
	rbcLa-R	GTAAAATCAAGTCCACCRCG
ITS2	ITS_S2F	ATGCGATACTTGGTGTGAAT
	ITS4	TCCTCCGCTTATTGATATGC

The utilization of the CCDB's advanced facilities and the adherence to rigorous protocols aimed to ensure the accuracy and reliability of the genomic data derived from the dried leaf (Fazekas et al. 2012). PCR amplification and sequencing were conducted at CCDB using the standard method developed by Maria Kuzmina and Natalia Ivanova (CCDB Protocols - www.ccdb.ca). PCR products were obtained for *rbcL* and ITS2 regions, employing the primers rbcLa-F (ATGTCACCACAAACAGAGACTAAAGC) and rbcLa-R (GTAAAATCAAGTCCACCRCG) for amplification and sequencing. Similarly, the primers ITS-S2F (ATGCGATACTTGGTGTGAAT) and ITS4 (TCCTCCGCTTATTGATATGC) were utilized for amplification and sequencing. The thermocycle programs for PCR were as follows: For *rbcL*, an initial denaturation at 94°C for 4 min, followed by 35 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min, with a final extension at 72°C for 10 min. For ITS2 (ITS2-S2F/ITS4), the protocol involved an initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 30 sec, 56°C for 30 sec, and 72°C for 45 sec, with a final extension at 72°C for 10 min. Purification and bidirectional sequencing of the obtained PCR products were carried out following the procedure outlined by Haibabaei et al. (2006). All the barcodes, images, and

other sample information have been uploaded to the Barcode of Life Data System (BOLD) (http://www.boldsystems.org) under the project titled "OLIVE DNA Barcoding of Flora of Olive ecosystem of Baluchistan, Pakistan" (https://v4.boldsystems.org/index.php/ MAS_Management_DataConsole?codes=OLIVE).

Sequence Analysis and Data Interpretation

The DNA sequences were analyzed, manually trimmed, and compared with the data deposited in the National Center for Biotechnology Information (NCBI) using the Basic Local Alignment Search Tool for Nucleotides BLASTN tool (https:// www.ncbi.nlm.nih.gov/ with Mega blast parameter (Altschul et al. 1990). The accuracy of barcodes was assessed at three taxonomic levels (Family, genus, and species). Both *rbcL* and ITS2 sequences were used, and a combination of both (*rbcL*+ITS2) in a dual-locus approach was assessed. A taxon was considered fully discriminated if *rbcL* and/or ITS2 resolved it in a BLAST analysis. The correctness of identification was based on the highest BLAST percent identity with the expected species. To ensure accuracy, no other species, even of the same genera, should have been hit with a similar score, so the species will be assigned as C (CORRECT) status. If any other species showed a similar highest identification score besides our concerned species, then that barcode(s) was assigned the status of ABM (Another Best Match). If the query sequence did not match our desired species with the highest score and related sequences of that specimen were already deposited, then it was assigned an INC (INCORRECT) barcode status. Additionally, the sequences were considered novel if specific *rbcL* or/and ITS2 sequences were not already present in reference databases such as NCBI and BOLD.

Lengths (bp) of *rbcL* and ITS2 regions and range of GC Content

Descriptive statistical analysis were performed using "ggplot2" package in R version 4.0.3 (https://www.r-project.org) of *rbcL* and ITS2 sequences. The minimum and maximum lengths (bp) of *rbcL* and ITS2 regions and the range of GC Content (%) were evaluated.

Phylogenetic analysis

Tree-based analyses were conducted using MEGA 7.0. Multiple sequence alignments were performed via CLUSTAL W function. Three different trees were generated using the *rbcL*, ITS2, and combined *rbcL*+ITS2 sequences of the plant species. To visualize patterns of sequence divergence between taxa, Neighbor-Joining (NJ) cluster analyses were performed using Molecular Evolutionary Genetics Analysis (MEGA7). NJ tree inferred from 1,000 replicates, and the evolutionary distances were computed using the Kimura 2 parameter (Tamura et al. 2013).

Results

Collected medicinal plants and Statistical analysis

In the present study, we systematically catalogued and categorized a total of 20 plant species, representing 19 genera across 14 distinct families, as outlined in Table 2. Within this taxonomic spectrum, the highest prevalence was observed for Solanaceae family, with three reported species, followed by the Lamiaceae and Zygophyllaceae families, each containing two species. Likewise, the Asteraceae, Euphorbiaceae, Moraceae, Nyctaginaceae, Malvaceae, Rubiaceae, Plantaginaceae, Phyllanthaceae, Apocynaceae, Aizoaceae, and Cucurbitaceae families each contributed one species to the overall collection (Figure. 2).

Table 2. Ethnobotanical uses of plants in Musakhel District, Balochistan, Pakistan

Botanical Name BOLD sample ID	**Family**	**Local Name**	**Part used**	**UR***	**UV** **	**FC** ***	**RFC** ****	**Medicinal Value/ Disease treated**
Trianthema portulacastrum BUITEMS-FKB-94	Aizoaceae	Bukan	Leaf	6	0.2	30	0.6	Relief constipation, Carminative effect, Relief Stomach pain, Laxative effect, Diuretic, Jaundice

Rhazya stricta BUITEMS-FKB-76	Apocynaceae	Izhwark	Leaves, legumes	8	0.2	39	0.78	Effective in treatment of intestinal worms, Sore throat, Sedative effect, Reduces muscle pain, Diabetic, Skin Allergies, Toothache, Constipation
Cirsium shansiense BUITEMS-FKB-112	Asteraceae	Marhee booti	Root	3	0.37	8	0.16	Diuretic, Urinary tract infection, Skin allergies
Pluchea arabica BUITEMS-FKB-71	Asteraceae	Washbo	Leaf and flowers	7	0.35	20	0.4	Liver inflammation, Mouth ulcer, Digestive diseases, Diarrhea, Astringent, Antipyretic, Urinary tract infection
Citrullus colocynthis BUITEMS-FKB-99	Cucurbitaceae	Guch	Fruit	6	0.14	43	0.86	Diabetes, Blood purifier, Diuretic, Asthma, Cough, Skin allergies
Euphorbia inaequilatera BUITEMS-FKB-19	Euphorbiaceae	Khontogh	Whole plant	5	0.17	28	0.56	Diuretic, kidney stone, Skin allergies, Blood purifier, Liver diseases
Sophora alopecuroides BUITEMS-FKB-116	Fabaceae	Gozhagh	Root	4	0.36	11	0.22	Use to treat intestinal parasite, Gastrointestinal pain, Mouth ulcer, Liver inflammation
Isodon rugosus BUITEMS-FKB-114	Lamiaceae	Janglee podna	Leaf	6	0.17	35	0.7	Carminative effect, Heat shock, Skin burns, Fever, Headaches, Antispasmodic
Phlomis aurea BUITEMS-FKB-120	Lamiaceae	Jhund	Leaf	6	0.33	18	0.36	Asthma, Cough, Diabetes, Stomach ache, Gastric problems, Carminative effect
Abutilon bidentatum BUITEMS-FKB-04	Malvaceae	Zarhdo booti	Leaf and shoot	4	0.22	18	0.36	Digestive problems, Stomach ulcer, Laxative effect, Jaundice
Ficus palmata BUITEMS-FKB-135	Moraceae	Jangle hinjeer	Fruit	5	0.1	48	0.96	Bone pain, Constipation, Tonic, Sap is used to treat warts, Chronic cough
Boerhavia procumbens BUITEMS-FKB-03	Nyctaginaceae	Baskhaprag	Leaf	5	0.16	30	0.6	Kidney diseases, Skin diseases, Cough, Asthma, Liver diseases

Phyllanthus maderaspatensis BUITEMS-FKB-28	Phyllanthaceae	Mesh-boti	Whole plant	4	0.16	25	0.5	Digestive diseases, Beneficial for lactating women, Stomach pain, Gastritis
Plantago major BUITEMS-FKB-15	Plantaginaceae	Koh-bhang	Leaf	10	0.24	41	0.82	Respiratory diseases, Chronic cough, Allergies, Sedative effect, Digestion diseases, Stomach cramps, Mouth ulcer, Constipation, Asthma, Insomnia
Triflorensia cameronii BUITEMS-FKB-14	Rubiaceae	Zaragh	Leaf, flower and stem	4	0.26	15	0.3	Skin diseases, Measles, Cough, Common cold,
Datura inoxia BUITEMS-FKB-137	Solanaceae	Jatora	Fruit	5	0.12	40	0.8	Cough, Asthma, Laxative effect, Intestinal worms, Skin fungal infections
Solanum cordatum BUITEMS-FKB-30	Solanaceae	Tolangoor	Fruit	5	0.25	20	0.4	Stomachache, Asthma, Cough, Chest congestion, Wound healer
Solanum xanthocarpum BUITEMS-FKB-80	Solanaceae	Racharhee	Fruits	7	0.14	48	0.96	Respiratory diseases, Cough, Asthma, Digestion diseases, Appetizer, Chronic skin wounds, Boils
Fagonia cretica BUITEMS-FKB-73	Zygophyllaceae	Khar khawagh, Dhamsa boti	Whole plant	7	0.15	45	0.9	Liver diseases, Effective in jaundice, Blood purification, Diuretic, Urinary tract infections, Thorns are used to heal ear and nose piercing wounds, Prevent pus formation in wounds
Tribulus terrestris BUITEMS-FKB-24	Zygophyllaceae	Maarkondee	Root and leaves	5	0.14	35	0.7	Kidney diseases, Diuretic, Skin diseases, Infertility problems, Skin burns

In our study, *Plantago major* exhibited the highest Use Report (UR) with a score of 10, indicating its prominent utilization for a variety of ailments. Following closely, *Rhazya stricta* demonstrated a UR of 8, while *Pluchea Arabica* and *Solanum xanthocarpum* both exhibited a UR of 7. In contrast, the lowest UR, at a mere 0.3, was observed in the case of *Cirsium shansiense*.

Additionally, we assessed these plant species' Use Value (UV), with *Cirsium shansiense* showing the highest UV at 0.37. This was followed by *Sophora alopecuroides* with a UV of 0.36 and *Pluchea Arabica* with a UV of 0.35. In contrast, *Ficus palmata* displayed the lowest UV at 0.1.

Figure 2. Medicinal plants used by local inhabitants and traditional healers in District Musakhel. (A) *Boerhavia procumbens* (B) *Abutilon bidentatum* (C) *Triflorensia cameronii* (D) *Plantago major* (E) *Euphorbia inaequilatera* (F) *Tribulus terrestris* (G) *Phyllanthus maderaspatensis* (H) *Solanum cordatum* (I) *Fagonia cretica* (J) *Pluchea Arabica* (K) *Rhazya stricta* (L) *Solanum xanthocarpum* (M) *Trianthema portulacastrum* (N) *Citrullus colocynthis* (O) *Cirsium shansiense* (P) *Isodon rugosus* (Q) *Sophora alopecuroides* (R) *Phlomis aurea* (S) *Ficus palmata* (T) *Datura inoxia*

Furthermore, *Solanum xanthocarpum* and *Ficus palmata* exhibited the highest Frequency of Citation (FC) values at 48, indicating their prevalence in the cited reports. This was followed by *Fagonia cretica* with an FC value of 45 and *Citrullus colocynthis* with an FC value of 43. Conversely, *Cirsium shansiense* had the lowest FC value at 8.
Finally, we calculated the Relative Frequency of Citation (RFC) index, which was highest for *Solanum xanthocarpum* and *Ficus palmata* at 0.96. Following closely, *Fagonia cretica* exhibited an RFC of 0.9, while *Citrullus colocynthis* and *Plantago major* had RFC values of 0.86 and 0.82, respectively.

In terms of their medicinal usage, these plants are either consumed as whole plant or as specific parts. Leaves and fruits, however, constitute the most employed components, each contributing to 25% of the reported medicinal uses.

Sequence Analysis and Data Interpretation
To comprehensively evaluate the performance of the *rbcL* and ITS2 barcodes individually and in combination, we calculated their success rates across distinct taxonomic ranks, including Family, Genus, and Species, for the entire set of specimens. At the family level, *rbcL* manifested 100% success rate in correctly affiliating each specimen with its respective family. However, at the more specific genus level, *rbcL* exhibited 80% success rate and further refinement to the species level yielded a success rate of 31%. The ITS2 barcode attained 80% success rate in accurately classifying specimens at the family level. ITS2 achieved a 75% and 47% success rate within these families at the genus and species level. A combined analysis followed these individual barcode assessments, where a perfect match was observed at the family level, signifying a 100% success rate. The combined barcode analysis yielded a 90% success rate at the genus level, while at the species level, a 58% success rate was achieved (Figure 3a). In addition to assessing the accuracy of identifications at varying taxonomic levels, our study considered other outcomes, namely, 'Correct' (C) identifications, 'Another Best Match' (ABM) identifications, and 'Incorrect' (INC) identifications (Figure 3b).

Lengths (bp) of rbcL and ITS2 regions and range of GC Content
We undertook a statistical analysis for the acquired *rbcL* and ITS2 sequences from all specimens. To represent the data visually, boxplots were generated employing the "ggplot2" package within the R statistical environment, version 4.0.3 (https://www.r-project.org) (Figure 3c).

The length distribution of the ITS2 barcode amplified region exhibited a range spanning from 305 to 360 base pairs (bp), whereas the *rbcL* region consistently measured 533 bp in length across all analyzed samples. The GC content analysis revealed distinct patterns for *rbcL* and ITS2, with *rbcL* GC content ranged from 41% to 44.3%, while ITS2 from 53.1% to 65.6%.

Phylogenetic analysis
We assessed the discriminatory capacity of the *rbcL* and ITS2 barcode regions for the 20 studied species and conducted phylogenetic tree-based analyses employing the Neighbor-Joining (NJ) method (Figure 4a, b and Figure 5).

The NJ tree constructed using *rbcL* sequences demonstrated notable performance, with 76.9% of nodes (10 out of 13) displaying bootstrap values equal to or greater than the established threshold of 50. This robust analysis successfully resolved 85% of the target species, encompassing 17 out of the 20 species studied. Conversely, the NJ tree generated from ITS2 sequences exhibited comparatively weaker performance, as only 53.8% of nodes (7 out of 13) surpassed the 50 bootstrap value threshold. Consequently, this analysis successfully resolved 65% of the examined species, corresponding to 13 out of 20 species.

Subsequently, a third phylogenetic tree was constructed by combining the information from both *rbcL* and ITS2 sequences (*rbcL*+ITS2). However, this combined analysis also displayed relatively modest performance, with 53.8% of nodes (7 out of 13) surpassing the 50 bootstrap value threshold. It successfully resolved 65% of the target species, corresponding to 13 out of the 20 species examined in this study.

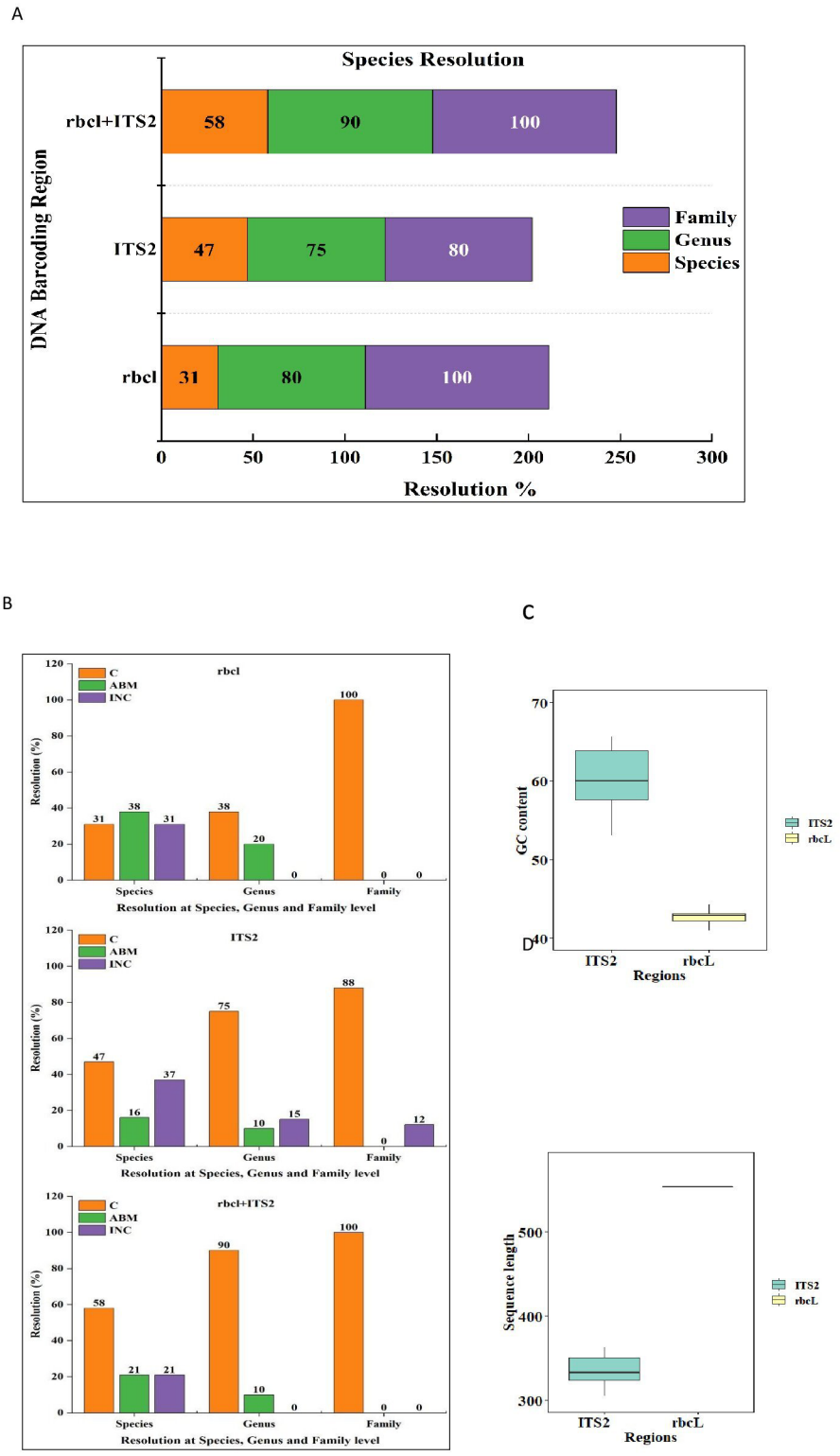

Figure 3. (A) Comparison on Species resolution of *rbcL*, ITS2 and *rbcl*+ITS2 at family, genus and species level. (B) Comparison of barcodes ITS2, *rbcL* and ITS2+*rbcl* to get Correct (C), Another Best Match (ABM) and Incorrect (INC) species identification on the basis of data similarity from NCBI. (C) Box plots of rbcL and ITS2 GC content (%) and (D) sequence length of *rbcL* and ITS2 plant species.

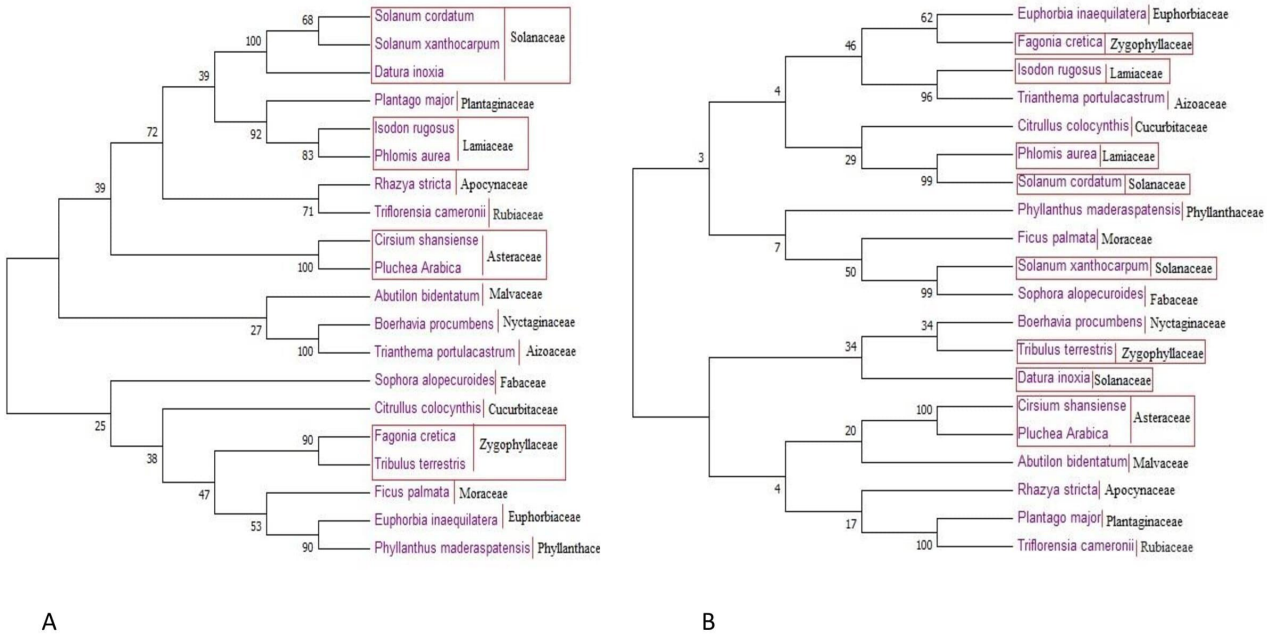

Figure 4. (A) Neighbor joining tree generated in the present study based on *rbcL* gene by using MEGA 7. (B) Neighbor joining tree generated in the present study based on ITS2 gene by using MEGA 7.

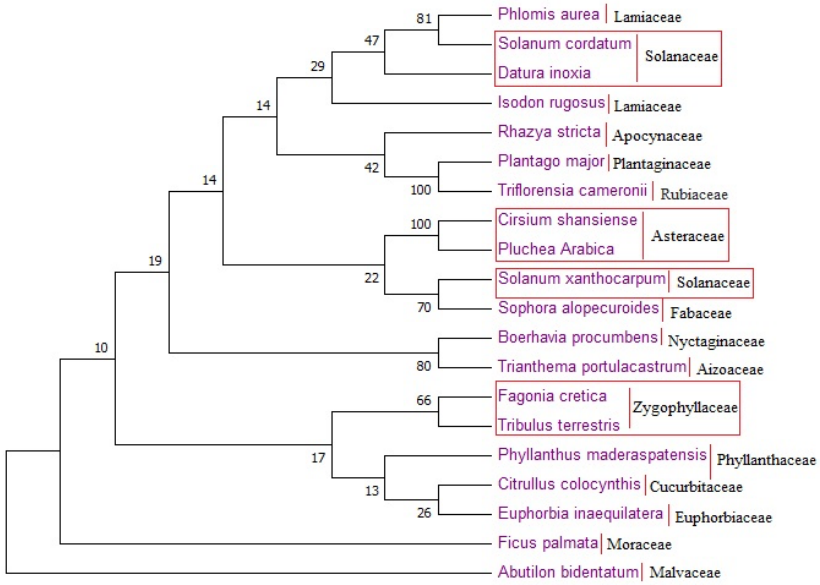

Figure 5. Neighbor joining tree generated in the present study based on rbcL+ITS2 gene by using MEGA 7.

Discussion

The research conducted in District Musakhel, Pakistan, presents a multifaceted exploration of its rich biodiversity, with a particular focus on previously uncharted medicinal plant species. As the study area, District Musakhel, is not fully explored, and there is a scarcity of literature on its plant biodiversity. According to a survey by BHC (https://bhc.gov.pk/district-judiciary/musakhail/introduction/history), nearly 211 plant species in the Musakhel area are recorded, signaling a baseline

understanding of the local flora. However, it also emphasizes the need for a more detailed study to comprehensively assess the diversity of both native and introduced medicinal plants. Our investigation employed a combination of traditional taxonomic methodologies and advanced DNA barcoding techniques, including using *rbcL* and ITS2 as molecular markers. The outcomes of this study hold significant implications for the fields of biodiversity conservation, ethnobotany, and molecular taxonomy.

Ethnobotanical Significance

This study delved into the ethnobotanical aspects of the 20 medicinal plants under investigation. The ethnobotanical data provide valuable insights into local communities' traditional knowledge and practices, shedding light on the importance of these species in indigenous medicinal systems.

In traditional medicine, a myriad of plant species spanning various taxonomic groups, families, and genera play a pivotal role. Our ethnobotanical analysis in this study unveiled the family Plantaginaceae as a predominant source of medicinal plants, with particular emphasis on *Plantago major*, locally referred to as Koh-bhang, with the highest Use Report (UR). Notably, the Plantago genus encompasses approximately 275 identified species, primarily distributed across Asia, Europe, and the Americas (Wang et al. 2015; Najafian et al. 2018). *Plantago major*, renowned for its historical utilization in traditional medicinal practices in regions such as Egypt and India, has garnered recognition for its effectiveness in alleviating symptoms associated with diarrhea and constipation. Its therapeutic repertoire encompasses a wide spectrum of ailments, including respiratory disorders, infections, circulatory disorders, digestive maladies, infertility, and various other health conditions (Samuelsen 2000). Our findings underscore the substantial therapeutic potential of *Plantago major* in addressing an extensive range of health concerns, such as digestive disorders, respiratory diseases, asthma, allergies, and insomnia. Furthermore, this botanical treasure trove boasts an abundance of essential phytochemicals, including phenylethanoids, glycosides, flavonoids, iridoids, phenolic acids, and other bioactive compounds, collectively contributing to its remarkable medicinal properties (Samuelsen 2000; Zhou et al. 2013)

Among the flora surveyed in our study, *Rhazya stricta*, belonging to the Apocynaceae family and locally known as Izhwark, emerged as the second most frequently reported species. This versatile plant boasts a multitude of significant medicinal applications, encompassing the treatment of intestinal worms, sore throat, muscle pain, diabetes, skin allergies, toothache, and constipation. *Rhazya stricta Decne* holds profound significance in indigenous herbal remedies, finding widespread usage in addressing various ailments not only in South Asian countries like Pakistan, India, and Afghanistan but also across the Middle East, including Saudi Arabia, Qatar, the United Arab Emirates (UAE), Iran, and Iraq. In the rural landscapes of Saudi Arabia, the leaves and legumes of this medicinal plant are specifically harnessed in traditional medicine to ameliorate conditions such as syphilis, chronic rheumatism, and bodily discomforts (Bhadane et al. 2018). Furthermore, traditional medicine practitioners tap into the therapeutic potential of *R. stricta* for the management of type 2 diabetes, inflammatory diseases, helminthiasis, and throat infections (Ali 1998; Albeshri et al. 2021).

Similarly, *Fagonia cretica*, also recognized by its synonym *Zygophyllum creticum* from the Zygophyllaceae family, exhibits noteworthy medicinal properties. This plant serves a diverse range of purposes, including treating liver diseases, jaundice, and urinary tract infections (UTIs). Its reputation also extends to its efficacy in blood purification and diuretic functions. Notably, the thorns of *Fagonia cretica* are employed in the healing of wounds resulting from ear and nose piercings. This botanical gem is primarily found in D.G Khan, Multan, and the surrounding regions of Pakistan. The presence of bioactive phytochemicals substantiates the significant medicinal value attributed to *Fagonia cretica* (Qureshi et al. 2015; Naz et al. 2021).
Furthermore, our study documented the presence of other valuable medicinal species, including *Tribulus terrestris* from the Zygophyllaceae family, *Boerhavia procumbens* from the Nyctaginaceae family, *Abutilon bidentatum* from the Malvaceae family, *Triflorensia cameronii* from the Rubiaceae family, *Euphorbia inaequilatera* from the Euphorbiaceae family, Phyllanthus maderaspatensis from the Phyllanthaceae family. Additionally, two species from the Asteraceae family, *Pluchea Arabica* and *Cirsium shansiense*, three species from the Solanaceae family, *Solanum cordatum*, *Solanum xanthocarpum*, and *Datura inoxia*, as well as *Trianthema portulacastrum* from the Aizoaceae family, *Citrullus colocynthis* from the Cucurbitaceae family, *Isodon rugosus* and *Phlomis aurea* from the Lamiaceae family, and *Ficus palmata* from the Moraceae family were documented in our study, highlighting their respective medicinal uses.

Additionally, *Cirsium shansiense* exhibited a high use value, emphasizing its paramount role in local traditional medicine. These ethnobotanical findings underscore the cultural significance of certain plant species and their potential value in pharmacological research and drug development. Furthermore, *Ficus palmata* and *Solanum xanthocarpum*, with their high Relative Frequency of Citation (RFC) values, reaffirm their importance in indigenous medicinal practices. The identification of these highly cited species underscores their potential as candidates for further phytochemical and pharmacological investigations.

Molecular Taxonomy and DNA Barcoding

One of the primary objectives of this study was to evaluate the utility of *rbcL* and ITS2 barcodes in resolving taxonomic relationships within the sampled medicinal plant species. The analysis revealed that *rbcL* exhibited robust performance in

distinguishing taxonomic groups at both the familial and generic levels. This outcome aligns with previous research showcasing the reliability of *rbcL* as a suitable barcode for plant species identification (Group 2009). The successful resolution of 85% of the studied species at various taxonomic levels underscores this marker's potential in plant identification, particularly in situations where rapid and accurate assessment is required.

On the other hand, the ITS2 barcode excelled in differentiating species-specific variations, although it encountered certain difficulties in clustering species within families. This finding is consistent with the known challenges associated with ITS2 due to its high intraspecific variation and potential difficulties in the presence of hybridization events (Chen et al. 2010). Nevertheless, ITS2 remains a valuable tool for species-level identification, especially when coupled with other markers.

A significant insight emerged from the combined analysis of *rbcL* and ITS2 data, demonstrating an enhanced species resolution rate of 58%. This amalgamated approach highlights the potential for improved taxonomic accuracy by integrating multiple markers, thereby minimizing the limitations associated with individual barcodes. Such integrative analyses underscore the importance of adopting a comprehensive approach in DNA barcoding studies.

Conservation Implications

The comprehensive documentation and precise identification of medicinal plant species native to District Musakhel contribute significantly to biodiversity conservation in this unique ecological niche. The accurate identification of these species is a fundamental step toward informed conservation initiatives. It enables the development of targeted strategies for the preservation of these invaluable resources, particularly in the face of environmental threats and habitat degradation. The medicinal plants that may need to be prioritized in terms of conservation are *Triflorensia cameronii*, *Pluchea Arabica*, *Cirsium shansiense*, *Isodon rugosus*, and *Fagonia cretica*. The decreasing population of these plants observed on each visit raises concerns about their sustainability.

Moreover, this study highlights the potential for future discoveries in the realm of medicinal plant diversity within District Musakhel. Identifying previously unexplored species underscores the need for continued exploration and research in this region, which may lead to the discovery of novel therapeutic agents and valuable contributions to ethnopharmacology.

Conclusion

In conclusion, this research amalgamates molecular taxonomy, ethnobotany, and conservation biology to comprehensively understand the medicinal plant diversity within District Musakhel. The findings underscore the potential of DNA barcoding as a powerful tool for species identification and the significance of traditional knowledge in guiding conservation efforts and drug discovery endeavors. Our research underscores the diverse array of medicinal plants from various botanical families and genera utilized in traditional medicine. These findings shed light on the multifaceted therapeutic potential of these botanical treasures, providing valuable insights into their applications in managing a wide spectrum of health conditions. This study serves as a foundational step toward preserving biodiversity and exploring untapped resources in this ecologically unique region.

Declarations

List of abbreviations: *rbcL*= ribulose-1,5-bisphosphate carboxylase, *matK*= maturase K, ITS= Internal Transcribed Spacer, BOLD**:** Barcode of Life Data Systems, POWO: Plant of the World Online, UR: Use Reported, UV: Use Value, FC: Frequency of Citation, RFC: Relative Frequency Citation, CCDB: Canadian Centre for DNA Barcoding, CTAB: Cetyltrimethyl ammonium bromide, PBB: Plant Binding Buffer, GF: Glass Fiber, PWB: Protein Wash Buffer, NCBI: National Center for Biotechnology Information, BLASTN: Basic Local Alignment Search Tool for Nucleotides, NJ: Neighbor-Joining,

Ethics approval and consent to participate: The Departmental Research Committee (DRC) of the Biotechnology Department and the Graduate Study Office (GSO) at Sardar Bahadur Khan Women's University thoroughly assessed and reviewed all ethical aspects of this research, granting permission to conduct the study. Prior to the interviews, participants were fully informed about the research on medicinal plants. Subsequently, interviews were conducted through questionnaires after obtaining their informed consent.

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