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MOLECULAR CHARACTERISATION OF SELECTED SMALL HERBS AND PHYTOCHEMICAL SCREENING BY HPTLC

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ABSTRACT

Medicinal and culinary herbs are widely utilized for their therapeutic and nutraceutical properties; however, accurate identification and quality assessment remain major challenges due to morphological similarities and possible adulteration. The present study aimed to perform molecular characterisation and phytochemical profiling of three selected small herbs, namely basil (*Ocimum basilicum* L.), mint (*Mentha* spp.), and oregano (*Origanum vulgare* L.), using an integrated analytical approach. Molecular authentication was carried out through DNA barcoding employing standard barcode regions, including the nuclear internal transcribed spacer (ITS) and chloroplast genes *rbcL* and *matK*, followed by BLAST and phylogenetic analyses, which confirmed accurate taxonomic identity of the selected herbs. In addition, phytochemical screening of methanolic/ethanolic extracts was performed using High-Performance Thin-Layer Chromatography (HPTLC) to generate characteristic chemical fingerprints. HPTLC analysis revealed the presence of major bioactive phytoconstituents, particularly phenolic compounds, with distinct Rf values and densitometric profiles for each herb, demonstrating variation in phytochemical composition among basil, mint, and oregano. The combined application of molecular characterisation and HPTLC-based phytochemical profiling provides a reliable, reproducible, and efficient strategy for authentication, quality control, and standardization of medicinal herbs, supporting the prevention of adulteration and the safe utilization of herbal raw materials in pharmaceutical and nutraceutical formulations.

KEYWORDS: DNA barcoding; High-Performance Thin-Layer Chromatography (HPTLC); *Ocimum basilicum* L.; *Mentha* spp.; *Origanum vulgare* L.; phytochemical profiling; molecular characterisation; herbal authentication; quality control.

1. INTRODUCTION

Medicinal and culinary herbs have been used since ancient times as an integral component of traditional healthcare systems and continue to serve as valuable sources of bioactive compounds for modern medicine (**World Health Organization [WHO], 2013; Newman & Cragg, 2016**). In recent decades, there has been renewed scientific interest in herbs due to their therapeutic efficacy, nutritional value, and role in the prevention and management of chronic diseases (**Gurib-Fakim, 2006**). Small herbs, in particular, are widely incorporated into food, pharmaceutical, and nutraceutical formulations owing to their rich phytochemical composition and ease of availability.

Among commonly used small herbs, basil (*Ocimum basilicum* L.), mint (*Mentha* spp.), and oregano (*Origanum vulgare* L.) are of significant importance due to their extensive culinary use and well-documented medicinal properties. These herbs exhibit a wide range of biological activities, including antioxidant, antimicrobial, anti-inflammatory, and anticancer effects (**Bakkali *et al.*, 2008; Kaefer & Milner, 2008**). The pharmacological potential of these herbs is primarily attributed to the presence of secondary metabolites such as phenolic acids, flavonoids, terpenoids, and essential oils, which contribute to both plant defense mechanisms and human health benefits (**Harborne, 1998; Pandey & Tripathi, 2014**).

Despite their widespread use, accurate identification and quality assessment of herbal materials remain major challenges. Morphological similarities among closely related species, phenotypic variations caused by environmental factors, and post-harvest processing often make conventional morphology-based identification unreliable (**van Vuuren & Viljoen 2011**). Moreover, the growing commercial demand for herbal raw materials has led to frequent cases of adulteration, substitution, and mislabeling, which can compromise therapeutic efficacy and pose potential health risks (**Ichim, 2019; Techen *et al.*, 2014**). These issues highlight the need for reliable and reproducible authentication techniques.

Molecular characterisation using DNA barcoding has emerged as a powerful tool for the accurate identification and authentication of medicinal plants. DNA barcoding employs short, standardized genomic regions such as the nuclear internal transcribed spacer (ITS) and

chloroplast genes rbcL and matK, which enable precise species-level identification independent of environmental and developmental variations (**Kress & Erickson, 2008; Hollingsworth *et al.*, 2009**). These molecular markers have been successfully applied for authentication of medicinal herbs, detection of adulterants, and conservation of plant genetic resources (**Chen *et al.*, 2010**; **Mishra *et al.*, 2016**).

While molecular methods ensure genetic authenticity, they do not provide information on the chemical composition and bioactive potential of herbal materials. Therefore, phytochemical profiling remains a critical component of herbal quality control and standardization. High-Performance Thin-Layer Chromatography (HPTLC) is a rapid, sensitive, and cost-effective analytical technique widely used for qualitative and quantitative analysis of phytoconstituents in medicinal plants (**Reich & Schibli, 2007; Wagner & Bladt, 2001**). HPTLC allows simultaneous analysis of multiple samples and generates characteristic chemical fingerprints useful for comparative evaluation and detection of adulteration (**Srivastava, 2011**).

The integration of molecular characterisation and HPTLC-based phytochemical profiling provides a comprehensive approach for authentication and quality assessment of medicinal herbs. Molecular techniques confirm species identity at the genetic level, while HPTLC profiling reveals phytochemical diversity and bioactive constituents. Therefore, the present study aims to perform molecular characterisation of basil (*Ocimum basilicum* L.), mint (*Mentha* spp.), and oregano (*Origanum vulgare* L.) using DNA barcoding techniques and to evaluate their phytochemical profiles through HPTLC analysis, thereby contributing to reliable authentication, quality control, and standardization of herbal raw materials.

2. MATERIALS AND METHODS

2.1 Collection and Identification of Plant Material

Fresh aerial parts of basil (*Ocimum basilicum* L.), mint (*Mentha* spp.), and oregano (*Origanum vulgare* L.) were procured from the local market at Chhapara, Bihar, India during their respective peak vegetative growth periods to ensure maximum leaf biomass and phytochemical content prior to flowering. Accordingly, *Ocimum basilicum* was collected in May, *Mentha* spp. in September (monsoon season), and *Origanum vulgare* in November. Plant collection was carried out in accordance with standard guidelines for medicinal plant research to ensure sustainability and sample integrity (**World Health Organization [WHO], 2013**). Only healthy plants free from visible signs of disease, pest infestation, or mechanical damage were selected for the study (**Jain, 2011**).

The collected plant materials were washed thoroughly with distilled water to remove adhering soil and debris and were preliminarily identified based on morphological characteristics using standard floristic keys. Final taxonomic identification and authentication were performed by a qualified taxonomist with reference to published taxonomic literature and regional floras (**Gurib-Fakim, 2006**). Voucher specimens of each plant species were prepared, assigned accession numbers, and deposited in the departmental herbarium for future reference and verification, following standard herbarium procedures (**Bridson & Forman, 1999**).

2.2 Molecular Characterisation

2.2.1 Genomic DNA Isolation

Genomic DNA was isolated from fresh, young leaf tissues of basil (*Ocimum basilicum* L.), mint (*Mentha* spp.), and oregano (*Origanum vulgare* L.) to ensure high-quality DNA suitable for molecular analysis. Approximately 100 mg of leaf tissue from each sample was surface-sterilized with distilled water, blotted dry, and ground to a fine powder using liquid nitrogen.

DNA extraction was carried out using the cetyltrimethylammonium bromide (CTAB) method with minor modifications to improve yield and purity, particularly for secondary metabolite–rich plant tissues (**Doyle & Doyle, 1987; Khanuja *et al.*, 1999**). The powdered tissue was incubated in a preheated CTAB extraction buffer, followed by chloroform–isoamyl alcohol purification and isopropanol precipitation of DNA. The DNA pellet was washed with 70% ethanol, air-dried, and resuspended in TE buffer.

The quality and integrity of the extracted DNA were assessed by electrophoresis on a 1% agarose gel, while DNA concentration and purity were determined spectrophotometrically using a NanoDrop spectrophotometer by measuring absorbance ratios at A260/A280. Only high-quality DNA samples were used for subsequent PCR amplification and sequencing.

2.2.2 PCR Amplification

Polymerase Chain Reaction (PCR) was performed to amplify standard DNA barcode regions for the molecular authentication of basil (*Ocimum basilicum* L.), mint (*Mentha* spp.), and oregano (*Origanum vulgare* L.). The nuclear internal transcribed spacer (ITS) region and chloroplast genes rbcL and matK were selected as target loci owing to their widespread application and effectiveness in species-level identification of medicinal plants (**Kress & Erickson, 2008; Hollingsworth *et al.*, 2009**).

PCR amplification was carried out in a total reaction volume of 25 µL containing approximately 50 ng of genomic DNA, 10 pmol each of forward and reverse primers, 12.5 µL of PCR master mix (comprising Taq DNA polymerase, dNTPs, $MgCl_2$, and reaction buffer), and nuclease-free water. Universal primer pairs specific to the ITS, *rbcL*, and *matK* regions were used as reported previously (**Chen *et al.*, 2010**).

The thermal cycling conditions consisted of an initial denaturation at 94–95 °C for 3–5 min, followed by 30–35 cycles of denaturation at 94 °C for 30 s, annealing at optimized temperatures ranging from 50–58 °C for 30–45 s, and extension at 72 °C for 1 min. A final extension step was carried out at 72 °C for 7–10 min to ensure complete synthesis of the amplified products.

2.2.3 Gel Electrophoresis

Agarose gel electrophoresis was employed to assess the quality of genomic DNA and to confirm successful amplification of PCR products obtained from basil (*Ocimum basilicum* L.), mint (*Mentha* spp.), and oregano (*Origanum vulgare* L.). Genomic DNA integrity was evaluated by resolving samples on a 1% (w/v) agarose gel, while PCR amplicons were analyzed on a 1.5% (w/v) agarose gel prepared in a 1× TAE buffer **(Sambrook & Russell, 2001; Green & Sambrook, 2012)**.

Prior to loading, DNA samples and PCR products were mixed with loading dye and electrophoresed alongside an appropriate DNA molecular weight marker to estimate fragment size. Electrophoresis was carried out at a constant voltage of 80–100 V for an appropriate duration until satisfactory separation was achieved **(Lee *et al.*, 2012)**. Following electrophoresis, gels were stained with ethidium bromide (0.5 µg/mL) and visualized under a UV transilluminator **(Sambrook & Russell, 2001)**.

Clear and intact genomic DNA bands indicated high-quality DNA suitable for downstream applications. The presence of distinct PCR bands corresponding to the expected sizes of the ITS, *rbcL*, and *matK* regions confirmed successful amplification and suitability of the products for sequencing and further molecular analyses **(Kress *et al.*, 2005; Hollingsworth *et al.*, 2011)**.

2.2.4 DNA Sequencing and BLAST Analysis

PCR-amplified products corresponding to the nuclear internal transcribed spacer (ITS) region

and chloroplast genes *rbcL* and *matK* obtained from basil (*Ocimum basilicum* L.), mint (*Mentha* spp.), and oregano (*Origanum vulgare* L.) were purified to remove residual primers and unincorporated nucleotides and subjected to bidirectional Sanger sequencing using the same primer pairs employed for PCR amplification (**Sanger *et al.*, 1977**). Raw sequence chromatograms were carefully examined, edited, and assembled to generate high-quality consensus sequences, and low-quality regions were trimmed to ensure sequence accuracy. The finalized nucleotide sequences were compared with reference sequences available in the NCBI GenBank database using the BLASTn (Basic Local Alignment Search Tool) algorithm for molecular identification and authentication (**Altschul *et al.*, 1997**). Species identity was confirmed based on percentage sequence similarity, query coverage, and E-values, with sequences showing ≥98% similarity considered reliable for species-level identification. The validated sequences were subsequently used for comparative and phylogenetic analyses to assess genetic relationships among the studied herbs and closely related taxa.

2.2.5 Phylogenetic Analysis

Phylogenetic analysis was performed to evaluate the evolutionary relationships of basil (*Ocimum basilicum* L.), mint (*Mentha* spp.), and oregano (*Origanum vulgare* L.) based on the ITS, *rbcL*, and *matK* DNA barcode sequences. The validated sequences obtained from BLAST analysis were aligned with closely related reference sequences retrieved from the NCBI GenBank database using the ClustalW multiple sequence alignment algorithm (**Thompson *et al.*, 1994**). Phylogenetic trees were constructed using MEGA software (version X) employing the Neighbor-Joining (NJ) method, which is widely used for plant DNA barcoding studies (**Kumar *et al.*, 2018**). The robustness of the inferred phylogenetic relationships was assessed by bootstrap analysis with 1,000 replicates, and bootstrap values ≥50% were considered significant and displayed on the tree. Clustering of the studied samples with their respective reference taxa further confirmed accurate molecular identification and taxonomic placement of the selected herbs.

2.3 Preparation of Plant Extracts

Fresh aerial parts of basil (*Ocimum basilicum* L.), mint (*Mentha* spp.), and oregano (*Origanum vulgare* L.) were washed thoroughly with distilled water to remove adhering impurities and shade-dried at room temperature to preserve thermolabile phytoconstituents. The dried plant materials were pulverized into coarse powder using a mechanical grinder and stored in airtight containers until extraction. Extraction was carried out using methanol and

ethanol as solvents, as these are widely employed for efficient recovery of phenolic compounds (**Harborne, 1998; Pandey & Tripathi, 2014**). Approximately 20–30 g of powdered plant material was subjected to Soxhlet extraction for 6–8 h or cold maceration for 48–72 h with intermittent shaking. The extracts were filtered and concentrated under reduced pressure using a rotary evaporator, and the dried residues were stored at 4 °C until further phytochemical analysis.

2.4 HPTLC Analysis

Phytochemical profiling of methanolic and ethanolic extracts of basil (*Ocimum basilicum* L.), mint (*Mentha* spp.), and oregano (*Origanum vulgare* L.) was carried out using High-Performance Thin-Layer Chromatography (HPTLC) following standard analytical procedures. Pre-coated silica gel 60 F_{254} plates were used as the stationary phase, and sample solutions were applied as uniform bands using a CAMAG Linomat applicator to ensure reproducibility (**Reich & Schibli, 2007**). Chromatographic development was performed in a twin-trough chamber pre-saturated with the optimized mobile phase. Developed plates were air-dried and visualized under UV light at 254 nm and 366 nm to detect separated phytoconstituents. Post-derivatization was carried out using suitable spraying reagents such as anisaldehyde–sulfuric acid and ferric chloride reagent to enhance visualization of phenolic compounds. Densitometric scanning was performed using a CAMAG TLC scanner, and chromatograms were recorded to generate characteristic chemical fingerprints. The presence of phytoconstituents was assessed based on Rf values and band color providing a reliable comparative profile for each herb (**Wagner & Bladt, 2001; Srivastava, 2011**).

2.5 Statistical and Data Analysis

Densitometric data obtained from HPTLC analysis, including peak areas and Rf values, were processed using CAMAG visionCATS software, and comparative phytochemical profiles of basil (*Ocimum basilicum* L.), mint (*Mentha* spp.), and oregano (*Origanum vulgare* L.) were evaluated based on peak number, intensity, and migration behavior (**Reich & Schibli, 2007**). For molecular studies, BLAST results were interpreted based on percentage similarity and query coverage, while phylogenetic tree robustness was assessed by bootstrap analysis with 1,000 replicates using MEGA software (**Kumar *et al.*, 2018**). All analytical procedures were conducted in accordance with accepted standards to ensure reliability and validity of the results.

3. RESULT AND DISCUSSION

3.1 Molecular Characterisation

The phylogenetic analysis of the three query sequences (Ms, Ob, and Ov) demonstrated clear clustering within their respective genera of the family Lamiaceae. Sample Ms clustered tightly with Mentha species, particularly with Mentha arvensis, showing very short branch lengths that indicate minimal genetic divergence and confirm its placement within the genus Mentha. Sample Ob grouped directly with authenticated Ocimum basilicum reference sequences and showed very small genetic distances, validating its species-level identity. Sample Ov clustered within the Origanum clade alongside Origanum vulgare and closely related taxa, where moderate branch lengths reflected expected intra-genus variation while still confirming correct taxonomic placement. Overall, the topology of the phylogenetic trees clearly supports the molecular identification of all three samples at the genus and species levels.

Table 1: BLAST-Based Closest Match and Percentage Identity of Plant.

S.No.	Sample Code	Closest Species	% Identity
1	Ms	*Mentha arvensis*	99.28
2	Ob	*Ocimum basilicum*	99.64
3	Ov	*Origanum vulgare*	98.93

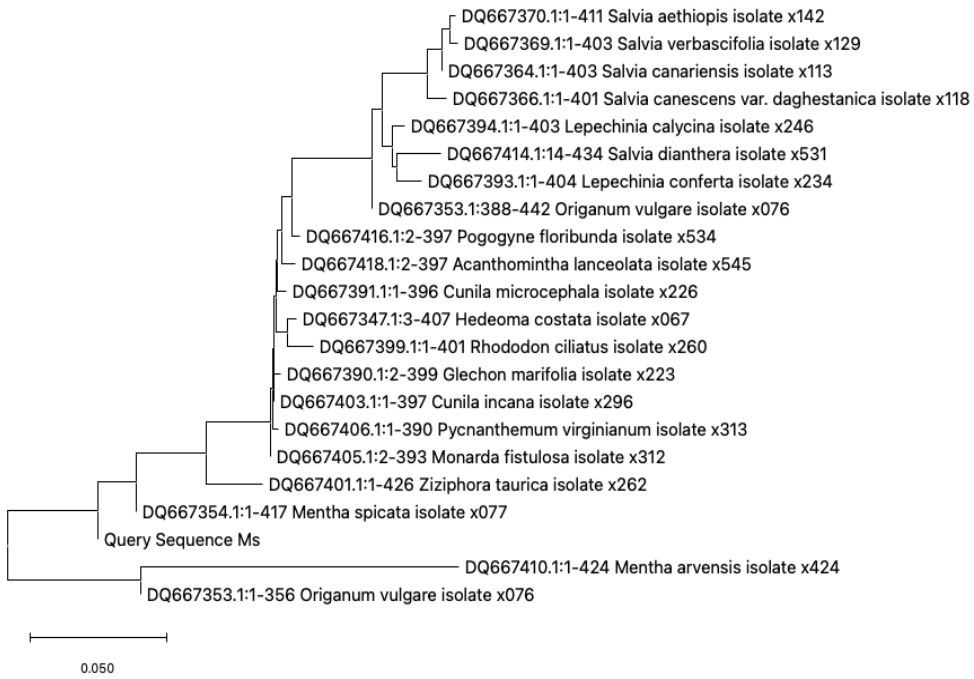

Figure 1: Phylogenetic tree showing Query Sequence Ms clustering closely with *Mentha arvensis*, confirming its identification within the genus *Mentha* (scale bar = 0.050).

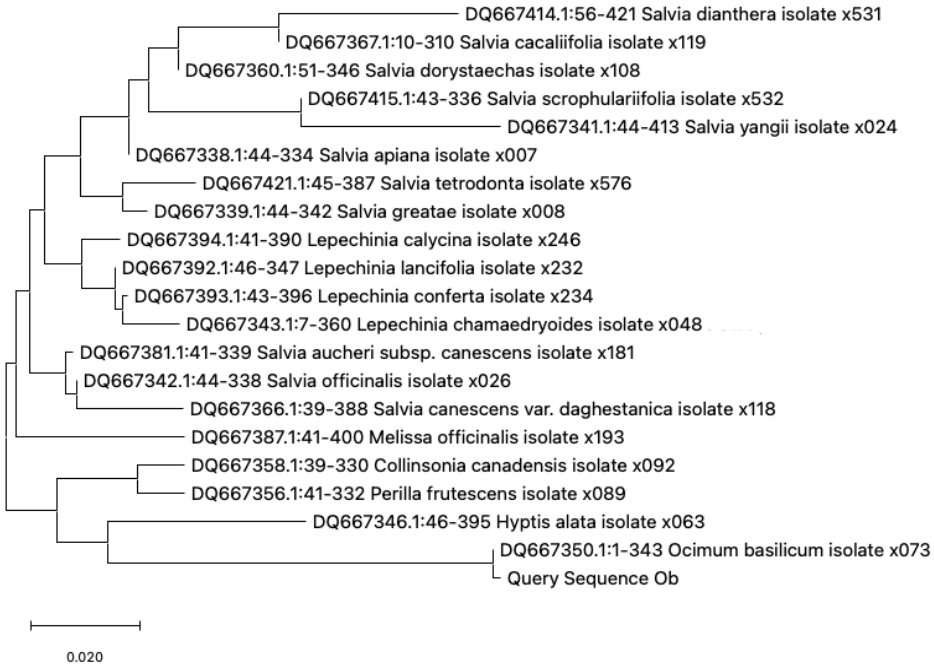

Figure 2: Phylogenetic tree showing Query Sequence Ob clustering with *Ocimum basilicum* and closely related Lamiaceae members, confirming its species identity (scale bar = 0.020).

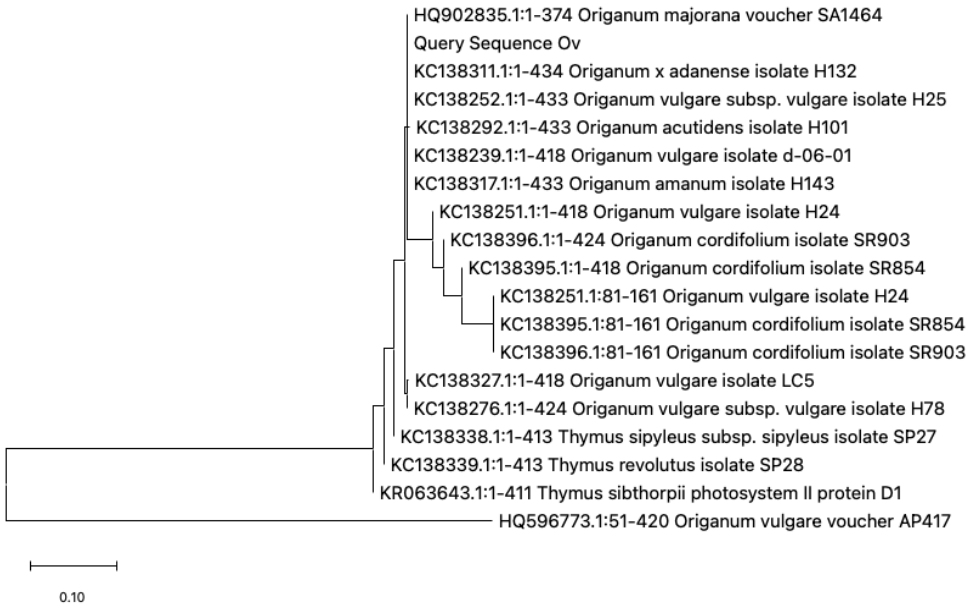

Figure 3: Phylogenetic tree showing Query Sequence Ov clustering within the *Origanum* clade, closely related to *Origanum vulgare*, confirming its species identity (scale bar = 0.10).

The molecular identification of the three medicinal plant samples was confirmed through the combined interpretation of BLAST similarity results and phylogenetic tree analysis. Sample Ms showed 99.28% sequence identity with Mentha arvensis and clustered within the Mentha group in the phylogenetic tree, confirming its identity as Mentha spicata. Sample Ob exhibited the highest sequence similarity (99.64%) with Ocimum basilicum and showed tight phylogenetic clustering with authenticated Ocimum sequences, validating precise species identification. Sample Ov demonstrated 98.93% identity with Origanum vulgare and grouped within the Origanum clade in the phylogenetic tree, confirming its correct taxonomic placement despite natural intra-genus variation. The strong agreement between BLAST percentage identity and phylogenetic clustering provides robust evidence for accurate molecular authentication of all three plant samples.

3.2 HPTLC Fingerprint Analysis for Gallic Acid

HPTLC analysis was carried out for the qualitative detection of gallic acid in methanolic extracts of OB (*Ocimum basilicum*), OC (*Origanum vulgare*), and MS (*Mentha spicata*) using silica gel 60 F_{254} plates. The mobile phase comprised ethyl acetate: formic acid: acetic acid: water (100:11:11:26 v/v/v/v). Densitometric scanning was performed at 254 nm.

The gallic acid standard (50 µg/mL) produced a sharp and well-resolved peak at Rf 0.857, which was taken as the reference. All three plant extracts exhibited peaks at or very near this Rf value, confirming the presence of gallic acid in the samples.

Table 2: Rf values and peak areas of gallic acid standard and plant extracts showing matching Rf (~0.857) that confirms the presence of gallic acid in OB (*Ocimum basilicum*), OC (*Origanum vulgare*), and MS (*Mentha spicata*).

Sample	Rf value	Peak Area (AU)
Gallic acid standard	0.857	0.00447
OB (Ocimum basilicum)	0.857	0.00581
OC (Origanum vulgare)	0.857	0.00777
MS (Mentha spicata)	0.855	0.00072

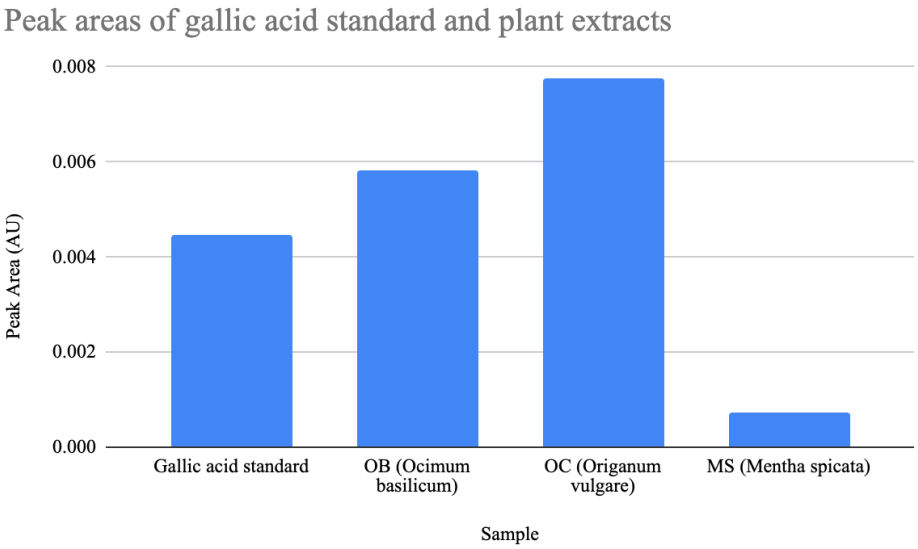

Figure 4: Peak areas of gallic acid standard and plant extracts.

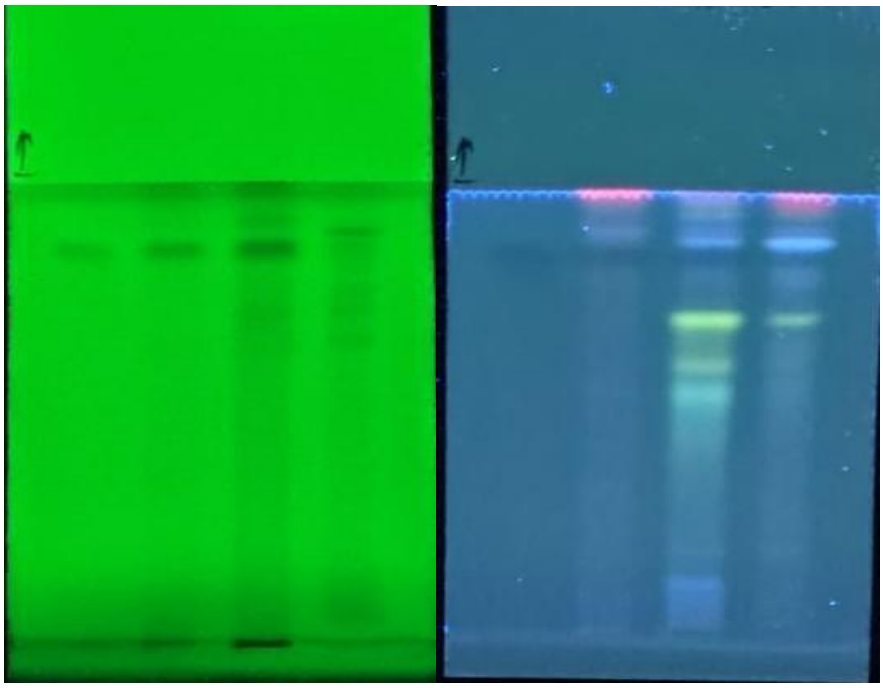

Figure 5: Plate observed at 254nm & 366nm.

The identical Rf values observed in the plant extracts and the gallic acid standard confirm the qualitative presence of gallic acid in all samples. Among the tested extracts, OC showed the highest peak area, indicating comparatively higher gallic acid content, followed by OB. MS exhibited a very small peak area, suggesting only a trace amount of gallic acid. The HPTLC fingerprint thus validates the occurrence of this important phenolic compound in the selected medicinal plants.

3.3 Data Analysis Results

Densitometric evaluation of the HPTLC chromatograms was carried out using CAMAG visionCATS software at 254 nm. The gallic acid standard produced a sharp and well-resolved peak at an Rf value of 0.857. Methanolic extracts of Ocimum basilicum, Mentha spicata, and Origanum vulgare also exhibited peaks at Rf values ranging from 0.855 to 0.857, indicating chromatographic correspondence with the standard and confirming the presence of gallic acid in all samples. Comparison of densitometric peak areas showed variation among the extracts. *Origanum vulgare* exhibited the highest peak area (0.00777 AU), followed by *Ocimum basilicum* (0.00581 AU), while *Mentha spicata* showed a very small peak area (0.00072 AU), suggesting only trace levels of gallic acid. The similarity in migration behavior and peak characteristics between the standard and plant extracts validated the qualitative detection of this phenolic marker. For molecular analysis, the ITS, rbcL, and matK sequences obtained from the samples were subjected to BLAST analysis against the NCBI GenBank database. High query coverage (>98%) and sequence similarity were observed with authenticated reference species. Sample Ob showed 99.64% similarity with *Ocimum basilicum*, sample Ms showed 99.28% similarity with *Mentha arvensis*, and sample Ov showed 98.93% similarity with *Origanum vulgare*.

Multiple sequence alignment using ClustalW and phylogenetic tree construction by the Neighbor-Joining method in MEGA X (with 1,000 bootstrap replicates) demonstrated clear clustering of each sample within its respective genus of the family Lamiaceae. The phylogenetic placement, supported by significant bootstrap values, confirmed the genetic identity and taxonomic position of the studied herbs.

4. DISCUSSION

The present study adopted an integrated analytical strategy combining DNA barcoding and HPTLC fingerprint profiling to achieve reliable authentication and quality evaluation of three widely used small herbs *Ocimum basilicum* (basil), *Mentha spicata* (mint), and *Origanum vulgare* (oregano). This dual approach addressed two fundamental requirements of herbal standardization: confirmation of genetic identity and assessment of phytochemical composition. Such integration is increasingly important in herbal research and industry, where morphological similarities, environmental variations, and market-driven adulteration frequently compromise the authenticity and quality of raw plant materials.

Molecular characterization using the ITS, rbcL, and matK barcode regions proved highly effective for precise species-level identification. The BLAST similarity values obtained for the samples (Ms: 99.28%, Ob: 99.64%, Ov: 98.93%) were well within the accepted range for reliable molecular authentication. In addition, phylogenetic tree analysis demonstrated distinct clustering of each query sequence within its respective genus in the family Lamiaceae. The minimal branch distances observed for *Mentha* and *Ocimum* indicated very low genetic divergence, while the moderate branch length in *Origanum* reflected expected intra-genus variation. The strong concordance between BLAST results and phylogenetic placement reinforces the robustness of DNA barcoding in distinguishing closely related medicinal and culinary herbs that are otherwise difficult to differentiate based on morphology alone. These findings align with previous studies highlighting the applicability of ITS and chloroplast markers for medicinal plant authentication and detection of adulterants.

Although molecular tools confirm species identity, they do not provide insight into the chemical constituents responsible for therapeutic activity. Therefore, HPTLC-based phytochemical profiling was performed using gallic acid as a marker phenolic compound. The HPTLC chromatograms revealed that all three plant extracts exhibited bands at an Rf value (~0.857) identical to the gallic acid standard, confirming its presence across samples. However, significant variation was observed in peak areas: *Origanum vulgare* showed the highest intensity, followed by *Ocimum basilicum*, while *Mentha spicata* displayed only a trace amount.

This variation reflects inherent phytochemical diversity among species within the same family. Oregano's higher gallic acid content corresponds with its documented phenolic richness and antioxidant capacity. Basil's moderate level supports its known medicinal relevance, whereas mint's minimal gallic acid presence suggests that its bioactivity may be primarily attributed to volatile compounds such as menthol rather than phenolic acids. Thus, HPTLC profiling not only confirmed phytoconstituent presence but also highlighted species-specific chemical differences.

5. CONCLUSION

The present study demonstrated a comprehensive approach for the authentication and quality assessment of three commonly used small herbs *Ocimum basilicum* (basil), *Mentha spicata* (mint), and *Origanum vulgare* (oregano) by integrating molecular characterization with HPTLC-based phytochemical profiling. DNA barcoding using ITS, rbcL, and matK regions,

supported by BLAST and phylogenetic analyses, confirmed accurate species-level identification and overcame the limitations of morphology-based methods, thereby enabling reliable detection of misidentification and adulteration. HPTLC fingerprint analysis revealed the presence of the phenolic compound gallic acid in all samples, with *Origanum vulgare* showing the highest relative content, followed by *Ocimum basilicum*, while *Mentha spicata* contained only trace levels, reflecting inherent phytochemical diversity. Together, DNA barcoding and HPTLC profiling provided complementary evidence of genetic authenticity and phytochemical quality, establishing a robust framework for herbal authentication, standardization, and quality control applicable to pharmaceutical and nutraceutical industries.

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