

Comprehensive Comparative Analysis of *Lippia citriodora* and *Stachys lavandulifolia* from Iran: Integrating GC-MS, Micromorphological, and 18S rRNA Phylogenetic Approaches

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Abstract

This study presents an integrated characterization of two medicinal Lamiaceae species, *Lippia citriodora* (lemon verbena) and *Stachys lavandulifolia* (mountain tea), cultivated and commercialized in Iran. Micromorphological observations revealed distinct anatomical features between academic and market samples. In *Lippia citriodora*, university-grown samples exhibited abundant glandular trichomes with multicellular globular heads, well-organized parenchymal cells, and uniformly distributed anomocytic stomata, indicating species purity and high quality, whereas these traits were underdeveloped in market samples. GC-MS analysis demonstrated qualitative and quantitative variations in essential oil composition: market samples showed limonene and citral dominance, while academic specimens contained higher carvacrol and citral levels with enhanced compound diversity. In *Stachys lavandulifolia*, samples from Shiraz and Shahreza showed quantitative differences in volatile profiles; thymol was dominant in the Shiraz specimen (69.03%) and n hexadecanoic acid (18.16%) in Shahreza. Molecular analysis of 18S rRNA sequences confirmed species identity and revealed minimal intra species variation (~97% identity), with conserved sequence blocks suitable for genus level primer design. Phylogenetic reconstruction validated the taxonomic classification and identified evolutionary differentiation across populations. Altogether, chemical, anatomical, and genetic evidence supports the authenticity of both plants and highlights environmental influences on their phytochemical characteristics. This integrated approach provides a reliable foundation for authenticity verification and quality control of medicinal plants in Iran's herbal market.

Keywords: *Stachys lavandulifolia*, *Aloysia citrodora*, GC-MS analysis, Micromorphology, Phylogenetic study, Authenticity.

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1. Introduction

The use of aromatic and medicinal

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plants has become integral to daily life, supported by advancements in modern medicine and a shift toward sustainable living (1). Due to their production of diverse phytochemicals for defense, plants are key sources for devel-

oping new herbal medicines (2). Despite the belief in their safety, ensuring quality, efficacy, and safety of herbal medicines remains essential due to regional differences in plant species (3, 4).

Stachys lavandulifolia Vahl, commonly known as “*Chai Kahi*” in Persian is a perennial flowering plant from the mint family (Lamiaceae), native to temperate regions of the Middle East, especially Iran. It is widely used in traditional medicine due to its analgesic, anti-inflammatory, and anxiolytic properties (5).

Extracts from the aerial parts of the plant are traditionally recommended for treating infections, asthma, inflammatory conditions, and rheumatism and is frequently used to alleviate joint pain, rheumatism, headaches, dizziness, and nerve-related discomfort (6).

Lemon verbena (*Aloysia citrodora* Paláu or *Lippia triphylla* Kuntze), is a perennial shrub from the *Verbenaceae* family. Native to South America—particularly Argentina, Bolivia, and Paraguay—it is now widely cultivated in warm, temperate, and tropical regions around the world. Its cultivation has expanded primarily due to its culinary uses and essential oil production, which is valued in both the food and cosmetic industries (7).

The chemical composition and quality of essential oils from medicinal plants are influenced by numerous factors such as geography, climate, genetic diversity, phenological stage, soil type, cultivation techniques, and extraction methods (1, 8). These variables significantly impact the oil's sensory and physiological properties. Key agricultural practices—like fertilization, irrigation, harvest timing, and drying—are also crucial for maximizing yield and maintaining essential oil quality (9, 10).

Variation in plant quality can arise from inter- or intraspecies genetic differences, environmental conditions (e.g., altitude, climate), the plant part used, and harvest timing, as active compounds can fluctuate throughout the plant's growth cycle or even within a day (11).

Standardization and reliable authentication methods are essential for ensuring the quality, safety, and efficacy of commercial

herbal products.

Various approaches have been developed to identify and prevent adulteration in herbal products and spices, depending on the processing methods involved. These include structural assessment through physical methods (e.g., macroscopic and microscopic analysis), physicochemical analyses, chemical profiling, and emerging biotechnological techniques (12, 13). Phytochemical profiling is essential for identifying bioactive constituents, using methods like chromatography, spectrophotometry, and biomarker-based fingerprinting (14, 15). Due to the limitations of traditional methods—especially for powdered or processed samples—modern DNA-based techniques such as PCR, DNA barcoding, and sequence alignment have gained prominence. These methods provide high specificity and are unaffected by morphological or environmental variations (16, 17). DNA barcoding has shown significant utility in detecting adulteration in various commercial products like saffron, turmeric, and olive oil (18–20), and bioinformatics tools like multiple sequence alignment (MSA) further enhance species identification accuracy (21).

The primary aim of this study is to conduct molecular identification, phylogenetic analysis, micromorphological examination, and GC-MS-based essential oil profiling of two *Stachys lavandulifolia* (chay-e kahi) and two *Aloysia citrodora* (lemon verbena) samples available in Iran's herbal market. The practical goal is to develop reliable tools for accurate identification and detection of potential adulteration in medicinal plants.

2. Materials and Methods

2.1. Materials and Equipment

All reagents and solvents used in this study were of analytical grade. DNA extraction kits were obtained from Pars Tous Biotechnology Company (Mashhad, Iran). PCR reagents including Taq DNA polymerase, dNTPs, and primers were supplied by Takapouzist Co. (Tehran, Iran). GC-MS analysis was performed using an Agilent 7890B gas chromatograph coupled with a 5977A mass spectrometer equipped with an HP-5MS col-

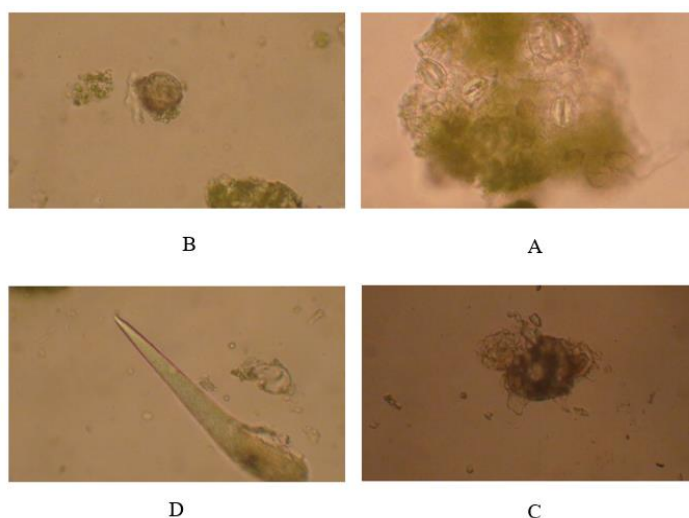


Figure 1. Micromorphological Analysis of *Lippia citriodora* (market sample) (a) anomocytic stomata; (b–c) surface view of the secretory head of glandular trichomes; (d) unicellular non-glandular trichomes.

umn (30 m×0.25 mm, 0.25 µm film thickness).

2.2. Plant Material and Identification

Dried powder of *Stachys lavandulifolia* Vahl and *Aloysia citriodora* Paláu were collected from different regions of Iran. Samples were authenticated by a botanist at the Herbarium of the Faculty of Pharmacy, and voucher specimens were deposited under specific accession numbers for future reference. The collected materials ground into fine powder for analysis.

2.3. Microscopic Studies

Powder microscopy was conducted following standard pharmacognosis procedures. Diagnostic features including trichomes, glandular hairs, calcium oxalate crystals, and stomatal types were observed using a light microscope (Leica DM500) after staining chloral hydrate and safranin. Representative micrographs were captured for identification and documentation (Figure 1).

2.4. Essential Oil Extraction

Essential oils were extracted from 100 g of powdered plant material by hydrodistillation using a Clevenger-type apparatus for 3 hours at approximately 100 °C. The obtained oils were dried over anhydrous sodium sul-

fate, stored in amber vials, and kept at 4 °C until analysis.

2.5. GC–MS Analysis

The essential oil constituents were analyzed using GC–MS under the following conditions: injector temperature 250 °C, split ratio 1:100, helium as carrier gas at 1 mL/min flow rate, and oven temperature programmed from 60 °C to 280 °C at 5 °C/min. Mass spectra were obtained in the electron impact mode at 70 eV with a scanning range of 40–500 m/z. Compounds were identified by comparison of their mass spectra with those in the NIST14 library and by calculating retention indices relative to a series of n-alkanes (C8–C25). Quantitative composition was determined by area normalization (%). (Table 1).

2.6. DNA Extraction

Genomic DNA was isolated from 100 mg of dried plant powder using the Pars Tous Plant DNA Extraction Kit (Pars Tous Biotechnology Co., Iran) according to the manufacturer's instructions. DNA purity and concentration were evaluated spectrophotometrically (NanoDrop 2000, Thermo Scientific) at 260/280 nm. Extracted DNA was stored at –20 °C until further analysis.

Table 1. GC-MS analysis of *Lippia triphylla* Market sample.

N.	Component	Area (%)	Retention Time	KI	KI _{ref}	Reference
1	α -Pinene	0.42	4.754	935.141	938	(30)
2	1-Octen-3-ol	0.71	5.581	978.259	979	(31)
3	Prenylacetone	0.68	5.745	986.809	988	(32)
4	dl-Limonene	17.92	6.864	1036.249	1035	(32)
5	1,8-Cineole	4.86	6.91	1038.175	1038	(32)
6	cis-Sabinene hydrate	0.96	7.656	1069.401	1070	(31)
7	β -Linalool	0.92	8.42	1101.266	1101	(33)
8	rose furan epoxide	0.51	10.384	1176.631	1177	(34)
9	4-Terpineol	0.47	10.483	1180.430	1180	(33)
10	α -Terpineol	2.3	10.886	1195.894	1197	(35)
11	trans-Carveol	0.72	11.573	1222.020	1220	(36)
12	Z-Citral	6.14	12.273	1248.595	1248	(37)
13	D-Carvone	0.79	12.337	1251.025	1254	(38)
14	Piperitone	0.6	12.558	1259.415	1260	(39)
15	E-Citral	7.81	13.077	1279.119	1279	(40)
16	Copaene	1.23	15.712	1381.097	1380	(41)
17	Geranyl acetate	1.63	15.84	1386.075	1386	(32)
18	β -Bourbonene	1.28	15.962	1390.821	1391	(42)
19	α -Cedrene	0.76	16.656	1418.618	1415	(43)
20	Caryophyllene	0.73	16.837	1425.976	1423	(44)
21	Aromadendrene	1.44	17.862	1467.642	1464	(38)
22	α -Curcumene	9.39	18.439	1491.098	1488	(45)
23	Calamenene	0.75	19.331	1528.590	1526	(43)
24	Nerolidol	1.36	20.427	1575.149	1577	(46)
25	Veridiflorol	1.08	21.121	1604.881	1600	(44)
26	α -Caryophylladienol	1.32	22.059	1646.888	1640	(47)
27	Acorenone B	0.53	23.271	1701.219	1700	(48)
28	Palmitic acid	0.71	28.669	1964.760	1964	(44)
29	Phytol	0.54	31.466	2113.700	2111	(49)
30	Monoterpenes	47.36%				
31	Sesquiterpenes	20.89%				
32	others	1.96%				
Identification		68.56				

2.7. PCR Amplification

The 18S rRNA gene fragment was amplified by PCR using universal primers synthesized by Takapouzist Co. (Tehran, Iran). The forward primer sequence was 5'-AACCTGGTTGATCCTGCCAGT-3' and the reverse primer 5'-TGATCCTTCTGCAG-GTTCACCTAC-3'. PCR reactions were carried out in a 25 μ L reaction mixture containing

12.5 μ L Master Mix (2 $\times$), 1 μ L of each primer (10 μ M), 2 μ L of DNA template, and nuclease-free water to volume. Thermal cycling conditions included an initial denaturation at 95 $^{\circ}$ C for 5 min, followed by 35 cycles of 95 $^{\circ}$ C for 30 s, 58 $^{\circ}$ C for 30 s, and 72 $^{\circ}$ C for 1 min, with a final extension at 72 $^{\circ}$ C for 10 min.

2.8. Agarose Gel Electrophoresis

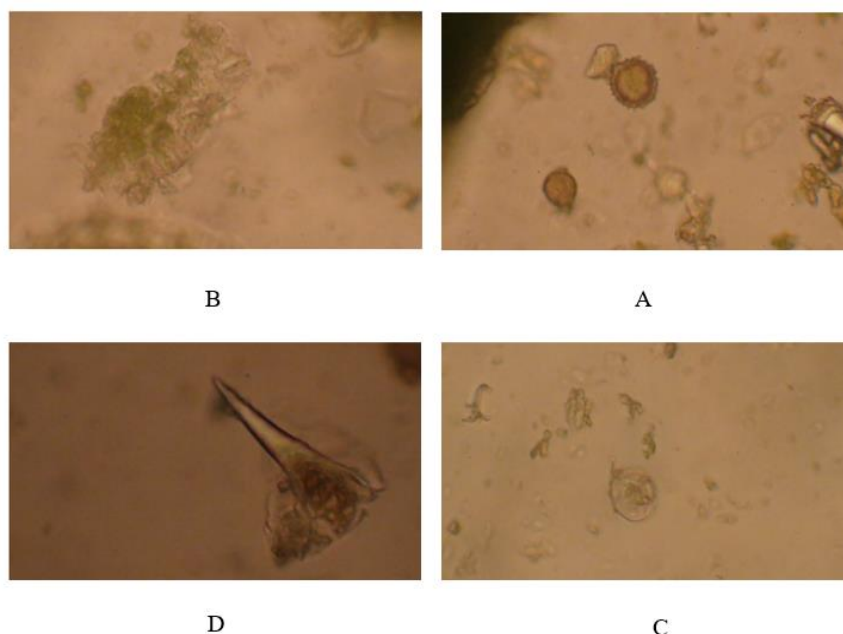


Figure 2. Micromorphological Analysis of *Lippia citriodora* (University sample) (a) pollen grain; (b) anomocytic stomata; (c) surface view of the secretory head of glandular trichomes; (d) unicellular non-glandular trichomes.

PCR products were analyzed by electrophoresis on 1.2% agarose gel stained with ethidium bromide and visualized under UV transilluminator (Vilber Lourmat, France). A 10 kbp DNA ladder was used as a molecular weight marker to verify the expected amplicon size (~700 bp) (Figure 2).

2.9. Sequencing and Phylogenetic Analysis

Purified PCR products were sequenced bidirectionally by a commercial sequencing service (Macrogen Inc., Seoul, South Korea). Sequences were trimmed and aligned using ClustalW in MEGA X software. BLAST analysis (NCBI) was performed to identify close matches in the GenBank database. Phylogenetic trees were constructed using the Neighbor-Joining method with 500 bootstrap replicates to determine genetic relationships among samples and reference taxa. CLC Sequence Viewer was used to visualize and annotate alignments (Figure 3).

3. Results

3.1. Microscopic Analysis of *Lippia citriodora*

In the market sample, scattered non-

glandular unicellular trichomes were observed, along with anomocytic stomata irregularly distributed on the leaf epidermis. Some images suggested the presence of structures resembling glandular trichomes; however, they lacked full clarity and the typical characteristics of *Lippia triphylla* (Figure 1).

In contrast, the university-grown sample exhibited well-defined glandular trichomes on the leaf epidermis, characterized by a multicellular spherical head and a distinct basal cell. Additionally, the presence of organized parenchymal cells with clearly visible nuclei and uniformly distributed anomocytic stomata on both sides of the leaf was noted. These features closely correspond to the anatomical descriptions of pure *Lippia triphylla* (Figure 2).

3.2. Microscopic Analysis of *Stachys lavandulifolia*

In the *Stachys lavandulifolia* sample from Shahreza, glandular trichomes with multicellular heads and stalks, regularly arranged anomocytic stomata, and parenchymal cells with distinct nuclei were clearly observed. Additionally, microscopic images revealed

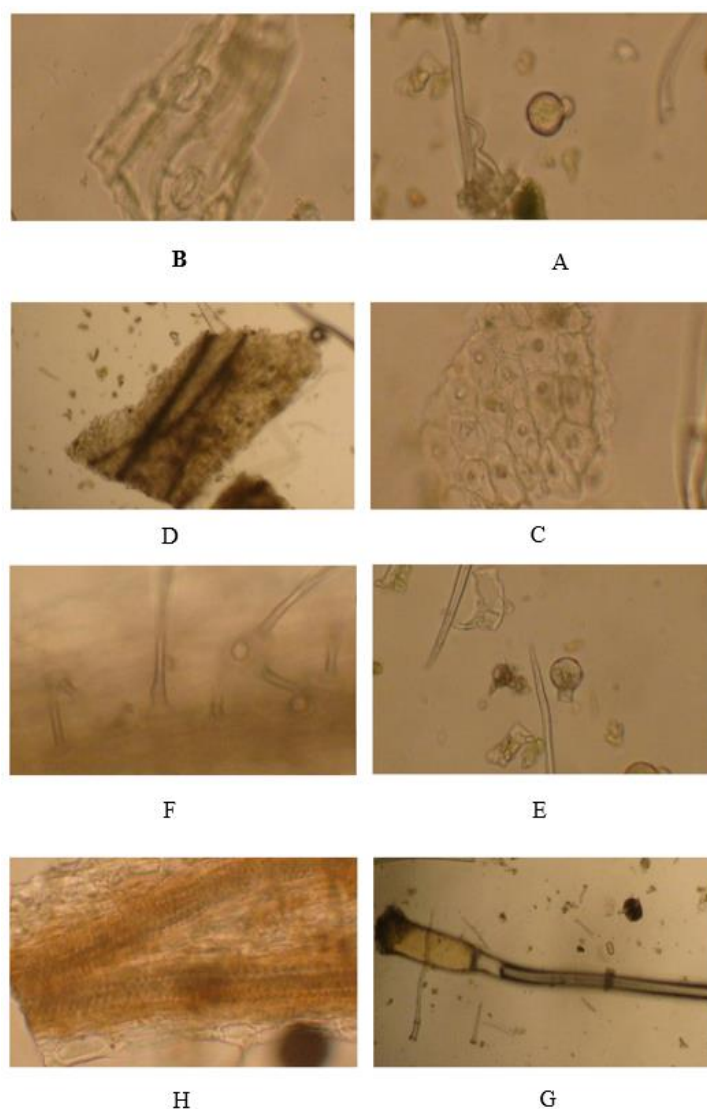


Figure 3. Micromorphological *Stachys lavandulifolia* (Shahreza sample) (a) pollen grain; (b) anomocytic stomata; (c) parenchyma cells with nuclei; (d) petal tissue section; (e) stalked glandular trichomes; (f) leaf surface trichomes; (g) surface view of the secretory head of glandular trichomes; (h) xylem vessels with reticulate thickenings.

xylem vessels with reticulate ornamentation in the leaf veins, which are consistent with the anatomical descriptions of the *Stachys lavandulifolia* species (Figure 3).

In the Shiraz sample, similar structures were observed, though the trichome density was lower and the cellular arrangement appeared more dispersed (Figure 4).

3.3. Essential Oil Composition Analysis (GC-MS)

A comparison of the volatile compounds in the market and faculty samples of

Lippia triphylla revealed significant differences. In the market sample (Table 1), the major constituents were limonene (17.92%), E-citral (7.81%), Z-citral (6.14%), and 1,8-cineole (4.86%). In contrast, the faculty-grown sample (Table 2), showed higher percentages of carvacrol (22.31%) and Z-citral (19.55%). Additionally, the total percentage of identified compounds was 68.56% in the market sample and 91.82% in the faculty sample.

In the GC-MS analysis of *Stachys lavandulifolia* from Shahreza (Table 3), 31 chemical compounds were identified, repre-

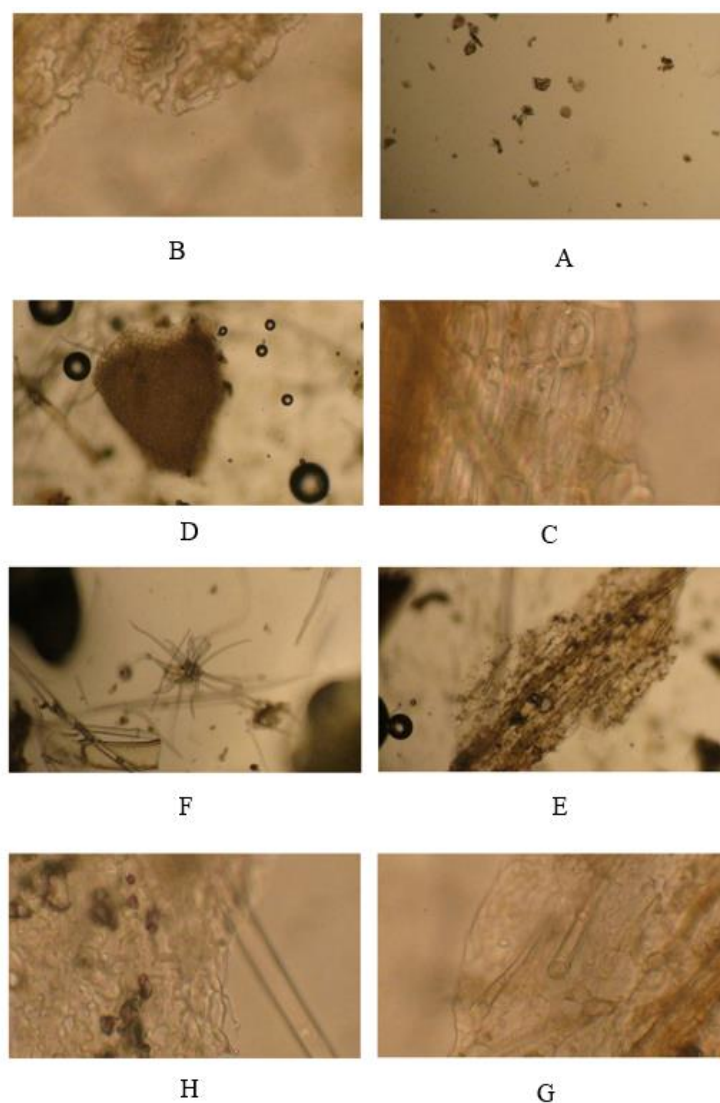


Figure 4. Micromorphological *Stachys lavandulifolia* (Shiraz sample) (a) pollen grain; (b) anomocytic stomata; (c) parenchyma cells with nuclei; (d) petal tissue section; (e) part of the leaf vein with xylem vessel; (f) branched non-glandular trichomes; (g) leaf surface view with non-glandular trichomes; (h) general leaf surface view.

senting 72.3% of the total composition. The major constituents were n-hexadecanoic acid (18.16%), thymol (12.54%), 2-pentadecanone (6.2%), and spathulenol (5.88%). These compounds primarily include fatty acids, terpenes, and phenols, indicating the plant's potential anti-inflammatory, antioxidant, and antibacterial properties.

In the GC-MS analysis of *Stachys lavandulifolia* from Shiraz (Table 4), 15 compounds were identified, accounting for 96.35% of the total composition. The dominant compound was thymol, at a remarkable

69.03%, followed by palmitic acid (6.32%), germacrene-D (3.84%), and spathulenol (3.54%). Most of these compounds belong to phenols, sesquiterpenes, and fatty acids, highlighting the strong medicinal potential of this sample, particularly its disinfectant and anti-inflammatory properties.

3.4. Molecular Phylogenetic Sequence

The 18S rRNA sequences obtained from the two commercial *Stachys lavandulifolia* (mountain tea) samples (Shiraz and Shahreza) were assembled into FASTA format

Table 2. GC-MS analysis of *Lippia triphylla* Faculty sample.

N.	Component	Area (%)	Retention Time	KI	KI _{ref}	Reference
1	α -Pinene	0.8	4.76	935.454	938	(50)
2	Sabinene	1.64	5.541	976.173	976	(30)
3	Prenylacetone	2.27	5.809	990.146	988	(32)
4	l-Limonene	13.69	6.899	1037.715	1031	(51)
5	1,8-Cineole	2.64	6.939	1039.389	1038	(32)
6	β -Ocimene	0.67	7.178	1049.393	1048	(52)
7	Sabinene hydrate	0.69	7.68	1070.406	1068	(53)
8	Linalool	0.65	8.455	1102.609	1101	(33)
9	Trans-chrysanthemal	0.82	9.778	1153.377	1153	(30)
10	Rose furan epoxide	1.31	10.437	1178.665	1172	(34)
11	α -Terpineol	1.8	10.897	1196.316	1197	(35)
12	Z-Citral	19.55	12.541	1258.770	1256	(54)
13	Carvacrol	22.31	13.398	1291.306	1294	(55)
14	Thymol	1.9	13.648	1300.817	1297	(56)
15	Copaene	0.82	15.758	1382.886	1380	(41)
16	Geranyl acetate	1.78	15.921	1389.226	1392	(56)
17	β -Bourbonene	0.49	16.003	1392.415	1391	(42)
18	Caryophyllene	3	16.936	1430.000	1429	(41)
19	Aromadendrene	0.81	17.897	1469.065	1468	(57)
20	ar-Curcumene	3.35	18.404	1489.675	1486	(31)
21	Bicyclogermacrene	0.82	18.801	1506.075	1505	(58)
22	Nerolidol	1.37	20.421	1574.894	1577	(46)
23	Spathulenol	8.64	20.922	1596.177	1594	(45)
24	Monoterpene	71.54%				
25	Sesquiterpenes	19.3%				
26	other	0				
Identification		91.82%				

and subjected to BLAST analysis in the NCBI database.

After filtering for the genus *Stachys*, eight reference sequences with the highest similarity were selected for multiple sequence alignment. Pairwise comparison revealed that the two Iranian samples were highly similar within the amplified 18S rRNA region, with a sequence identity of approximately 97.1%, indicating minimal genetic variation and confirming that they belong to the same genus and species.

When compared with reference sequences from the *Stachys* genus, the similarity

values varied from ~57% to ~71%. For example, the Shiraz sample showed 58.2% similarity with *S. cretica* (KF577385.1) and 70.95% with *S. arrecta* (OR878468.1), suggesting the presence of interspecific differences.

Position-by-position alignment using CLC software revealed several conserved blocks across all sequences (defined as regions $\geq 90\%$ conservation and ≥ 5 nt in length). The most conserved block was located at positions 625-676 (52 bp), followed by 234-278 (45 bp), 147-181 (35 bp), and 703-726 (24 bp). These conserved regions represent functionally critical parts of the ribosomal gene, which

Table 3. GC-MS analysis of *Stachys lavandulifolia* Shahreza sample.

N.	Component	Area (%)	Retention Time	KI	KI _{ref}	Reference
1	α -Terpinolene	0.36	8.391	1100.153	1097	(50)
2	Propanal, 2-methyl-3-phenyl	0.6	12.104	1242.179	1244	(59)
3	Thymol	12.54	13.456	1293.508	1293	(60)
4	Carvacrol	1.81	13.678	1301.984	1301	(60)
5	α -Copaene	0.7	15.677	1379.736	1377	(61)
6	trans-Caryophyllene	0.76	16.796	1424.309	1423	(44)
7	Aromadendrene	0.66	17.286	1444.228	1441	(62)
8	β -Farnesene	0.44	17.624	1457.967	1458	(63)
9	α -Amorphene	0.64	18.183	1480.691	1482	(64)
10	α -Curcumene	0.42	18.3	1485.447	1486	(31)
11	β -Ionene	0.4	18.381	1488.740	1488	(30)
12	γ -Murolene	0.78	18.632	1498.943	1502	(65)
13	α -Murolene	0.84	18.923	1511.257	1508	(66)
14	γ -Cadinene	0.87	19.104	1518.946	1515	(67)
15	Selina-3,7(11)-diene	0.54	19.215	1523.662	1523	(68)
16	δ -Cadinene	2.75	19.314	1527.867	1526	(69)
17	α -Cadinene	0.43	19.652	1542.226	1541	(70)
18	Lauric acid	1.34	20.212	1566.015	1567	(31)
19	Spathulenol	5.88	20.649	1584.579	1582	(44)
20	Caryophyllene oxide	2.59	20.777	1590.017	1589	(44)
21	Dillapiole	0.41	21.651	1628.616	1625	(71)
22	Isospathulenol	0.71	21.983	1643.484	1641	(72)
23	t-Murolol	1.06	22.356	1660.188	1654	(73)
24	Tetradecanoic acid	3.19	24.641	1765.448	1765	(74)
25	Hexahydrofarnesyl acetone	6.2	26.332	1846.903	1847	(75)
26	Pentadecanoic acid	0.37	26.606	1860.374	1863	(76)
27	Isobutyl phthalate	1.93	26.821	1870.944	1870	(77)
28	n-Hexadecanoic acid	18.16	28.815	1972.282	1972	(78)
29	Linolic acid	3.33	31.898	2137.001	2134	(44)
30	Stearic acid	0.76	32.353	2161.543	2162	(79)
31	Monoterpene	14.71%				
32	Sesquiterpenes	25.27%				
33	other	31.49%				
Identification		71.47%				

have remained structurally stable over evolutionary timescales. Such regions are ideal targets for designing genus-wide primers and for molecular assays such as qPCR or DNA barcoding. In addition to conserved domains, several variable regions and short indels were observed, including a notable indel around

position ~240. These variable regions could serve as molecular markers for species-level differentiation.

Alignment with unrelated plant taxa (e.g., *Panax*, *Capsicum*, *Vitex*, *Salvia*, *Plantago*) demonstrated minimal sequence similarity outside of highly conserved ribosomal

Table 4. GC-MS analysis of *Stachys lavandulifolia* Shiraz sample.

N.	Component	Area (%)	Retention Time	KI	KI _{ref}	Reference
1	β-Pinene	0.72	5.605	979.510	978	(56)
2	1,8-Cineole	2.01	6.794	1033.319	1033	(80)
3	D-Carvone	1.22	12.465	1255.885	1256	(38)
4	Thymol	69.03	13.683	1302.178	1302	(42)
5	Carvacrol	1.35	13.8	1306.729	1305	(60)
6	α-Copaene	0.86	15.706	1380.863	1380	(81)
7	trans-Caryophyllene	0.99	16.825	1425.488	1423	(44)
8	Germacrene-D	3.84	18.364	1488.049	1485	(31)
9	Bicyclogermacrene	1.91	18.719	1502.591	1505	(58)
10	σ-Cadinene	1.49	19.331	1528.590	1528	(44)
11	Spathulenol	3.54	20.678	1585.811	1582	(44)
12	Caryophyllene oxide	1.15	20.8	1590.994	1581	(82)
13	Hexahydrofarnesyl acetone	1.92	26.337	1847.148	1847	(75)
14	Palmitic acid	6.32	28.82	1972.540	1972	(78)
15	Monoterpene	74.33%				
16	Sesquiterpenes	15.7%				
17	others	6.32%				
Identification		96.35%				

regions, with frequent gaps and penalties observed across the alignment. This indicates that while phylogenetic confirmation of *S. lavandulifolia* is possible, genetic similarity does not necessarily correlate with pharmacological potency, which may vary significantly depending on the region of harvest.

Similarly, 18S rRNA sequences extracted from *Aloysia citrodora* (lemon verbena) samples—one from the academic collection and one from the commercial market—were analyzed. Pairwise alignment showed a sequence identity of 95.78%, suggesting that both samples belong to the same species. Comparisons with other *Aloysia* species showed similarity values ranging from 84.5% to 98.5%, with the highest similarity to *L. filifolia* and *L. florida*. Detailed alignment revealed that 578 nucleotide positions (~79.7%) were ≥95% conserved. Thirty conserved blocks (≥6 bp) were identified, with the longest located at positions 27–50 (24 bp), 52–74 (23 bp), 100–114 (15 bp), and 137–152 (16 bp). Variable regions and short indels were also detected, particularly within positions

200–240 and around 400–430 bp, which could serve as evolutionary markers for species discrimination or population-level studies.

As observed in *Stachys*, cross-alignment with distantly related taxa showed similarity only in short, highly conserved ribosomal regions (10–20 bp), confirming that 18S rRNA is most suitable for broad phylogenetic comparisons and genus-level identification rather than fine-scale differentiation.

Notably, *Aloysia* sequences exhibited longer, and more numerous conserved regions compared to *Stachys*, and phylogenetic trees generated from the aligned sequences displayed more branches with bootstrap values >50%, indicating higher confidence in phylogenetic relationships. In contrast, *Stachys* sequences showed fewer well-supported branches, likely due to limited reference data and greater intraspecific genetic diversity.

In *Lippia citriodora*, the Genus-level comparison, Both school and market samples clustered closely with *Lippia citriodora* reference sequences (bootstrap support: 98%), confirming their correct taxonomic identifica-

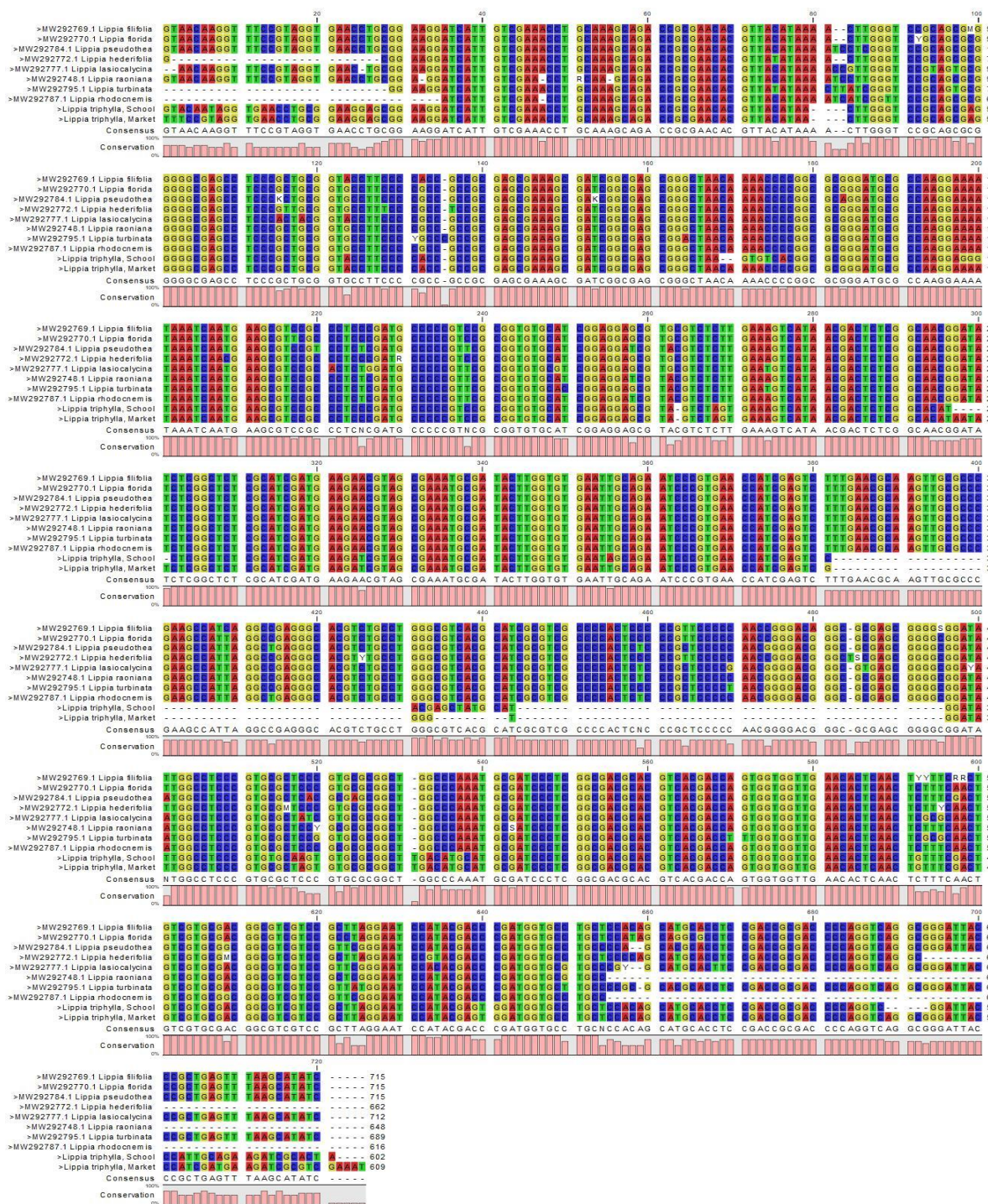


Figure 5. Multiple sequence alignment results of the 18S rRNA gene sequences within the genus *Lippia* (including two analyzed samples: School and Market, and eight reference sequences retrieved from the NCBI GenBank database shown on the left panel). The pink columns indicate the percentage of sequence conservation across aligned regions.

tion (Figure 5). In the green plant comparison, the analyzed samples showed clear genetic divergence from non-*Lippia* green plants, supporting species-level specificity (Figure 6). In the market adulterant comparison, both popu-

lations were genetically distinct from the 20 commercial herbal samples analyzed, indicating no evidence of adulteration (Figure 7).

In *Stachys lavandulifolia*, the genus-level comparison, both Shiraz and Shahreza



Figure 6. Multiple sequence alignment results of the 18S rRNA gene sequences among green plant species (including two analyzed samples: Faculty and Market, and eight reference sequences retrieved from the NCBI GenBank database shown on the left panel). The pink columns indicate the percentage of sequence conservation across aligned regions.

samples clustered closely with *Stachys lavandulifolia* reference sequences (bootstrap support: 98%), confirming their correct taxonomic identification (Figure 8). In the Green

plant comparison, the analyzed samples showed clear genetic divergence from non-*Stachys* green plants, supporting species-level specificity (Figure 9). In the market adulter-

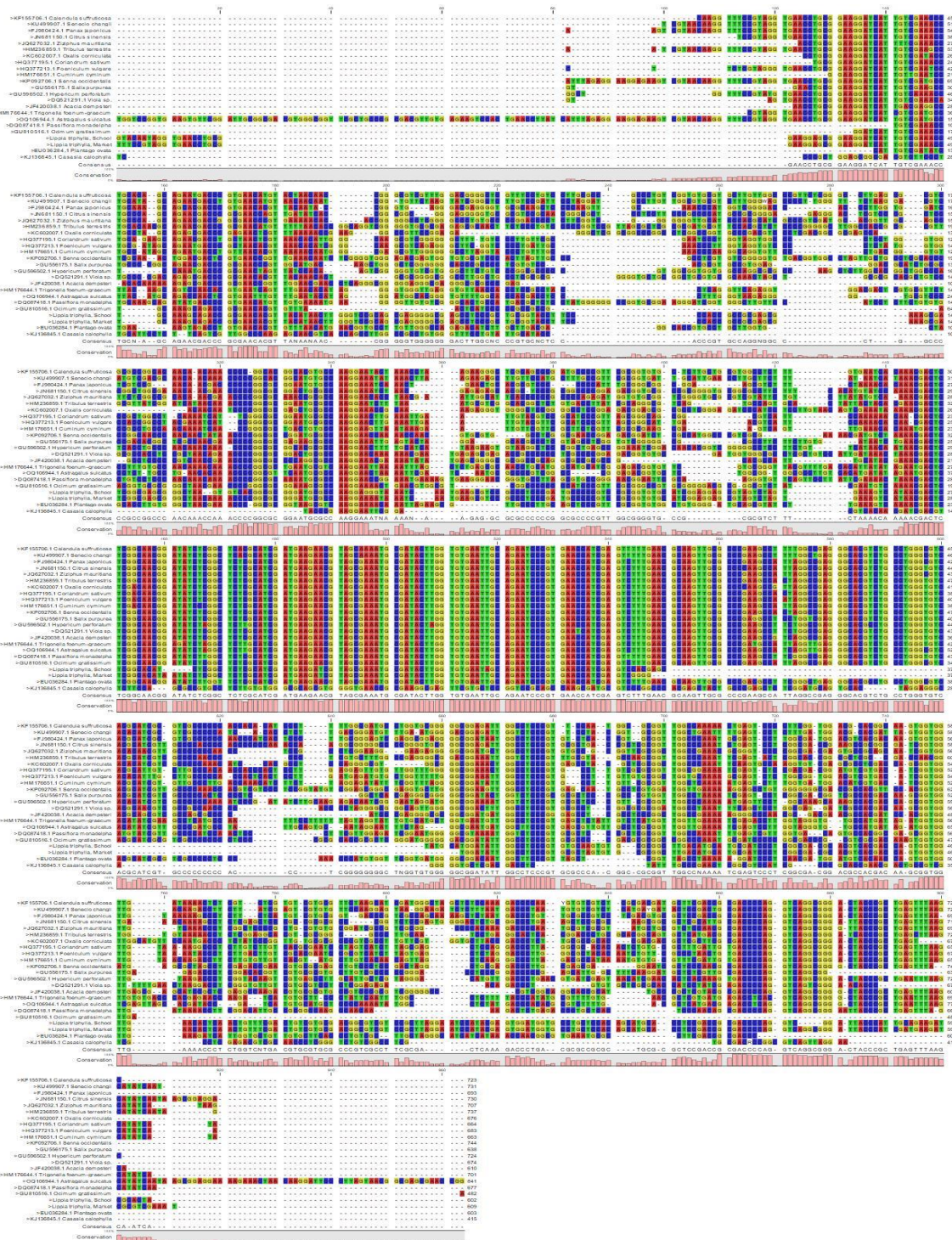


Figure 7. Multiple sequence alignment results of the 18S rRNA gene sequences among various medicinal plant species available in the Iranian herbal market (including two analyzed samples: Faculty and Market, and related reference sequences retrieved from the NCBI GenBank database shown on the left panel). The pink columns indicate the percentage of sequence conservation across aligned regions.

ant comparison, both populations were genetically distinct from the 20 commercial herbal samples analyzed, indicating no evidence of adulteration (Figure 10). The figures demon-

strating the MSA and conserved areas are presented in supplementary A.

3.5. Phylogenetic Analysis



Figure 8. Multiple sequence alignment results of the 18S rRNA gene sequences within the genus *Stachys* (including two analyzed samples: Shiraz and Shahreza, and eight reference sequences retrieved from the NCBI GenBank database shown on the left panel). The pink columns indicate the percentage of sequence conservation across aligned regions.

To elucidate the evolutionary relationships among the studied samples, phylogenetic trees were constructed using the Maximum Likelihood (ML) method. The analysis was based on the multiple sequence alignment

matrix of the 18S rRNA gene, which included the two *Stachys lavandulifolia* samples from Shiraz and Shahreza along with eight reference sequences from the *Stachys* genus. A separate analysis was performed for *Aloysia citrodora*



Figure 9. Multiple sequence alignment results of the 18S rRNA gene sequences among green plant species (including two analyzed samples: Shiraz and Shahreza, and eight reference sequences retrieved from the NCBI GenBank database shown on the left panel). The pink columns indicate the percentage of sequence conservation across aligned regions.

(lemon verbena), including the academic collection sample (school) and the commercial market sample, together with eight reference sequences from the *Lippia* genus.

The phylogenetic tree was reconstructed using the Tamura–Nei nucleotide substitution model (1993). The tree with the highest log-likelihood value (Log Likelihood =



Figure 10. Multiple sequence alignment results of the 18S rRNA gene sequences among various medicinal plant species available in the Iranian herbal market (including two analyzed samples: Shiraz and Shahreza, and related reference sequences retrieved from the NCBI GenBank database shown on the left panel). The pink columns indicate the percentage of sequence conservation across aligned regions.

–2214.77) was selected as the final tree. The reliability of branching patterns was assessed through bootstrap analysis with 500 replications, and the bootstrap values are displayed next to the corresponding branches.

During the heuristic search phase, the

initial tree was generated and compared using both Neighbor-Joining (NJ) and Maximum Parsimony (MP) methods. The tree topology with the highest likelihood score was used as the starting point for ML analysis. The NJ tree was constructed based on a pairwise distance

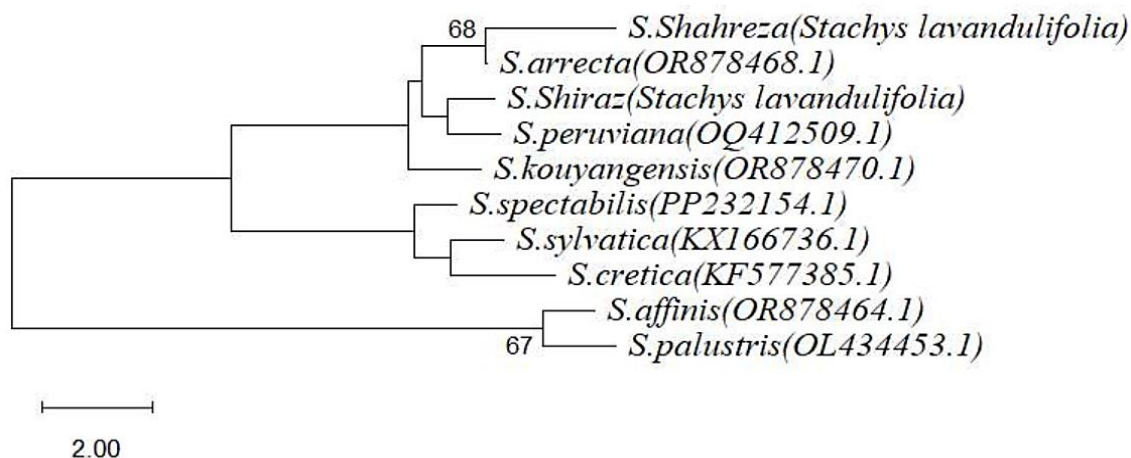


Figure 11. Phylogenetic tree of *Stachys lavandulifolia* (mountain tea) generated by Maximum Likelihood analysis based on 18S rRNA gene sequences.

matrix calculated using the p-distance model, while the MP tree was selected from the ten shortest trees generated from independent random starting trees. Figures 11 and 12 demonstrate phylogenetic trees of *Stachys lavandulifolia* and *Aloysia citrodora* respectively using maximum likelihood analysis based on 18S rRNA gene sequences.

The dataset consisted of 10 nucleotide sequences, including coding positions (first, second, and third codons) as well as non-coding regions, resulting in a total of 823 nucleotide positions used in the final analysis. All evolutionary analyses were performed using MEGA version 12 (22) with up to seven par-

allel processing threads to enhance computational efficiency.

4. Discussion

This study provides a comprehensive insight into the phytochemical composition and microscopic structural characteristics of two widely used medicinal plants: *Lippia triphylla* (lemon verbena) and *Stachys lavandulifolia* (mountain tea). The aim was to evaluate the quality of commercial samples compared to authenticated academic specimens and to explore the key factors influencing the observed differences in their chemical profiles and anatomical features.

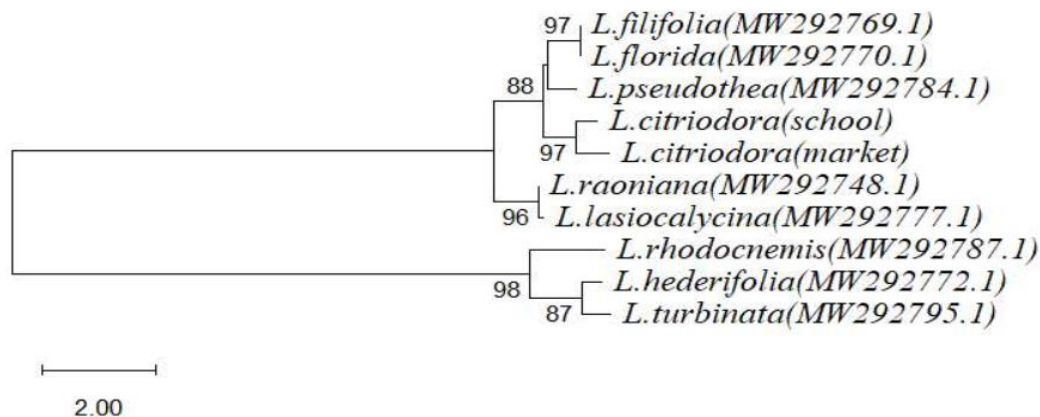


Figure 12. Phylogenetic tree of *Aloysia citrodora* (lemon verbena) generated by Maximum Likelihood analysis based on 18S rRNA gene sequences.

Microscopic (microtomy) analysis revealed distinct structural differences between commercial and academic samples in both species. In *Lippia triphylla*, the authenticated university samples displayed well-defined secretory glandular trichomes with multicellular globular heads and uniformly distributed anomocytic stomata—morphological markers that reflect species authenticity and high-quality standards. In contrast, these features were either underdeveloped or entirely absent from commercial samples. Previous studies have identified the presence and morphology of glandular trichomes as critical diagnostic tools for identifying essential oil-bearing species, where their degradation or absence may indicate quality loss or adulteration (23). Notably, due to the limited previous research on the detailed anatomy of *Lippia triphylla*, the microscopic insights provided here may offer valuable benchmarks for its botanical authentication.

For *Stachys lavandulifolia*, micromorphological differences were observed between samples collected from Shahreza and Shiraz. The Shahreza samples demonstrated a higher density of glandular trichomes and more organized parenchyma cells and stomatal arrangements, suggesting a potential influence of environmental factors such as light intensity, humidity, and soil composition. These observations are consistent with earlier studies that reported micromorphological variability in *S. lavandulifolia* populations as a result of ecological and climatic diversity across regions (24, 25).

The phytochemical analysis using GC-MS further highlighted significant variation in essential oil composition between the samples. In *Lippia triphylla*, the university specimen exhibited greater chemical diversity and higher concentrations of biologically active compounds such as carvacrol and Z-citral, while the commercial sample contained predominantly limonene and fewer bioactive constituents. Such discrepancies are likely

attributable to differences in post-harvest processing, storage conditions, or even adulteration. Moreover, variations in extraction methods and geographic origin are known to significantly influence essential oil composition, which aligns with previous findings on the chemical variability of *Lippia triphylla* (26, 27).

Similarly, the GC-MS analysis of *Stachys lavandulifolia* revealed pronounced regional differences. The sample from Shiraz showed a remarkably high thymol content (~69%), while the Shahreza sample contained moderate levels (~12%). In contrast, populations from northwestern Iran and Khorramabad were previously reported to be rich in sesquiterpenes such as α -cadinol and spathulenol (28, 29). These results underscore the strong influence of geographical and climatic conditions on the phytochemical profiles of medicinal plants. For instance, the dominance of phenolic compounds like thymol in southern regions and the prevalence of sesquiterpenes in the northwest reflect region-specific biosynthetic pathways shaped by local environmental stressors.

It is also important to note that the nature of dominant compounds directly affects both the biological activity and industrial applicability of medicinal plants. Phenolic-rich chemotypes (e.g., thymol and carvacrol) are associated with stronger antimicrobial and sedative effects, making them suitable for pharmaceutical and sanitizing applications. Conversely, sesquiterpene-rich profiles are often more effective in anti-inflammatory and antioxidant formulations.

In alignment with the practical goal outlined in the introduction, to develop reliable tools for accurate identification and detection of potential adulteration in medicinal plants, this study successfully integrates micromorphological, GC-MS, and 18S rRNA phylogenetic approaches to provide a multifaceted framework for authenticity verification. Our results demonstrate that micromorphological

features, such as the presence and structure of glandular trichomes and anomocytic stomata, serve as visual markers to distinguish high-quality academic samples from potentially adulterated market samples in *L. citriodora*. The GC-MS profiling revealed significant qualitative and quantitative differences in essential oil compositions, offering chemical fingerprints that can detect variations indicative of adulteration or environmental influences. Furthermore, the 18S rRNA sequence analysis confirmed species identity with high similarity ($\approx 97\%$ intraspecies) and identified conserved blocks suitable for designing genus-specific primers, enabling DNA-based barcoding for rapid, non-morphological authentication even in processed herbal products. Collectively, these methods form a robust, integrated toolkit that addresses adulteration risks in Iran's herbal market, as evidenced by the discrepancies observed between commercial and cultivated samples. This approach not only verifies authenticity but also highlights the impact of cultivation practices on phytochemical quality, providing actionable insights for regulatory standards and quality control protocols.

5. Conclusion

This study successfully combined pharmacognosy, phytochemical, and molecular techniques to authenticate and characterize *Stachys lavandulifolia* and *Lippia citriodora* from different Iranian sources. Morphological and microscopic observations revealed distinct structural features between populations, while GC-MS profiling confirmed qualitative and quantitative differences in essential oil composition, reflecting environmental and genetic influences.

The molecular results based on 18S rRNA sequencing and phylogenetic analysis further validated the taxonomic identity of all samples and highlighted genetic differentiation between populations collected from distinct locations or markets. Importantly, comparative analyses with closely related species,

green plants, and market adulterants provided clear molecular evidence of authenticity and potential adulteration risks in commercial herbal products.

Overall, the integrated biotechnological and pharmacognosy approach presented here provides a reliable framework for the authentication and quality control of medicinal plants in Iran's herbal market. Such multidisciplinary methods can be extended to other plant species to ensure the efficacy, purity, and reproducibility of herbal-based pharmaceuticals.

Authors contributions

MMZ: Resources, Conceptualization, Methodology, Project administration, Visualization, Funding acquisition, Supervision, Validation. NT: Investigation, Writing, Review, Data curation, Editing, Visualization, Formal analysis. MHM: Resources, Conceptualization, Methodology, Project administration, Visualization, Supervision, Validation.

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Data availability

Data supporting the findings of this study can be obtained from the corresponding author upon reasonable request.

Conflict of Interest

The authors declare that they have no conflict of interest.

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