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Integrated Molecular and Chemical Authentication of *Thymus* Species: Implication for Industrial Quality Control

Mostafa Ebadi[1]*, Shaghayegh Valinejad[1] and Saeed Mollaei[2]

[1] *Department of Biology, Faculty of Science, Azarbaijan Shahid Madani University, Tabriz, Iran*
[2] *Department of Chemistry, Faculty of Science, Azarbaijan Shahid Madani University, Tabriz, Iran*

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***Corresponding author**
Mostafa Ebadi
Department of Biology, Faculty of Science, Azarbaijan Shahid Madani University, Tabriz, Iran
ebadi2023@gmail.com

ABSTRACT

This study integrates molecular, phytochemical, and morphological approaches to accurately identify and characterize *Thymus* species commonly sold in local herbal markets of northwestern Iran, aiming to support industrial standardization and quality control of thyme-based products. Dried plant samples from Ahar (TA1, TA2) and Kaleybar (TK) were analyzed using internal transcribed spacer region of ribosomal DNA (ITS-rDNA) barcoding, essential oil profiling, and leaf morpho-anatomy. DNA barcoding identified two *Thymus* species: *T. kotschyanus* (TA1, TA2) and *T. pubescens* (TK), with sequence similarity exceeding 98%. The Maximum Likelihood phylogenetic tree supported their genetic divergence, consistent with chemotypic differentiation observed in essential oil composition. Gas Chromatography-Mass Spectrometry (GC–MS) analysis revealed thymol (52.11–67.51%) as the dominant compound in *T. kotschyanus*, classifying it as a thymol chemotype, whereas *T. pubescens* exhibited a Linalool–thymol chemotype characterized by high Linalool (29.81%) and 1,8-cineole (12.15%) levels. Principal Component Analysis (PCA) distinctly separated the two species based on their chemical profiles, accounting for 85.3% of the total variance. Morphological comparison further confirmed diagnostic traits: *T. pubescens* with two pairs of lateral veins and narrow lanceolate leaves, and *T. kotschyanus* with three pairs of lateral veins and broader ovate leaves. The integrative evidence highlights the significance of molecular authentication and chemotaxonomy in preventing species misidentification, which may affect both the therapeutic efficacy and commercial value of thyme products. These findings provide a scientific foundation for the industrial application and quality control of *Thymus*-derived essential oils. The proposed integrated workflow offers a practical and cost-effective solution for routine quality assurance in the herbal product industry.

Keywords: Thyme, DNA barcoding, Essential oil composition, Chemotaxonomy, PCA

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INTRODUCTION

Medicinal plants are important natural resources that have been widely used in traditional medicine due to their diverse therapeutic properties. They contain bioactive compounds such as phenolics, flavonoids, alkaloids, and terpenoids, which contribute to anti-oxidant, anti-inflammatory, anti-microbial, and other health-promoting effects [1-3]. The genus *Thymus* (Lamiaceae) is recognized as one of the most valuable medicinal plant groups worldwide. Pharmacological investigations have demonstrated its remarkable antimicrobial, anti-inflammatory, antioxidant, and anticancer properties [4-7]. Iran hosts more than 14 native *Thymus* species, which exhibit considerable diversity in their essential oil composition, with profiles varying among species, populations, and chemotypes [8]. This diversity constitutes both a valuable reservoir of bioactive compounds and a significant taxonomic challenge in species identification. In traditional markets and herbal shops, *Thymus* is typically sold in dried or powdered forms, often mixed with other plant materials. Such processing eliminates key morphological traits such as leaf shape, flower arrangement, and microscopic structures, making correct

identification difficult [9]. For example, Rabiei *et al.* [10] reported that nearly 30% of commercial samples marketed as "mountain thyme" in Iran were misidentified. Misidentification not only reduces therapeutic efficacy due to mismatched phytochemical profiles but may also cause undesirable side effects and erode consumer trust.

Different *Thymus* species, such as *T. vulgaris*, *T. daenensis*, and *T. kotschyanus*, exhibit distinct volatile compound profiles (e.g., thymol, carvacrol, and *p*-cymene), which directly determine their pharmacological activities [11]. Moreover, the industrial application of *Thymus* essential oils has been explored in various food products; for instance, yogurt blended with *T. kotschyanus* essential oil demonstrated enhanced antimicrobial properties and extended shelf life [12]. For instance, *T. vulgaris* with a thymol-rich profile is a potent antimicrobial, whereas *T. serpyllum*, rich in carvacrol, has demonstrated stronger anti-inflammatory activity. These differences highlight the necessity of precise species authentication in both traditional and industrial applications. Since conventional morphological approaches are often ineffective for processed plant materials, DNA barcoding has emerged as a reliable molecular tool for species authentication [13, 14]. Standard DNA regions such as rbcL, matK, and nuclear ribosomal ITS (Internal Transcribed Spacer) are commonly employed for this purpose. Among them, the ITS region is a widely used molecular marker for plant phylogenetic analysis and authentication, due to its relatively high mutation rate, the presence of conserved flanking regions suitable for universal primer design, and proven effectiveness in discriminating closely related *Thymus* species [15-17].

Beyond molecular identification, integration with phytochemical profiling offers a more comprehensive approach. Advanced analytical techniques such as gas chromatography–mass spectrometry (GC-MS) and high-performance liquid chromatography (HPLC) provide quantitative and qualitative insights into essential oils and phenolic metabolites, thereby linking genetic identity with functional properties [11, 18]. Given the rich diversity of medicinal plants in Iran, several studies have evaluated their phytochemical and antibacterial properties, emphasizing the need for rigorous quality control and standardization of herbal products [19].

Therefore, the present study was designed to address the challenges of *Thymus* authentication in the herbal supply chain. We applied ITS-based DNA barcoding to identify commercial *Thymus* samples collected from Iranian markets and subsequently characterized the chemical profiles of the authenticated species using GC-MS. This integrated molecular–chemical approach not only confirms species identity but also establishes a direct relationship between genetic authentication and phytochemical composition, underscoring its potential as a quality control protocol in industrial production of thyme-based products. To the best of our knowledge, this is the first study to combine ITS-based DNA barcoding with chemometric analysis specifically for commercial Thymus samples from the Arasbaran region of Iran.

MATERIALS AND METHODS

Study Area and Sample Collection

Dried thyme samples were purchased from local herbal shops (known as Attari) in the Arasbaran region of northwestern Iran, specifically from the cities of Ahar (TA) and Kaleybar (TK). These regions were selected due to their rich biodiversity and the natural distribution of native *Thymus* species. Each collected sample was properly labeled with detailed information (collection site, date, and seller identity) and stored under dry and dark conditions until DNA extraction.

Genomic DNA Extraction

Genomic DNA was extracted from small amounts of leaf tissue using a modified cetyltrimethylammonium bromide (CTAB) method [20]. Briefly, leaf tissues were ground in liquid nitrogen and incubated with CTAB extraction buffer at 65 °C for 45 min. An equal volume of chloroform: isoamyl alcohol (24:1) was added, followed by centrifugation at 13,000 rpm for 10 min. The aqueous phase was transferred to a fresh tube, and DNA was precipitated with cold isopropanol and incubated at −20 °C for 1 h. After centrifugation, the DNA pellet was washed with 70% ethanol, air-dried, and dissolved in sterile DNase-free water. DNA integrity was checked by agarose gel electrophoresis.

DNA Amplification and Sequencing

The nuclear ribosomal ITS region was amplified by polymerase chain reaction (PCR) using the universal primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) [21].

PCR reactions were performed in 25 µL volumes containing ~10 ng template DNA, 1X reaction buffer supplemented with 1.4 mM $MgCl_2$ (final concentration), 0.2 mM (200 µM) of each dNTP, 0.4 µL of each primer (20 pmol), 0.5 U Taq DNA polymerase, and nuclease-free water. The thermal profile included an initial denaturation at 94 °C for 2 min, followed by 35 cycles of 94 °C for 20 s, 54.5 °C for 30 s, and 72 °C for 2 min, with a final extension at 72 °C for 10 min. PCR products were separated on 1.2% agarose gels (85 V), stained with ethidium bromide, and visualized under UV illumination.

The amplified products were purified and sequenced bidirectionally using the same primers. DNA barcoding was performed by comparing sequences against the NCBI (National Center for Biotechnology Information) database using the BLASTn algorithm. A match was considered reliable when a single species showed >97% sequence similarity [22].

Phylogenetic Analysis

Sequences were aligned using MUSCLE, and phylogenetic analyses were conducted in MEGA v6.0 [16]. Maximum Likelihood (ML) trees were constructed under the Tamura–Nei substitution model, and node support was evaluated with 1,000 bootstrap replications.

Leaf Morphology Analysis

For morphological characterization, mature leaves were collected from flowering branches of each *Thymus* accession prior to essential oil extraction. Leaf samples were carefully examined under a stereomicroscope, and diagnostic traits including leaf shape, apex form, glandular trichome distribution, and the number of lateral veins were recorded following the taxonomic descriptions in Flora of Iran. Illustrative sketches were prepared to highlight interspecific variation, particularly the number and arrangement of secondary veins, which serve as reliable taxonomic markers even in fragmented or powdered herbal materials. All illustrations were adapted from *Flora of Iran* and verified by direct morphological observation. The number of lateral veins was recorded as a key diagnostic trait, as this feature remains observable even in partially fragmented leaf materials commonly encountered in commercial samples.

Essential Oil Extraction

Essential oil was extracted from 10 g of dried plant material using the hydro-distillation method. The extraction was performed for 3 hours in a round-bottom flask connected to a Clevenger-type apparatus, with 100 mL of distilled water serving as the distillation medium, in accordance with the standard procedure described in the British Pharmacopoeia [24]. After distillation, traces of moisture were removed by drying the oil over anhydrous sodium sulfate. The resulting essential oil was transferred to amber vials and stored at -20 °C until further chemical analysis. This standardized approach ensured the efficient isolation and preservation of volatile constituents for subsequent GC-based characterization.

GC–FID and GC–MS Analysis

The chemical composition of the essential oils was analyzed using both gas chromatography–flame ionization detection (GC–FID) and gas chromatography–mass spectrometry (GC–MS), following a slightly modified procedure described by Adams [25]. Separation of volatile compounds was carried out using a capillary column (e.g., HP-5MS, 30 m × 0.25 mm, film thickness 0.25 µm). Identification of the essential oil constituents was based on a combination of methods: First, comparison of retention indices (RI) relative to a homologous series of n-alkanes (C8–C30) analyzed under identical conditions. Second, matching of the obtained mass spectra with those in the NIST and Wiley libraries. The relative percentages of individual components were calculated from GC–FID peak areas without applying correction factors. This combined analytical strategy provided a comprehensive and reliable chemical profile of the essential oils examined.

RESULTS

The ITS rDNA region was successfully amplified from all collected samples, producing a clear single band of approximately 500-600 bp. Sequence alignment and BLASTn analysis confirmed a high similarity (>98%) of the obtained sequences with reference accessions of *Thymus* species available in the NCBI GenBank database.

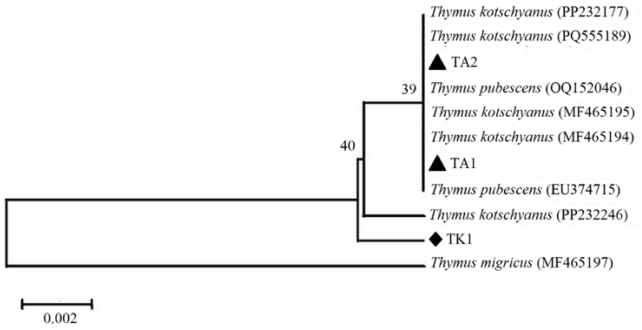


Fig. 1 Maximum Likelihood phylogenetic tree of *Thymus* accessions based on ITS-rDNA sequences. Bootstrap values (1000 replicates) are shown at nodes. Samples TA1 and TA2 cluster within the *T. kotschyanus* clade, while sample TK groups with *T. pubescens* references.

In contrast, sample TK (PV876123) grouped strongly with reference sequences of *T. pubescens* (OQ152046, EU374715), forming a separate, distinct clade. This clear phylogenetic separation, supported by bootstrap values, provides conclusive molecular evidence for the identification of TK as *T. pubescens*. *T. migricus* (MF465197) was included as an outgroup and was clearly separated from the other *Thymus* taxa. All sequences generated in this study were deposited in the NCBI GenBank under accession numbers PV876120-23. A Maximum Likelihood phylogenetic tree was constructed to evaluate the genetic relationships among the sequenced samples (TA1, TA2, and TK) and their closest reference sequences (Fig. 1). The phylogenetic analysis revealed two distinct, well-supported clades corresponding to species identity. Samples TA1 (PV876120) and TA2 (PV876121) clustered firmly within a clade containing reference sequences of *T. kotschyanus* (PP232177, PQ555189, MF465195, MF465194, PP232246), with strong bootstrap support, confirming their taxonomic assignment to this species.

Essential Oil Composition

The essential oil composition of the three Thymus samples - two samples of *T. kotschyanus* (TA1, TA2, collected from Ahar) and one of *T. pubescens* (TK, collected from Kaleybar)- is presented in Table 1. A total of eight major constituents were identified, accounting for over 97% of the total oil composition in all samples (Fig. 2).

Table 1 Chemical composition (%) of the essential oil from plants collected from Ahar and Kaleybar regions

No.	Compounds	TA1	TA2	TK
1	α-pinene	10.28	3.00	1.75
2	*p*-cymene	16.14	18.94	14.54
3	1,8-cineole	2.28	3.24	12.15
4	γ-terpinene	2.28	1.32	0.28
5	Linalool	4.67	1.32	29.81
6	Thymol	52.11	67.51	36.99
7	Carvacrol	8.97	1.32	0.74
8	(E)-caryophyllene	1.67	1.56	1.66
		98.40	98.20	97.90

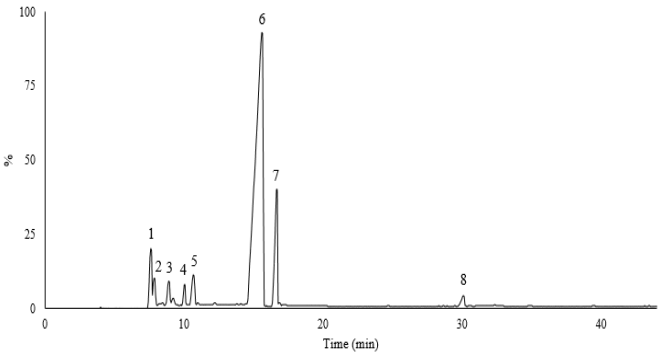


Fig. 2 GC chromatogram of *T. pubescens* (TK, collected from Kaleybar). 1: α-pinene; 2: *p*-cymene; 3: 1,8-cineole; 4: γ-terpinene; 5: Linalool; 6: Thymol; 7: Carvacrol; 8: (E)-caryophyllene

Thymol was the predominant compound across all accessions, with the highest concentration detected in *T. kotschyanus* TA2 (67.51%), followed by TA1 (52.11%) and *T. pubescens* TK (36.99%). Significant amounts of *p*-cymene and α-pinene were also present, particularly in the Ahar samples, while Linalool and 1,8-cineole were notably higher in TK.

The two *T. kotschyanus* accessions exhibited a characteristic thymol chemotype, supported by the co-occurrence of its biogenetic precursors *p*-cymene (16.14–18.94%) and γ-terpinene (1.32–2.28%). However, an intraspecific variation was observed between the two: TA1 showed a higher carvacrol content (8.97%) compared to TA2 (1.32%).

In contrast, *T. pubescens* (TK) displayed a distinct chemical profile dominated by Linalool (29.81%) and 1,8-cineole (12.15%), accompanied by a reduced thymol content (36.99%) and very low γ-terpinene (0.28%). This composition defines TK as a Linalool–thymol chemotype, markedly different from the thymol-rich profiles of *T. kotschyanus*. The presence of Linalool as a major constituent (29.81%) in T. pubescens is particularly noteworthy, as this compound is highly valued in aromatherapy and cosmetic applications for its pleasant floral scent and mild antimicrobial properties.

Principal Component Analysis (PCA)

Principal Component Analysis (PCA) was performed to explore the chemical relationships among the essential oils of the three *Thymus* accessions (TA1, TA2, and TK) based on the relative abundance of their major constituents (Fig. 3). The first two principal components accounted for more than 85.3% of the total variance, effectively summarizing the main differences in chemical composition.

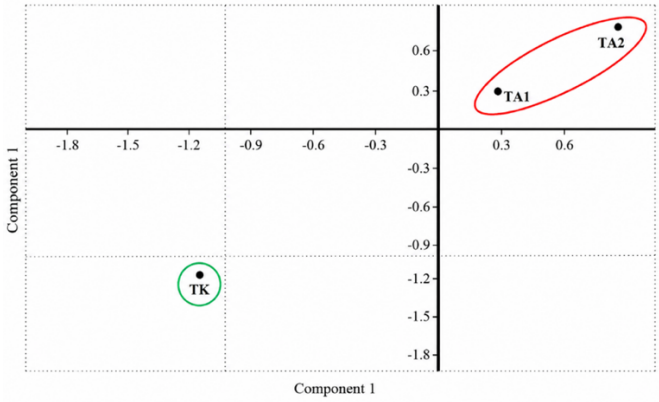


Fig. 3 Principal component analysis (PCA) score plot based on the relative abundance of major essential oil components in three *Thymus* accessions (TA1, TA2, and TK). The first two principal components (PC1 and PC2) explained 85% of the total variance, clearly separating *T. kotschyanus* samples (TA1, TA2) from *T. pubescens* (TK) according to their chemotypic composition.

The resulting biplot revealed two distinct clusters corresponding to species identity. The *T. kotschyanus* accessions (TA1 and TA2) grouped closely together on the positive side of PC1, forming a well-defined cluster characterized by high levels of thymol, *p*-cymene, and γ-terpinene. This tight clustering reflects their strong chemical similarity and supports their classification as thymol-type chemotypes.

In contrast, the *T. pubescens* sample (TK) was positioned separately on the negative axis of PC1, clearly discriminated from the *T. kotschyanus* group. This separation is primarily explained by its elevated contents of Linalool and 1,8-cineole, which are the key loading variables contributing to PC1. The distinct positioning of TK highlights its Linalool–thymol chemotype, confirming the marked interspecific variation inferred from the compositional data.

Overall, the PCA results reinforce the chemotaxonomic differentiation between *T. kotschyanus* and *T. pubescens*, illustrating the robustness of multivariate analysis for distinguishing species and chemotypes within the *Thymus* genus. The clear separation of the two species in the PCA biplot demonstrates that chemical profiling alone can serve as a reliable tool for species authentication, complementing molecular data in quality control workflows.

Morphological Comparison

The comparative leaf morphology clearly distinguished *T. pubescens* from *T. kotschyanus* (Fig. 4). *T. pubescens* exhibited an elongated, lanceolate leaf shape with a pointed apex and predominantly two pairs of secondary veins branching from the midrib. In contrast, *T. kotschyanus* displayed broader, ovate leaves with three pairs of well-defined lateral veins. The glandular trichomes were more abundant and darker in *T. kotschyanus* (reddish-brown) compared to the lighter, translucent glands observed in *T. pubescens*.

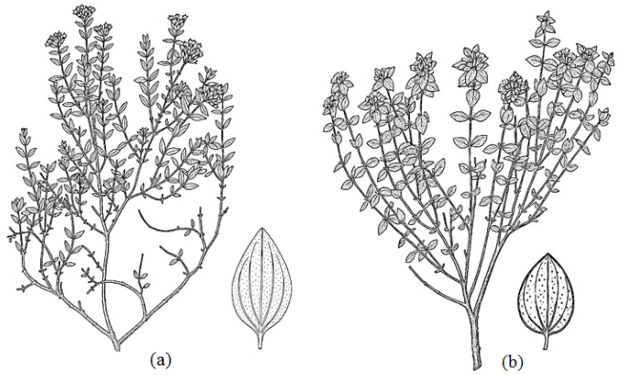


Fig. 4 Comparative leaf morphology of *Thymus kotschyanus* (TA1, TA2) and *Thymus pubescens* (TK). The leaves of *T. pubescens* are more elongated and pointed, with two pairs of lateral veins (a), whereas *T. kotschyanus* exhibits an ovate to triangular shape with three pairs of lateral veins (b). Glandular trichomes are denser and more pigmented in *T. kotschyanus*, while *T. pubescens* displays fewer and lighter-colored glands. These diagnostic morphological traits support the molecular and chemical differentiation observed between the two taxa. (Source: adapted from Flora of Iran)

These morphological distinctions correspond closely with the taxonomic keys provided in *Flora Iranica* and confirm the species-level separation inferred from DNA barcoding and essential oil profiles. Notably, the number of secondary veins can serve as a practical diagnostic feature for species identification, particularly in commercial or powdered herbal materials where other diagnostic traits are often lost.

DISCUSSION

The combined evidence from DNA barcoding, essential oil profiling, PCA, and morphological observations robustly supports the taxonomic distinction between *T. kotschyanus* (TA1, TA2) and *T. pubescens* (TK). The phylogenetic analysis based on ITS sequences provided unambiguous molecular authentication, with samples clearly separating into two well-defined clades corresponding to each species.

This genetic differentiation is mirrored in the volatile profiles: both *T. kotschyanus* samples displayed a thymol-dominant chemotype, with thymol percentages exceeding 52% and supported by significant quantities of *p*-cymene and γ-terpinene-hallmarks of the monoterpene biosynthetic pathway leading to phenolic monoterpenes [26]. The presence of elevated carvacrol in TA1 (8.97%) suggests possible differential enzyme specificity or regulation within *T. kotschyanus*.

In contrast, *T. pubescens* (TK) exhibited a substantially different chemotype. It's essential oil featured high levels of Linalool (29.81%) and 1,8-cineole (12.15%), accompanied by reduced *p*-cymene and γ-terpinene, indicative of a shift toward oxygenated monoterpene pathways. This metabolic reallocation likely reflects altered expression levels or substrate channeling of terpene synthases [15, 17].

The antimicrobial potential of thymol-rich essential oils from *T. kotschyanus* has been demonstrated in food products, such as yogurt, where it significantly inhibited bacterial growth and extended product shelf life [12]. Moreover, essential oils from indigenous Iranian plants, including *Thymus* species, have been reported as effective natural agents against multidrug-resistant *Escherichia coli* strains [20], reinforcing the clinical and industrial relevance of accurate species identification.

Principal Component Analysis further reinforced the separation: TA1 and TA2 clustered closely along positive PC1, associated with thymol, *p*-cymene, and γ-terpinene loadings, while TK was clearly separated on the negative side of PC1, driven by Linalool and 1,8-cineole (Fig. 3). This pattern illustrates a strong chemotaxonomic signal, reinforcing species-level differences in volatile composition.

Morphological traits derived from the *Flora of Iran* also corroborate these findings. *T. kotschyanus* typically features more glandular stems, a higher leaf length-to-width ratio, and darker secretory glands, whereas *T. pubescens* exhibits narrower, acuminate leaves, paler glandular dots, and notably two pairs of lateral veins (vs. commonly three in *T. kotschyanus*) [8]. The venation pattern, in particular, is highly practical: even in powdered or fragmented commercial samples (as typically encountered in herbal markets), the count of lateral veins remains discernible. In this study, TK displayed two pairs of lateral veins, aligning with *T. pubescens*, while TA1 and TA2 had predominantly three pairs, supporting their assignment to *T. kotschyanus*.

From an application standpoint, these chemotype differences bear considerable implications. Thymol-rich oils (as in *T. kotschyanus*) are well-known for potent antimicrobial, antioxidant, and preservative properties, making them suitable for pharmaceutical formulations, natural preservatives, and antiseptics [28]. Conversely, oils rich in Linalool and cineole (as in *T. pubescens*) tend to have milder antimicrobial activity but are highly valued in perfumery, aromatherapy, and cosmetic applications due to their fragrance and lower irritancy [11]. Misidentification, such as substituting *T. pubescens* for *T. kotschyanus* or vice versa, could result in reduced bio-efficacy or unwanted side effects, particularly when high antimicrobial potency is expected.

Previous studies [9] have demonstrated that misidentification of *Thymus* species in commercial preparations can lead to significant variation in phenolic content, antioxidant capacity, and therapeutic outcomes. Their work emphasizes that DNA-based authentication is essential to mitigate such inconsistencies and ensure product quality and safety. The integration of molecular, chemical, and morphological analyses in this study presents a robust framework to authenticate raw materials and standardize industrial *Thymus* products. The findings of this study align with the growing body of evidence supporting the need for systematic quality control in the herbal industry, particularly for widely consumed medicinal plants such as *Thymus* [19].

CONCLUSION

In conclusion, *T. kotschyanus* and *T. pubescens* represent genetically, chemically, and morphologically distinct taxa with clearly divergent biosynthetic pathways and industrial potentials. The distinct chemotypes observed (thymol-rich versus Linalool–thymol types) emphasize the importance of species-specific utilization to ensure consistency in biological efficacy and product quality. Integrating DNA barcoding with essential oil profiling and morphological markers such as leaf venation proved to be a reliable and cost-effective approach for accurate species authentication. These findings provide valuable insights for the standardization, quality control, and sustainable industrial application of *Thymus* essential oils in pharmaceutical and food industries. We recommend that future studies expand this integrated approach to include a wider range of Thymus species and larger sample sizes, enabling the development of comprehensive databases for chemotype classification and quality assurance.

Consent for Publication

Not Applicable.

Data availability

The data used and analyzed for the current study can be obtained from the corresponding author.

Conflict of Interest

The authors have no conflict of interest to declare.

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There is no financial support.

Authors' contributions

Ebadi Mostafa: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation, Data curation, Conceptualization. Valinejad Shaghayegh: Methodology, Investigation, Data curation. Mollaei Saeed: Writing – review & editing, Visualization, Validation, Methodology, Investigation, Data curation, Conceptualization.

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