



Development of Pharmacognostic and Molecular Identification Standards for *Thesium viride* A.W. Hill

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Abstract

Background and Objectives: *Thesium viride* A.W.Hill is a hemiparasitic subshrub from the family Santalaceae, used in the treatment of jaundice, hepatomegaly, splenomegaly, and ulcers. This research aimed to establish some identification parameters, such as pharmacognostic characters and the DNA barcode of *T. viride*, to aid in standardization and identification of the plant.

Materials and Methods: The plant material was examined microscopically for some diagnostic structures, and its physicochemical parameters, such as moisture content, extractive values, and ash values, were also determined. DNA extraction from the plant material and amplification of the DNA using chloroplast barcode markers *rbcL* and *matK* were carried out.

Results: Microscopical examination of the aerial parts of *T. viride* revealed the presence of unicellular covering trichomes, straight-walled polygonal epidermal cells, paracytic stomata, cork and sclerenchyma cells, and a distinguishable cambium layer. The powdered plant showed moisture content (5.33%), total ash (10.33%), acid-insoluble ash (3.17%), water-soluble ash (4.93%), alcohol-soluble extractives (4.87%), and water-soluble extractives (5.93%). Polymerase chain reaction amplification produced clear bands of approximately 512 bp and 817 bp for *rbcL* and *matK*, respectively. The amplified products were sequenced using the Sanger sequencing method and analyzed using the Basic Local Alignment Search Tool against sequences deposited in the GenBank database, and the submitted sequences were assigned GenBank accession numbers PZ051249 and PZ804900 for *rbcL* and *matK*, respectively. The obtained sequences showed high similarity with related *Thesium* species, supporting the molecular identification and authentication of *T. viride*.

Conclusion: Combined use of pharmacognostic evaluation and DNA barcoding provided useful identification of *Thesium viride* and established reference data for its authentication. These findings contribute to the standardization, quality control, and safe utilization of this medicinal plant.

Keywords: DNA barcoding, Molecular authentication, Pharmacognostic evaluation, Standardization, *Thesium viride*

Introduction

Plants have long been used as a primary source of medicinal agents for the treatment of numerous illnesses worldwide and continue to play a vital role in modern medicine. More than 80% of the

world population depends on plants for their primary healthcare needs (1). Since more than 25% of conventional drugs are derived from plant sources, many countries have established national policies, enacted legislation, and developed



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regulatory frameworks to oversee the use of medicinal plants in the management of various illnesses (2).

The problem of adulteration, substitution, and misidentification has been a bottleneck in the reliability and safety of the use of plants in herbal medicine practices (3). Therefore, correct identification and quality control of medicinal plant materials is an essential step to overcome such significant challenges (4). Conventional identification methods, such as the use of morphological, organoleptic, and chemical characteristics, are generally inadequate for accurate identification. This is the case particularly when plant materials are processed into powdered form or when their chemical profiles vary due to geographic origin or storage conditions (5).

The plant *Thesium viride* A.W.Hill is a hemiparasitic subshrub belonging to the family Santalaceae (6). It is widely distributed in Africa, Madagascar, and South America and has also been reported under the names *Thesium hararensis* A. G. Mill and *Thesium unyikense* Engl. (7). This plant is locally known as “Huntu” among the Hausa people of northern Nigeria (8). It has been used for the treatment of jaundice, liver enlargement, splenomegaly, and ulcers (9). Moreover, it is used to treat arthritis, inflammation, hypertension, and pediatric diseases (10).

According to previous studies, *T. viride* contains phenolics, flavonoids, glycosides, and alkaloids (11). Several bioactive compounds, including bis-(2-ethylhexyl) phthalate, octadecenoic acid isomers, and diethyl phthalate, have been detected using gas chromatography/mass spectrometry and reported to exhibit various biological activities (12).

Traditional identification of plants has largely relied on morphological characters of leaves, flowers, and fruits (13). This approach has been utilized in plant classification for centuries but is often constrained by problems of

misidentification and ambiguity, especially among closely related species (14). Due to these limitations, there is a need to incorporate molecular techniques, such as DNA barcoding, to enhance accuracy and resolve taxonomic uncertainties (15).

However, to our knowledge, available pharmacognostic standards and DNA barcode information for the accurate authentication of *T. viride* appear to be limited. The present study aimed to establish pharmacognostic characteristics and DNA barcode data for *T. viride* to facilitate its accurate identification, authentication, and standardization as a medicinal plant.

Methods

Plant collection and authentication

An intact plant of *T. viride* was collected at Karaukarau of Giwa Local Government Area of Kaduna State, Nigeria (11°07'40"N 7°30'04"E). The plant was identified by Mr. U. S. Gallah at the herbarium unit of the Department of Pharmacognosy and Drug Development, Faculty of Pharmaceutical Sciences, Kaduna State University, with a voucher number of KASU/PCG/HERB/300. The aerial part of the plant was dried under shade at room temperature for one week, pulverised, and kept in a dried and airtight container for future use.

Pharmacognostic evaluation

The pharmacognostic evaluation was carried out as described by Evans (16). A transverse section of the stem of *T. viride* was prepared and cleared in chloral hydrate solution by boiling in a water bath. The section was subsequently stained with phloroglucinol and concentrated hydrochloric acid, mounted in dilute glycerol, and examined under a microscope. For powdered microscopy, a small quantity of ground *T. viride* material was similarly cleared in chloral hydrate solution by boiling in a water bath, then mounted in dilute glycerol and observed microscopically. Moisture

content, ash, and extractive values of powdered *T. viride* were determined as outlined by World Health Organization guidelines (17).

DNA Extraction

DNA was extracted using the Accuprep Plant Genomic DNA Extraction Kit (Bioneer, South Korea). This was performed following the instructions of the manufacturer with some modifications. Lysis of 100 mg of dried, powdered *T. viride* was performed using plant lysis buffer containing Proteinase K and RNase A. Purification of the DNA included centrifugations to separate out cellular debris, while the DNA was finally eluted using 50 µL of the elution buffer. Concentration and purity of the DNA were determined using ultraviolet-visible Spectrophotometer by taking readings of absorbance values at 260 and 280 nm.

High levels of DNA purity were indicated by absorbance ratios (A₂₆₀/A₂₈₀) ranging from 1.8 to 2.0, indicating pure genomic DNA that could undergo polymerase chain reaction (PCR) amplification. Quantification of the amount of DNA extracted was conducted in ng/µL using an ultraviolet-visible spectrophotometer. Integrity of the DNA was confirmed using agarose gel electrophoresis (18). DNA yield was recorded in ng/µL. The isolated DNA was stored at -20°C for further use.

DNA amplification

The PCR amplification was performed in a 20 µL reaction volume containing 2 µL of template DNA, 1 µL each of forward and reverse primers, 16 µL of PCR master mix containing DNA polymerase, PCR buffer, MgCl₂, dNTPs, and nuclease-free water to make up the final volume to 20µL. The *rbcL* region was amplified using *rbcLa-F* (5'-ATGTCACCACAAACAGAGACTAAAGC-3') and *rbcLa-R* (5'-GTAAAATCAAGTCCACCRCG-3'), while the *matK* region was amplified using *matK-1RKIM-f*

(5'-CCCAGTCCATCTGGAAATCTTGGTTC-3') and *matK-3FKIM-r* (5'-CGTACAGTACTTTTGTGTTTACGAG-3').

The PCR amplification was performed using a C1000 Touch™ Thermal Cycler (Bio-Rad). The thermal cycling conditions consisted of an initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 54 °C for 30 s, and extension at 72 °C for 1 min, followed by a final extension at 72 °C for 5 min (19). The PCR products were assessed by agarose gel electrophoresis using a 1-kb DNA ladder (Thermo Fisher Scientific, USA).

Gel electrophoresis

The PCR-amplified fragments were subjected to agarose gel electrophoresis using a 1.3% agarose gel. The gel was stained with ethidium bromide for 20 min and fluoresced under UV light to visualize the banding pattern indicating DNA fragment size. Electrophoresis was performed at a voltage of 110-120 V for 30 min. The PCR products were visualized on an agarose gel, and amplification was confirmed by the presence of bands in the expected size range of 500–600 bp for *rbcL* and 800–1,000 bp for *matK* under UV illumination (19).

DNA sequencing and analysis

Following gel electrophoresis, the purified PCR products of the *rbcL* and *matK* regions were analysed by Sanger sequencing. The obtained sequences were validated and viewed using the FinchTV software program. Searches for sequence similarities were carried out by comparing against the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) database to determine the closest sequence matches for *T. viride* and other *Thesium* species (20).

Nucleotide base composition, including percentage adenine-thymine (AT) and guanine-cytosine (GC) content, was determined. Upon successful submission, each sequence received a

unique GenBank accession number, which serves as a permanent identifier for the sequence in public databases and allows for reproducibility and comparative analyses. These accession numbers were cited in subsequent results, tables, and phylogenetic analyses.

Phylogenetic analysis

Phylogenetic analyses were performed in MEGA software (version 12) to construct trees using aligned sequences, following the method of Tamura (21). Evolutionary relationships of *T. viride* within the genus *Thesium* were inferred using independent phylogenetic analyses of *rbcL* and *matK* sequences. Nucleotide sequences of the 10 most similar species were retrieved from GenBank using the multiple sequence comparison by log-expectation algorithm, implemented in MEGA software. Sequences were aligned and inspected. Phylogenetic trees were constructed using the maximum likelihood method based on the best-fit nucleotide substitution model and the Tamura-Nei model selected in MEGA software.

Robustness of the inferred phylogenetic relationships was assessed using standard bootstrap at 1,000 replicates (22). The bootstrap values were displayed at the corresponding nodes of the tree and were calculated as the percentage of replicate trees in which a specific clade was recovered. Values were categorized as follows: < 50% (weak support), 50–69% (moderate support), and $\geq 70\%$ (strong support), with values > 90% indicating very strong confidence in the recovered clades (23).

Results

The powdered *T. viride* was dark greenish in color, wool-like in appearance, with a characteristic odor and taste. The transverse section of the stem (Figure 1) shows cuticularised

epidermal cells, unicellular trichomes, and stem cortex made up of 4-5 layers of oval cells. The sclerenchymatous sheath was located above the vascular bundles, with a distinct cambium and large pith cells made up of parenchyma cells. Microscopically, the powdered *T. viride* revealed paracytic stomata, straight-walled polygonal epidermal cells, and cork and sclerenchyma cells. Photomicrographs of these features are presented in Figure 2.

The powdered *T. viride* showed physicochemical constants of $5.33 \pm 0.27\%$ moisture content, $10.33 \pm 0.24\%$ total ash, $3.17 \pm 0.24\%$ acid-insoluble ash, $4.93 \pm 0.47\%$ water-soluble ash, $4.87 \pm 0.09\%$ alcohol-soluble extractives, and $5.93 \pm 0.19\%$ water-soluble extractives, as presented in Table 1.

Table 1. Physicochemical constants of powdered *Thesium viride* A.W.Hill

Physicochemical constants	Percentage (w/w %)
Moisture	5.33 ± 0.27
Total ash	10.33 ± 0.24
Acid-insoluble ash	3.17 ± 0.24
Water-soluble ash	4.93 ± 0.47
Alcohol-soluble extractives	4.87 ± 0.09
Water-soluble extractives	5.93 ± 0.19

All values are expressed as mean $\pm$ standard error of the mean (n=5)

Genomic DNA was successfully extracted from the powdered *T. viride*, yielding a DNA concentration of 48.15 ng/ μ L with a high purity of 2.0 and quality suitable for further molecular analysis. The extracted DNA showed no signs of degradation, indicating that it remained intact and was appropriate for PCR amplification and sequencing. The *rbcL* and *matK* barcode regions of *T. viride* were successfully amplified using *rbcL* and *matK* primers, generating amplicons of 512 bp and 817 bp, respectively (Figure 3).

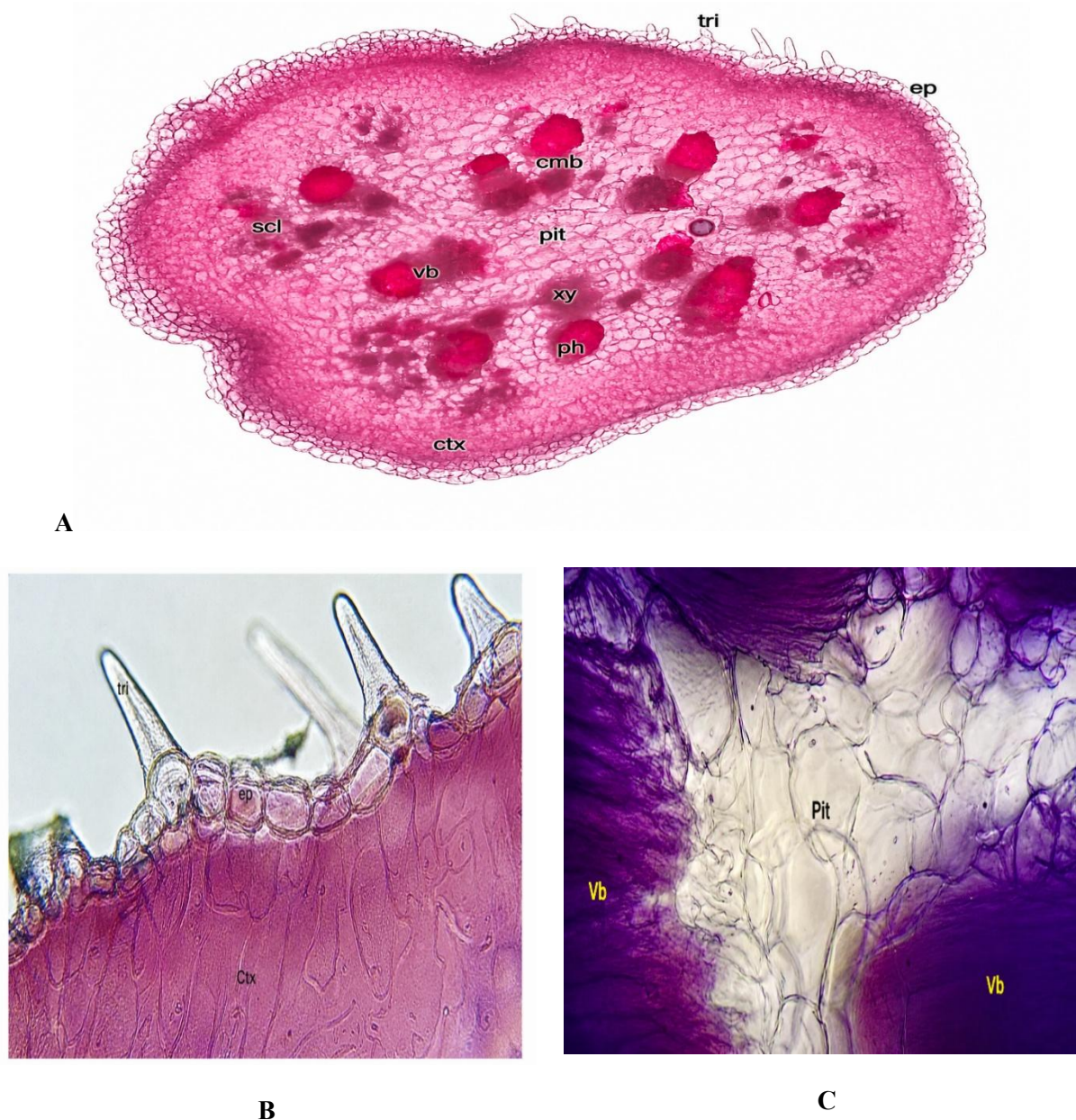


Figure 1. Transverse section of the stem of *Thesium viride*.

A. Epidermis (Ep), cortex (Ctx), phloem (Ph), xylem (Xy), vascular bundle (Vb), sclerenchyma cells (Scl), and pith (Pit) (Mag. $\times 100$).

B. Tissue system consisting of covering trichomes (Tri), epidermis (Ep), and Ctx (Mag. $\times 400$).

C. Section stained with phloroglucinol–hydrochloric acid showing Pit surrounded by Vb (Mag. $\times 400$).

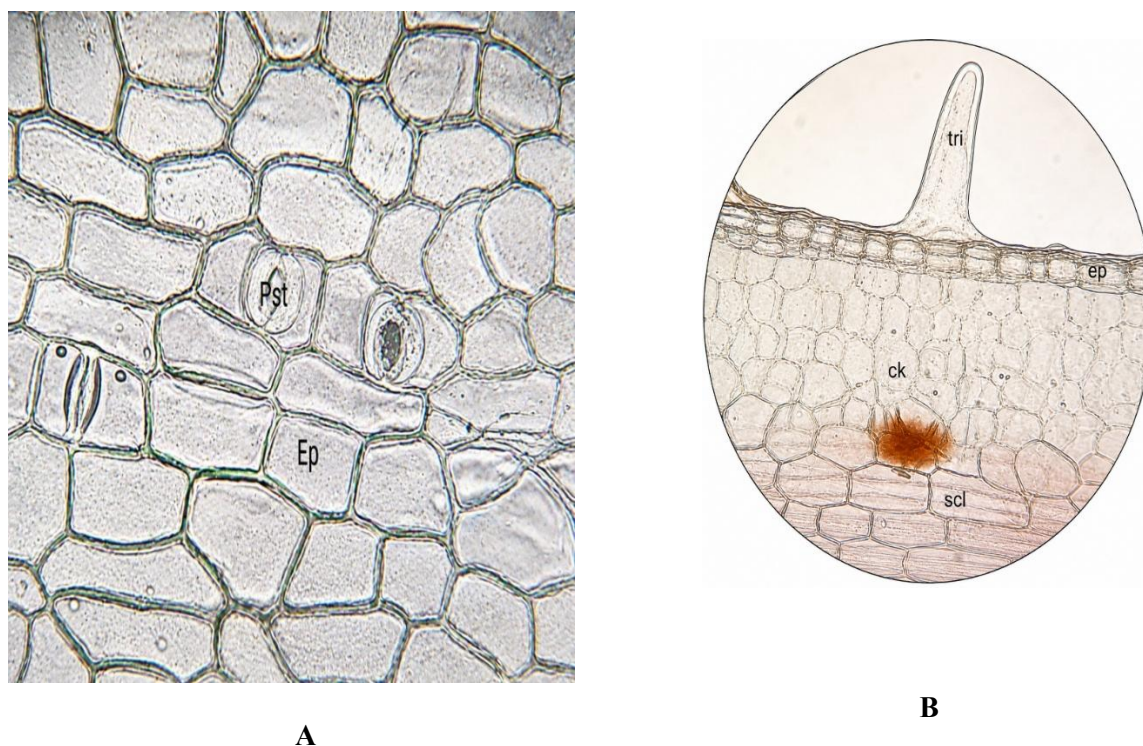


Figure 2. Photomicrograph of cleared powdered sample of *Thesium viride*.

A. Polygonal, straight-walled epidermal cells (Ep) and paracytic stomata (Pst) (Mag. $\times 640$)

B. Unicellular trichomes (Tri), cuticularized Ep, cortex (Ctx), calcium oxalate crystals (CaOx), and sclerenchyma cells (Scl) (Mag. $\times 400$).

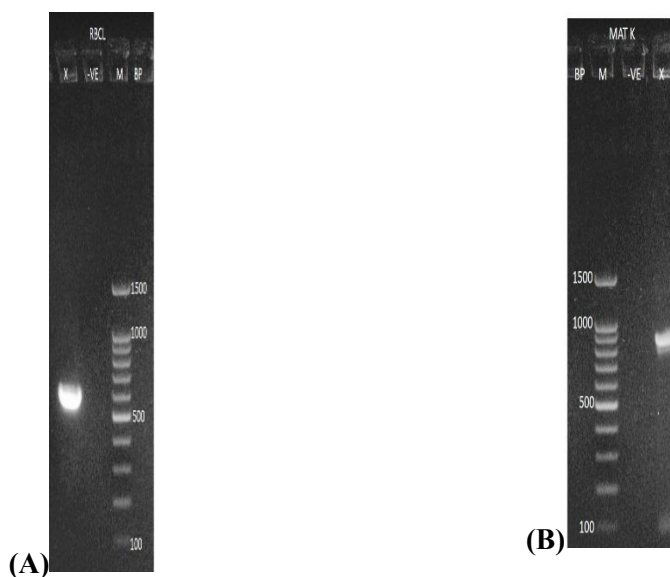


Figure 3. Agarose gel electrophoresis of polymerase chain reaction amplicons obtained from amplification of the *rbcL* (A) and *matK* (B) barcode regions of *Thesium viride*. M: DNA molecular marker (100-bp ladder); BP: blank control; -VE: negative control; X: *T. viride* DNA sample. The expected amplicon sizes were approximately 512 bp for *rbcL* and 817 bp for *matK*.

Subsequent sequencing of the amplicons yielded high-quality reads for both loci. Nucleotide composition analysis indicated variation in AT and GC content between the markers: *rbcL* exhibited 56.25% AT and 43.75% GC, while *matK* showed 66.87% AT and 33.13% GC. Overall, both loci demonstrated higher AT than GC content (Table 2).

The BLAST analysis of the *rbcL* sequence of *T. viride* showed high query coverage, ranging from

95–98%, with sequence identities of 92.15–92.90% among the top 10 NCBI matches (Table 3). The highest similarity (92.90%) was obtained with *Thesium decaryanum* (OR712484) and *T. ericifolium* (PP129907), while all matches had an E-value of 0.0, indicating highly significant sequence similarity. The results confirmed that the obtained *T. viride* sequence is closely related to other members of the genus *Thesium*.

Table 2. Nucleotide composition (AT% and GC%) of *rbcL* and *matK* sequences in *T. viride*.

Marker	A	T	C	G	Total	AT	GC	AT%	GC%
<i>rbcL</i>	143	145	113	111	512	288	224	56.25	43.75
<i>matK</i>	306	240	134	137	817	546	271	66.82	33.17

AT: guanine-cytosine, GC: guanine-cytosine

Table 3. Top 10 BLAST results of the *rbcL* sequence of *T. viride* from the NCBI BLASTN RID-7GSGRE8K014.

Scientific name	Query cover %	E-value	Identity %	Accession number
<i>Thesium decaryanum</i> Cavaco and Keraudren	98	0.0	92.90	OR712484
<i>Thesium strictum</i> P. J. Bergius	98	0.0	92.71	AM235126
<i>Thesium fruticosum</i> A.W. Hill	98	0.0	92.71	EF584609
<i>Thesium fragile</i> L. f.	98	0.0	92.34	EF584608
<i>Thesium fragile</i> L. f.	98	0.0	92.34	AM235125
<i>Thesium ericifolium</i> A. DC.	96	0.0	92.90	PP129907
<i>Thesium euphorbioides</i> P. J. Bergius	95	0.0	92.88	KP110494
<i>Thesium kilimandscharicum</i> Engl.	96	0.0	92.75	ON951210
<i>Thesium schimperianum</i> Hochst.	98	0.0	92.15	MH390680
<i>Thesium subsucculentum</i> J. C. Manning and F. Forest	98	0.0	92.15	EF584596

For the *matK* sequence, the BLAST results showed 92–100% query coverage and considerably higher sequence identity, ranging from 95.80–96.77% (Table 4). The highest identity (96.77%) was observed with *Thesium euphorbioides* (KP110165), followed by other *Thesium* species with identities above 95%. All

matches had an E-value of 0.0, further supporting the reliability of the sequence similarity. Overall, the *matK* marker demonstrated greater sequence discriminatory power than *rbcL*, as indicated by its higher percentage identity (95.80–96.77% versus 92.15–92.90%).

Table 4. Top 10 BLAST results of the *matK* sequence of *T. viride* from the NCBI BLASTN RID-7GPJV0UR014.

Scientific name	Query cover %	E-value	Identity %	Accession number
<i>Thesium euphorbioides</i> P. J. Bergius	97	0.0	96.77	KP110165
<i>Thesium decaryanum</i> Cavaco and Keraudren	99	0.0	96.08	OR712484
<i>Thesium nigroperianthum</i> Zhigila, Verboom, and Muasya	92	0.0	96.99	PP197590
<i>Thesium minus</i> (A.W.Hill) J. C. Manning and F.Forest	95	0.0	96.31	MN382710
<i>Thesium ericifolium</i> A. DC.	92	0.0	96.73	PP197587
<i>Thesium ramosum</i> Hayne	100	0.0	94.42	MH390638

<i>Thesium carinatum</i> A. DC.	92	0.0	96.48	PP197585
<i>Thesium corymbuligerum</i> Sond.	95	0.0	95.80	MN382661
<i>Thesium rhizomatum</i> Zhigila, Verboom, and Muasya	95	0.0	95.80	MN382682
<i>Thesium stirtonii</i> Zhigila, Verboom, and Muasya	95	0.0	95.80	MN382662

Sequences with the highest similarity (10 *Thesium* species) were retrieved for phylogenetic tree construction with *T. viride* shown in Figures 4 and 5. In the *rbcL* tree, *T. viride* (PZ051249) and *T. decaryanum* (OR712484) formed a sister pair with 44% bootstrap support, indicating a weakly supported relationship. In contrast, the *matK* tree

showed the same sister relationship with 100% bootstrap support, providing strong evidence for the close genetic affinity between the two species. Other well-supported relationships included *T. strictum*–*T. fragile* (94%) and *T. schimperianum*–*T. subsucculentum* (93%) in the *rbcL* tree.

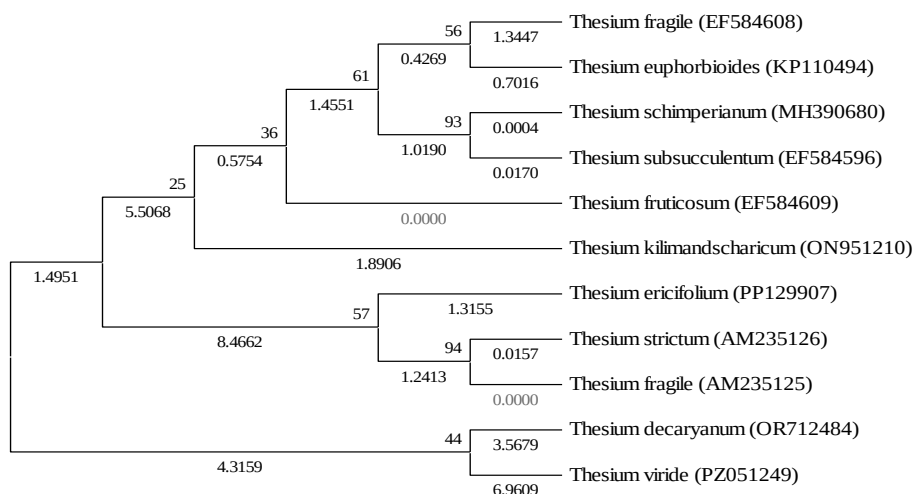


Figure 4. Phylogenetic tree of *Thesium viride* nucleotide sequences for *rbcL* and the 10 most similar sequences of the genus *Thesium* in GenBank.

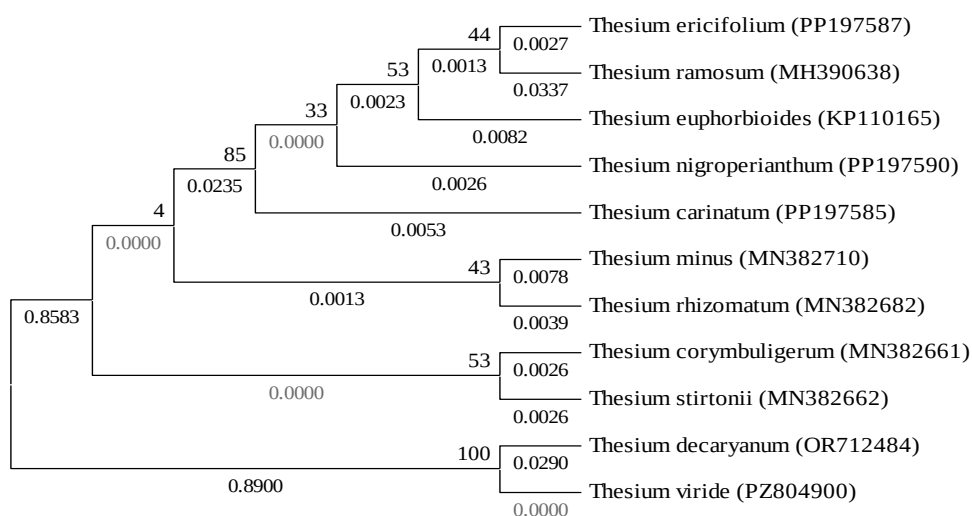


Figure 5. Phylogenetic tree of *Thesium viride* nucleotide sequences for *matK* and the 10 most similar sequences of the genus *Thesium* in GenBank.

Discussion

Plant pharmacognostic evaluation is the first step that provides important information on the morphology, microscopy, and physical features (24). This information on *T. viride* revealed diagnostic features that can be used for plant identification (25).

The powdered *T. viride* is dark greenish in color, wool-like in appearance, with a characteristic odor and taste. Some of these sensory features are characteristic and could aid in the identification of the plant material. Microscopic examination of the powdered *T. viride* revealed paracytic stomata, a straight-walled polygonal epidermal, cork, and sclerenchyma cells. It has been previously reported that the paired guard cells of Santalaceae are accompanied by several columns of parallel subsidiary cells, resulting in a paracytic type of stomata (26). These microscopic characters may serve as useful diagnostic features for the identification of *T. viride* in powdered form, particularly the presence of a covering trichome, which distinguishes it from the glabrous forms of other *Thesium* species (26).

Furthermore, the structures and tissue arrangements in the stem of *T. viride* exhibit a distinguishable cambium layer compared with *T. ebracteatum*, which is a characteristic anatomical feature of the stems of hemiparasitic plants. This is the first time the transverse anatomical features of *T. viride* have been reported.

The physico-chemical constant determination according to WHO guidelines (2011) showed a low moisture content (5.33 ± 0.27). Generally, moisture content in crude drugs should not be more than 14% (27); therefore, it is crucial to minimize moisture, as high moisture in plant material may lead to deterioration resulting in biodegradation of bioactive constituents (28).

Ash values are indications of inorganic residues when vegetable drugs are incinerated. Physiological and non-physiological ash are referred to as total ash, while acid-insoluble ash represents a fraction of the total ash and an

indicator of silica present, especially in earthy substances (29). It was observed that *T. viride* showed the presence of lower acid-insoluble ash (3.17 ± 0.24) than the water-soluble ash (4.93 ± 0.47). This may serve as important standards for quality, authenticity, and purity determination (30) and could be useful in *T. viride* monograph development.

The percentage extractive values of *T. viride* in aqueous cold extraction are higher ($5.93 \pm 0.19\%$), compared with ethanol cold extraction ($4.87 \pm 0.09\%$). This is an indication that *T. viride* contains more highly polar constituents, such as flavonoids, polyphenols, and glycosides, than non-polar constituents. These are valuable tools that could aid in the determination of chemical constituents and the detection of exhausted and previously utilized plant materials that could be fraudulently used as substitutes or adulterants (31).

The successfully isolated genomic DNA from *T. viride* has a purity assessment based on the A_{260}/A_{280} ratio of 2.0 and a concentration of 48.15 ng/ μ L. This concentration indicates that the DNA is sufficient for PCR amplification and sequencing. The purity also falls within the acceptable range of 1.8-2.0, indicating minimal protein contamination. Sufficient and high-quality DNA is critical for DNA analysis, as impurities and degraded DNA would lead to inefficient amplification and inaccurate sequencing (32). The high purity and concentration obtained in this study were the result of optimized DNA extraction protocols employed in medicinal plant authentication for efficient PCR amplification and further DNA sequencing (33). Distinct bands of 512 and 817 bp were produced from agarose gel electrophoresis of *rbcL* and *matK*, respectively, which are consistent with the expected fragment sizes of these barcode regions of *T. viride*. These clear, distinct bands confirm successful PCR amplification and suggest the suitability of the extracted DNA for analysis (34).

The *rbcL* nucleotide sequence obtained from *T. viride* was 512 bp, with a GC content of 43.75% and an AT content of 56.25%. This profile falls within the expected range for chloroplast *rbcL* sequences in higher plants, supporting the reliability and stability of the sequence. Analysis of the nucleotide composition indicated a moderate GC content in both markers, consistent with the typical features of chloroplast genomes in higher plants.

Generally, chloroplast genes exhibit conserved base composition patterns in which variation in AT-rich regions contributes to evolutionary divergence and species differentiation (35). The observed nucleotide composition is consistent with previously reported *rbcL* sequences within the family Santalaceae, thereby reinforcing its suitability for molecular authentication of *T. viride* (36).

The phylogenetic analyses based on the *rbcL* and *matK* chloroplast DNA barcode regions provided putative molecular evidence supporting the taxonomic placement of *T. viride* within the genus *Thesium*. In both analyses, *T. viride* was recovered as a distinct lineage closely associated with *T. decaryanum*. The occurrence of the same sister relationship in the two independent marker analyses is particularly noteworthy as it provides congruent molecular evidence for the close genetic affinity between these two taxa. The sequences analysed included *T. viride* and closely related *Thesium* species retrieved from GenBank.

The *rbcL* tree showed *T. viride* (PZ051249) and *T. decaryanum* (OR712484) as sister taxa, although the relationship was supported by a relatively low bootstrap value of 44%. This moderate-to-low support suggests that *rbcL* alone may have limited resolving power for confidently establishing relationships between some closely related *Thesium* species. Other relationships in the *rbcL* tree were more strongly supported, as in the case of the *T. strictum*–*T. fragile* pair (94%) and *T. schimperianum*–*T. subsucculentum* pair (93%). The contrasting bootstrap values

demonstrate that evolutionary relationships within the sampled taxa are not equally resolved by the *rbcL* marker.

In contrast, the *matK* tree provided stronger resolution of the relationship involving *T. viride* (PZ804900), which formed a distinct sister pair with *T. decaryanum* (OR712484), supported by a 100% bootstrap value. Such strong support indicates that the association was highly stable within the inferred *matK* topology. This finding is particularly important because the same *T. viride*–*T. decaryanum* relationship was recovered in the *rbcL* analysis, despite the weaker support obtained with that marker. Therefore, the agreement between the two markers strengthens the inference that *T. decaryanum* represents the closest sampled relative of *T. viride* in the present dataset.

The comparatively higher resolution obtained with *matK* may reflect its greater discriminatory capacity among closely related taxa in this study. The substantially stronger support for the *T. viride*–*T. decaryanum* clade in the *matK* tree suggests that *matK* may provide additional phylogenetic information for resolving relationships within *Thesium*. However, the presence of weakly supported deeper nodes in the *matK* tree, including values of 4% and 33%, indicates that not all relationships among the sampled species are well resolved. Therefore, the results should be interpreted as putative evidence of particular relationships rather than as a completely resolved evolutionary history of the genus.

Phylogenetic position of *T. viride* is also relevant to its molecular authentication and standardisation. The species was consistently recovered as a member of the *Thesium* clade in both marker analyses, while its association with *T. decaryanum* was consistently supported across both loci. This provided additional confidence that the sequence generated from the investigated plant represents a genuine *Thesium* taxon rather than an unrelated or misidentified sequence. This

is particularly valuable for *T. viride*, for which reliable identification is important since the plant is used medicinally and accurate authentication is necessary for pharmacognostic and phytochemical investigations.

Nevertheless, the differences in bootstrap support between the two markers demonstrate the importance of using multiple DNA barcode loci rather than relying exclusively on a single chloroplast region (37). The 44% support obtained for the *T. viride*–*T. decaryanum* relationship in *rbcL* contrasts with the 100% support obtained from *matK*. Therefore, the combination of the information from both markers provides a more robust basis for assessment of the molecular identity and relationships of *T. viride*.

The results also indicated that additional loci and a broader sampling of *Thesium* species could further improve resolution of relationships that remain weakly supported in the present phylogenetic trees. Such relationships may be better resolved using a nuclear marker, such as the internal transcribed spacer (26) and Angiosperm353 approach, that focuses on 353 low-copy nuclear genes that are broadly conserved across angiosperms (38).

Overall, the phylogenetic findings provide molecular support for the identity and taxonomic placement of *T. viride*. Consistent recovery of *T. viride* and *T. decaryanum* as sister taxa in both trees, particularly the strong 100% bootstrap support in the *matK* analysis, suggests a close evolutionary relationship between the two species. The results further demonstrate that *matK* provided stronger resolution than *rbcL* for the focal relationship, while the combined use of both markers increases confidence in the putative molecular authentication of *T. viride*. These findings complement the pharmacognostic characteristics of the species and support the application of DNA barcoding as an additional tool for the standardization and identification of *T. viride*.

Conclusion

This study successfully established the pharmacognostic characters and generated the DNA profile of *T. viride* A.W.Hill with GenBank accession numbers PZ051249 and PZ804900 for *rbcL* and *matK*, respectively. These profiles can be used for proper identification and standardization of the plant for medicinal and pharmacological applications.

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Conflict of interests

The authors declare no competing interests.

Author contributions

- Study conceptualization: Sani Shehu and Abdulazeez Abdullahi Muhammed,
- Investigation, methodology, and formal analysis: Sadiya Umar Turaki, Sani Shehu, Abdulazeez Abdullahi Muhammed, Uwaisu Iliyasu and Yushau Musa Hadi.
- Writing (Original manuscript draft): Sani Shehu, Aisha Ibrahim Barau, and Abdulrahman Mahmoud Dogara.
- Writing (Critical revision and editing): Sani Shehu and Murjanatu Abubakar Ahmed.
- All the authors read and approved the final manuscript.

Ethics Statement

Not applicable.

Data Availability

All the data generated during this study are included in this article. Further enquiries on the data can be directed to the corresponding author.

Artificial Intelligence Statement

No artificial intelligence was used in the design, experimentation, analysis, and writing of this manuscript.

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