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Putative occurrence of amentoflavone across *Selaginella* species inferred from HPLC Retention Time analysis

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Abstract. *Setyawan AD, Chikmawati T, Miftahudin, Lotulung PDN, Sutarno, Sugiyarto, Sunarto. 2025. Putative occurrence of amentoflavone across* Selaginella *species inferred from HPLC Retention Time analysis. Biodiversitas 26: 6490-6501.* Amentoflavone is a prominent biflavonoid frequently reported in the genus *Selaginella* and widely studied for its diverse biological activities. However, information on its distribution across species remains fragmented and largely restricted to isolated reports on individual taxa. This study aims to provide a comparative, compound-centric assessment of the putative occurrence of amentoflavone across multiple *Selaginella* species using a standardized Retention Time (RT)-based HPLC approach. Plant materials representing 20 samples from 17 *Selaginella* species were analyzed under uniform extraction and chromatographic conditions. An authentic amentoflavone reference standard was used to establish an external RT benchmark, and chromatograms recorded at 270 nm were qualitatively evaluated for RT correspondence within a predefined RT screening window centered on the reference peak. Occurrence was assessed using a presence–absence framework without quantitative estimation or multivariate analysis. Putative RT correspondence consistent with amentoflavone was detected in 9 of the 17 analyzed species, while several taxa consistently lacked detectable RT-matched peaks. The observed RT ranges among positive species were narrowly clustered, indicating stable chromatographic behavior of the detected signal across different plant matrices. Intraspecific comparisons revealed contrasting presence–absence outcomes among populations of the same species, highlighting population-level variability in detectable compound occurrence. The results demonstrate that putative amentoflavone occurrence in *Selaginella* is species-specific and not universally distributed across the genus. This study underscores the value of RT-based HPLC as an exploratory, hypothesis-generating screening tool for mapping compound occurrence across diverse taxa, while explicitly acknowledging its limitations in structural confirmation and sensitivity. By providing a qualitative baseline, this screening-based framework offers an initial biotechnological entry point for prioritizing *Selaginella* taxa for targeted metabolite validation and subsequent investigation using structure-confirming analytical techniques.

Keywords: Amentoflavone, bioflavonoids, HPLC–UV, retention time matching, *Selaginella*

INTRODUCTION

Amentoflavone is one of the most widely reported biflavonoids in the genus *Selaginella* and has attracted sustained attention due to its structural uniqueness and broad spectrum of biological activities. As a dimeric flavonoid composed of two apigenin units, amentoflavone represents a characteristic class of secondary metabolites frequently associated with early-diverging vascular plants, particularly lycophytes and ferns (Markham 1988; Wollenweber et al. 1998). Within *Selaginella*, amentoflavone has been repeatedly isolated from several species and is often cited as a chemotaxonomically informative compound (Harborne and Williams 2000; Setyawan 2011).

Pharmacological studies have demonstrated that amentoflavone exhibits diverse bioactivities, including antioxidant, anti-inflammatory, antiviral, cytotoxic, and neuroprotective effects (Lin et al. 2000; Cao et al. 2010; Shim et al. 2018; Křížkovská et al. 2021). These properties have positioned amentoflavone as a key candidate for natural product research and drug discovery, particularly in the context of traditional medicinal plants. In *Selaginella*, species such as *S. tamariscina*, *S. doederleinii*, and *S. moellendorffii* have been repeatedly investigated for their biflavonoid content, reinforcing the prominence of amentoflavone within the genus (Cao et al. 2010; Zhao et al. 2013; Yao et al. 2017).

Beyond its pharmacological relevance, amentoflavone is also thought to play ecological and physiological roles, including protection against oxidative stress and environmental pressures such as high irradiance and temperature fluctuations (Close and McArthur 2002; Agati et al. 2012). These functional attributes suggest that the occurrence of amentoflavone in *Selaginella* may be linked not only to phylogenetic constraints but also to adaptive strategies across diverse habitats.

Despite extensive interest in amentoflavone as an individual compound, current knowledge of its distribution across *Selaginella* species remains fragmented. Most phytochemical studies have focused on compound isolation or bioactivity assessment in one or a few species, often selected based on ethnomedicinal use or prior reports of biological activity (Lin et al. 2000; Cao et al. 2010; Shim et al. 2018). As a consequence, available data provide limited insight into whether amentoflavone is consistently present across the genus or restricted to particular taxonomic lineages or ecological groups.

Reports of amentoflavone in *Selaginella* are frequently species-specific and methodologically heterogeneous, making interspecific comparison difficult. In many cases, absence of detection is not explicitly discussed, leaving uncertainty as to whether amentoflavone is genuinely absent or simply undetected due to methodological limitations. Moreover, few studies have examined multiple *Selaginella* species under standardized analytical conditions, a prerequisite for meaningful comparison of compound occurrence patterns (Harborne 1994; Wollenweber et al. 1998). This gap in knowledge limits the interpretation of amentoflavone as a chemotaxonomic or ecological marker within *Selaginella*. Without comparative data, it remains unclear whether amentoflavone represents a broadly conserved biochemical trait, a lineage-specific feature, or a compound whose presence is modulated by environmental factors. Addressing this gap requires a comparative yet compound-focused approach that evaluates the occurrence of amentoflavone across multiple species using consistent analytical criteria.

High-Performance Liquid Chromatography coupled with ultraviolet detection (HPLC–UV) remains one of the most widely applied techniques for flavonoid analysis due to its robustness, reproducibility, and accessibility (Stalikas 2007; Snyder et al. 2010). For compounds such as amentoflavone, which exhibit strong UV absorbance and relatively stable chromatographic behavior, Retention Time (RT) comparison with a reference standard provides a practical means of preliminary detection (Liang et al. 2004; Xie et al. 2006).

RT-based matching does not constitute definitive structural identification, which would require complementary techniques such as LC–MS or NMR. However, when applied under strictly standardized chromatographic conditions and supported by a reference standard, RT correspondence is widely accepted as an exploratory screening tool for assessing putative compound occurrence across multiple samples (Gong et al. 2003; Tistaert et al. 2011). This approach is particularly useful for comparative surveys aimed at identifying presence–absence patterns rather than quantifying compound concentrations or elucidating molecular structures. In the context of *Selaginella*, RT-based HPLC screening provides an efficient strategy to assess the distribution of amentoflavone across multiple species, offering a first-order perspective on interspecific occurrence while minimizing analytical complexity and resource demands.

Given the prominence of amentoflavone in *Selaginella* research and the lack of comparative data on its interspecific distribution, a focused investigation of its occurrence across multiple species is warranted. A compound-centric, RT-based HPLC approach allows systematic evaluation of putative amentoflavone presence under standardized conditions, thereby addressing an important gap in current phytochemical knowledge. The objectives of this study are therefore to (i) assess the putative occurrence of amentoflavone across selected *Selaginella* species using RT-based HPLC analysis, (ii) evaluate the consistency of RT correspondence among species and among samples collected from different localities, and (iii) discuss the potential chemotaxonomic and ecological implications of observed occurrence patterns. By explicitly framing RT matching as a preliminary screening tool, this study aims to provide a robust exploratory foundation for future targeted analyses employing advanced structural and quantitative techniques. Based on the fragmented and species-specific nature of existing reports, we hypothesized that the occurrence of amentoflavone in the genus Selaginella is not universally conserved, but instead shows species-specific and population-dependent patterns. We further posited that such patterns can be preliminarily identified through standardized retention time–based HPLC screening under uniform analytical conditions.

MATERIALS AND METHODS

Plant materials and species identification

This study builds upon an existing HPLC dataset generated under standardized analytical conditions, previously used for comparative flavonoid profiling across several *Selaginella* species (Setyawan et al. 2025). While chromatographic conditions, sample preparation, and raw data acquisition remain identical, the present study adopts a distinct compound-centric analytical framework, focusing specifically on the putative occurrence of amentoflavone inferred from retention time correspondence with an authentic reference standard.

Plant materials analyzed in this study consisted of 20 samples representing 17 *Selaginella* species collected across a broad range of ecological settings and geographic regions in Indonesia. Species selection was designed to capture interspecific variation within the genus rather than to represent exhaustive taxonomic coverage. The analyzed samples encompass lowland, montane, and high-altitude habitats, including forest understories, rocky slopes, riverbanks, and anthropogenically influenced environments. A detailed list of species, collection localities, habitat descriptions, elevation ranges, and voucher information is provided in Table 1.

Field collections were conducted using standard botanical sampling procedures to ensure taxonomic reliability and reproducibility of phytochemical analyses (Bridson and Forman 1992; Singh et al. 2019). Only healthy and mature aerial parts were collected to minimize variability associated with ontogenetic stage or tissue degradation. Habitat type and elevation was recorded in situ using handheld GPS devices or altimeters to provide

ecological context for each sample.

Species identification was based primarily on morphological characters traditionally used in *Selaginella* taxonomy, including growth form, stem architecture, leaf dimorphism, arrangement of lateral and median leaves, and characteristics of strobili when present. Identification followed classical and authoritative taxonomic treatments of *Selaginella* in the Malay Archipelago and surrounding regions, particularly the works of Alston (1934, 1935a, b, 1937, 1940), complemented by more recent regional floristic accounts and revisions (Wong 1982, 2010; Tsai and Shieh 1994; Li and Tan 2005; Setyawan 2012, et al. 2013; Zhang et al. 2013; Weststrand and Korall 2016).

To ensure taxonomic accuracy, collected specimens were compared with authenticated reference material housed at Herbarium Bogoriense (BO), Indonesia, including specimens determined by A.G.H. Alston. Voucher specimens for all analyzed samples were prepared and deposited at the Herbarium of Universitas Sebelas Maret, Indonesia (UNS; herbarium code: SO), with complete collection data including locality, elevation, collector name, and collection number. Voucher accession numbers corresponding to each sample are listed in Table 1. Selected duplicate specimens are intended for deposition at BO to facilitate future taxonomic verification and comparative studies.

Numeric suffixes appended to species names (e.g., "-01", "-02") indicate distinct samples of the same species collected from different localities or collection events, following the original sample labeling (Table 1). These paired samples were included to allow assessment of intraspecific consistency in putative amentoflavone occurrence under standardized analytical conditions, while maintaining taxonomic traceability through voucher documentation.

Sample preparation and extraction

Plant materials of each *Selaginella* sample listed in Table 1 were processed using a standardized extraction protocol to ensure comparability of putative amentoflavone detection across species. Fresh aerial parts, consisting mainly of stems and leaves, were cleaned to remove adhering soil and debris and subsequently air-dried under shade at ambient temperature. Shade drying was chosen to minimize photodegradation and oxidative loss of phenolic compounds, including biflavonoids, which are known to be sensitive to prolonged exposure to light and high temperatures (Stalikas 2007; Dai and Mumper 2010).

Table 1. List of *Selaginella* species, collection sites, and ecological characteristics

Species name	Collection locality	Province (Indonesia)	Habitat type	Elevation (masl)	Voucher specimen
Selaginella opaca	Karangtengah: Sikidang Crater	Central Java	*Albizia lophantha* forest above a volcanic crater	2,124	ADS 22
S. remotifolia	Karangtengah: Sikidang Crater	Central Java	Dirt inspection road of the geothermal steam pipeline	2,071	ADS 23
S. intermedia-01	Citalahab-Malasari: Nirmala Tea Plantation	West Java	Forest trail and tea plantation margin	1,118	ADS 117
S. subalpina-01	Banyukuning: Gedongsongo Temple	Central Java	Shaded area under the bamboo canopy	1,309	ADS 45
S. involvens-01	Kledung Village: Temporary tobacco field	Central Java	Drainage edge of the tobacco field	1,493	ADS 38
S. wildenowii	Citalahab-Malasari: Nirmala Tea Plantation	West Java	Forest trail and tea plantation margin	1,118	ADS 116
S. mayeri	Bogor Botanical Gardens	West Java	Wild growth among botanical garden collections	262	ADS 111
S. ornata	Citalahab-Malasari: Nirmala Tea Plantation	West Java	Forest trail and tea plantation margin	1,118	ADS 118
S. plana	Gondowido: Lake Ngebel	East Java	Lake margin	786	ADS 52
S. frondosa	Bogor Botanical Gardens	West Java	Wild growth among botanical garden collections	262	ADS 112
S. subalpina-02	Gunung Bunder 2: Mount Halimun Salak National Park	West Java	Cliff in Damar Forest	869	ADS 136
S. intermedia-02	Gunung Bunder 2: Mount Halimun Salak National Park	West Java	Cliff in Damar Forest	869	ADS 135
S. aristata	Berjo: Ngargoyoso Grand Forest Park	Central Java	Roadside cliff	1,220	ADS 8
S. ciliaris	Gentan: Batuseribu Park	Central Java	Small river cliff	182	ADS 46
S. repanda	Kulurejo: Riparian agroforestry	Central Java	Hills near a small stream	185	ADS 134
S. involvens-02	Baturiti: Batu Kahu Nature Reserve	Bali	Montane forest floor	1,403	ADS 155
S. ketra-ayam	Mount Menumbing	Bangka Belitung Islands	Hill forest	400	ADS 146
S. velutina	Cycloops Mountain Nature Reserve	Papua	Lower montane forest	281	ADS 141
Selaginella sp. (Mt. Meja)	Mount Meja Protected Forest	West Papua	Lowland protected forest	102	ADS 145
S. caudata	Mount Meja Protected Forest	West Papua	Lowland protected forest	102	ADS 144

Note: Elevation is expressed in meters above sea level (masl)

Dried plant materials were ground into a fine, homogeneous powder using a laboratory mill and stored in airtight containers until extraction. For each sample, approximately 0.5 g of powdered material was extracted with 10 mL of analytical-grade methanol. Methanol was selected as the extraction solvent due to its broad efficiency in solubilizing flavonoids and biflavonoids and its frequent use in phytochemical studies targeting phenolic compounds (Stalikas 2007; Dai and Mumper 2010). Extraction was assisted by sonication for 30 min at room temperature to enhance solvent penetration and compound release, followed by static maceration for 24 h in the dark.

After extraction, the mixtures were centrifuged at 4,000 rpm for 10 min to separate particulate material. The supernatants were carefully collected and filtered through 0.45 µm PTFE syringe filters to remove residual solids and prevent clogging or damage to the chromatographic system (Snyder et al. 2010). Filtrates were transferred into amber glass vials and stored at 4°C prior to HPLC analysis to minimize chemical degradation.

No additional purification or fractionation steps were applied, as the objective of this study was to assess the putative occurrence of amentoflavone under comparable extraction conditions rather than to isolate or quantify individual compounds. All samples were prepared using identical procedures to reduce methodological bias and to ensure that observed differences in chromatographic profiles reflect interspecific variation rather than differences in sample handling.

HPLC instrumentation and analytical conditions

The HPLC analysis was performed to evaluate the putative occurrence of amentoflavone in *Selaginella* extracts under standardized and reproducible analytical conditions. Chromatographic separation was carried out using a reversed-phase HPLC system equipped with a quaternary pump, an autosampler, a column oven, and a UV–visible detector. Separation was achieved on a C18 reversed-phase column (250 × 4.6 mm, 5 µm particle size), a stationary phase widely applied for the analysis of flavonoids and biflavonoids due to its robustness and broad selectivity (Stalikas 2007; Snyder et al. 2010).

The mobile phase consisted of solvent A (water containing 0.1% formic acid) and solvent B (methanol). A gradient elution program was applied to ensure adequate resolution of biflavonoid constituents, particularly in the retention time region corresponding to amentoflavone. The gradient was programmed as follows: 60% B at initial conditions, linearly increased to 80% B over 25 min, then held for 10 min before re-equilibration to initial conditions. The flow rate was maintained at 1.0 mL min^{-1}, the column temperature was set at 30°C, and the injection volume was 10 µL for all samples.

Detection was performed at 270 nm, a wavelength commonly used for monitoring biflavonoids and related phenolic compounds due to their strong UV absorbance in this region (Lin et al. 2000; Cao et al. 2010). Chromatograms recorded at 270 nm were used consistently for all analyses to ensure comparability of RT data across samples.

An authentic reference standard of amentoflavone was analyzed under identical chromatographic conditions to establish a reference retention time and to evaluate system stability. The standard consistently produced a single, well-resolved peak at approximately 7.27 min, serving as an external RT reference for subsequent sample comparisons (Figure 1). Method stability was assessed through replicate injections of the reference standard and selected samples, with RT variation remaining within ±0.2 min across analytical runs. This level of RT stability is considered acceptable for RT-based screening approaches and supports reliable comparison of putative amentoflavone occurrence among samples (Liang et al. 2004; Xie et al. 2006).

All samples and standards were analyzed under identical chromatographic conditions to minimize instrumental bias. No internal standard was added, as the study focused on qualitative RT correspondence rather than quantitative determination.

Amentoflavone reference standard and RT matching criteria

An authentic amentoflavone reference standard (purity ≥98%) was used to establish a chromatographic benchmark for RT comparison. The standard was dissolved in methanol to prepare a working solution at a concentration of 100 µg mL^{-1} and analyzed under the same HPLC conditions applied to all *Selaginella* extracts. The purpose of analyzing the reference standard was to provide an external RT reference for preliminary screening of putative amentoflavone occurrence, rather than to perform definitive compound identification.

Under the standardized chromatographic conditions applied in this study, the amentoflavone reference consistently produced a single, symmetrical peak with a stable retention time of approximately 7.27 min (Figure 1). Replicate injections of the standard were conducted at the beginning, middle, and end of analytical sequences to monitor system performance and RT stability. Across all analytical runs, RT variation of the amentoflavone standard remained within ±0.2 min, indicating satisfactory chromatographic reproducibility and justifying the use of RT matching as a screening criterion (Liang et al. 2004; Xie et al. 2006).

For sample analysis, chromatographic peaks detected in *Selaginella* extracts were evaluated for correspondence with the amentoflavone reference RT. A peak was considered to indicate the putative occurrence of amentoflavone if its RT fell within the predefined tolerance window of ±0.2 min relative to the reference standard and exhibited comparable peak shape and resolution. This tolerance range was selected based on common practice in chromatographic fingerprinting and RT-based screening studies to accommodate minor instrumental fluctuations while minimizing false-positive matching (Gong et al. 2003; Tistaert et al. 2011).

RT correspondence alone was not interpreted as definitive evidence of compound identity. No claims were made regarding structural confirmation, absolute purity, or concentration of amentoflavone in the analyzed extracts. The term “putative” is therefore used throughout this study

to acknowledge the preliminary nature of RT-based inference explicitly. Potential co-elution of structurally related biflavonoids or other phenolic compounds cannot be fully excluded without complementary spectrometric or spectroscopic analyses. By explicitly defining RT matching criteria and analytical limitations, this study adopts a conservative and transparent framework for assessing the putative occurrence of amentoflavone across *Selaginella* species. This approach provides a reproducible exploratory baseline for future targeted investigations employing advanced analytical techniques.

Data interpretation and qualitative assessment

Chromatographic data obtained from HPLC analysis were interpreted using a qualitative, presence–absence framework focused exclusively on the putative occurrence of amentoflavone. For each *Selaginella* sample, chromatograms recorded at 270 nm were visually and analytically examined for the presence of a peak whose RT corresponded to that of the amentoflavone reference standard within the predefined tolerance window of ±0.2 min, as defined in this study. Samples meeting this criterion were recorded as "present," whereas samples lacking such a peak were recorded as "absent."

No quantitative evaluation of peak area, height, or relative abundance was performed. This deliberate omission was intended to avoid biases associated with differences in extraction efficiency, matrix effects, or detector response, and to maintain the exploratory nature of the study. Consequently, the resulting dataset reflects only qualitative occurrence patterns rather than concentration-dependent variation.

Interpretation of results was conducted at two complementary levels. First, interspecific occurrence patterns were evaluated by comparing presence–absence data across different *Selaginella* species. This comparison allowed identification of species exhibiting consistent RT correspondence with the amentoflavone standard as well as species in which such correspondence was not detected. Second, intraspecific consistency was assessed for species represented by multiple samples collected from different localities. Agreement or divergence in presence–absence outcomes among conspecific samples was used to infer the stability of putative amentoflavone occurrence under standardized analytical conditions.

No multivariate analyses, similarity indices, clustering procedures, or fingerprint-based comparisons were applied. The absence of such analyses was intentional and reflects the compound-centric focus of this study. All interpretations were confined to qualitative RT correspondence and descriptive comparison of occurrence patterns.

The presence–absence dataset generated through this approach forms the basis for subsequent presentation of results and discussion of potential chemotaxonomic and ecological implications. By restricting interpretation to qualitative screening outcomes, this study maintains a clear distinction between preliminary RT-based inference and definitive structural or quantitative analyses that would require additional analytical confirmation.

RESULTS AND DISCUSSION

Chromatographic characteristics of amentoflavone reference

The chromatographic behavior of the amentoflavone reference standard was evaluated under the standardized HPLC conditions applied in this study to establish a reliable RT benchmark for subsequent qualitative screening of *Selaginella* extracts. Analysis of the reference solution at 270 nm produced a single, well-defined chromatographic peak with a symmetrical shape and stable baseline separation from adjacent regions of the chromatogram (Figure 1). No secondary peaks or shoulders were observed, indicating adequate chromatographic resolution under the applied conditions.

The amentoflavone standard consistently eluted at an RT of approximately 7.27 min across replicate injections. Minor RT variation was observed between runs; however, this variation remained within a narrow range (±0.2 min), confirming stable chromatographic performance under controlled analytical conditions. This level of reproducibility supports the use of the reference peak as a reliable RT anchor for qualitative comparison of plant extracts.

Peak shape characteristics further supported the suitability of the analytical conditions. The amentoflavone peak exhibited a sharp apex and minimal tailing, suggesting efficient interaction between the analyte and the stationary phase. Such chromatographic behavior is consistent with previous reports describing biflavonoids analyzed on C18 reversed-phase columns under acidic mobile phase conditions (Lin et al. 2000; Stalikas 2007). Clear resolution of the reference peak reduces ambiguity during RT-based matching and minimizes the risk of misinterpretation due to overlapping signals.

For the purpose of putative occurrence screening, a relatively broad RT window (7.07-7.47 min) was defined around the reference RT of amentoflavone (≈7.27 min). This window was intentionally selected to accommodate minor RT variability associated with matrix effects, peak broadening, and subtle instrumental fluctuations commonly encountered in complex plant extracts. The use of this inclusive RT range is appropriate for hypothesis-generating screening aimed at identifying samples that may contain amentoflavone or closely related biflavonoids, while reducing the likelihood of false-negative detection. Accordingly, RT correspondence within this window is interpreted as indicative of putative occurrence rather than definitive compound identification.

The chromatographic characteristics of the amentoflavone reference demonstrate that the HPLC method applied in this study provides sufficient resolution, stability, and reproducibility to support RT-based qualitative screening. These characteristics form the analytical foundation for evaluating the putative occurrence of amentoflavone across Selaginella species, while clearly distinguishing exploratory screening from confirmatory identification.

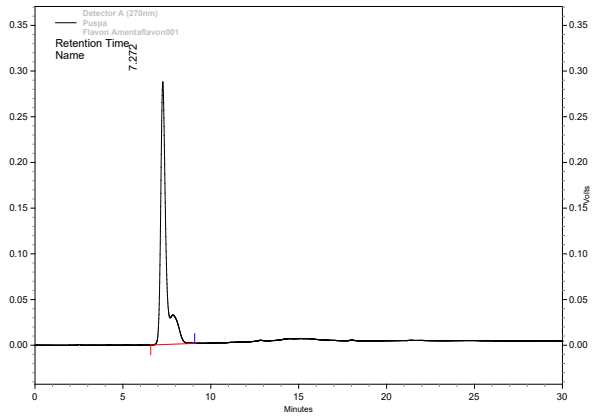

Figure 1. HPLC chromatogram of amentoflavone reference standard recorded at 270 nm under the chromatographic conditions applied in this study, showing a single, well-resolved peak with a retention time of approximately 7.27 min

Putative occurrence of amentoflavone across *Selaginella* species

Qualitative screening of Selaginella extracts using RT-based HPLC analysis revealed variable patterns of putative amentoflavone occurrence across the analyzed species. Evaluation was conducted by examining chromatograms recorded at 270 nm for the presence of peaks eluting within the predefined RT window corresponding to the amentoflavone reference standard (approximately 7.27 ± 0.2 min). Species-level occurrence was inferred when at least one sample of a given species exhibited RT correspondence within the defined screening window. Based on this criterion, samples were classified into two categories: species exhibiting a peak consistent with the reference RT (presence) and species in which no such peak was detected (absence).

Putative amentoflavone occurrence was detected in several *Selaginella* species spanning different geographic regions and habitat types (Table 2). In these species, a distinct chromatographic peak was consistently observed within the expected RT window, with peak shapes comparable to that of the reference standard. The RT values recorded for these peaks showed only minor variation among species, remaining within the predefined tolerance range. This consistency suggests that the detected signals represent a recurrent chromatographic feature compatible with amentoflavone under the applied analytical conditions.

In contrast, a number of species did not exhibit any detectable peak within the reference RT window. For these taxa, chromatograms displayed either no peak or only minor baseline fluctuations in the relevant RT region. The absence of a corresponding peak was consistently observed across replicate injections, supporting the interpretation that putative amentoflavone was not detected in these samples under the applied conditions. Importantly, absence in this context does not imply absolute absence of amentoflavone, but rather indicates that no signal compatible with the reference RT was observed within the sensitivity and resolution limits of the method.

The distribution of putative amentoflavone across species did not show an obvious association with geographic proximity. Species collected from different islands or distant regions sometimes shared similar occurrence patterns, whereas species sampled from nearby localities could differ in presence–absence outcomes. This observation suggests that putative amentoflavone occurrence may reflect intrinsic biochemical traits rather than simple spatial or environmental gradients. However, given the qualitative nature of the analysis, such interpretations remain preliminary.

The RT range associated with detected peaks showed limited interspecific variation (Table 2), further supporting the stability of the chromatographic behavior of the putative compound across different *Selaginella* matrices. This stability is a prerequisite for meaningful comparative screening and reinforces the suitability of RT-based HPLC analysis for exploratory surveys of compound occurrence.

The presence–absence patterns summarized in Table 2 demonstrate that putative amentoflavone is not uniformly distributed across the analyzed *Selaginella* species. Instead, its occurrence appears to be species-specific, with some taxa consistently exhibiting RT correspondence with the reference standard and others lacking detectable signals. These findings provide the first comparative overview of putative amentoflavone distribution across multiple *Selaginella* species under standardized analytical conditions and establish a qualitative baseline for subsequent discussion of chemotaxonomic and ecological implications.

Table 2. Presence or absence of putative amentoflavone in *Selaginella* species based on RT-based HPLC analysis

Species name	Voucher	Putative amentoflavone	RT range (min)
Selaginella opaca	ADS 22	Present	7.25-7.31
S. remotifolia	ADS 23	Absent	-
S. intermedia-01	ADS 117	Absent	-
S. subalpina-01	ADS 45	Present	7.24-7.30
S. involvens-01	ADS 38	Absent	-
S. wildenowii	ADS 116	Absent	-
S. mayeri	ADS 111	Absent	-
S. ornata	ADS 118	Absent	-
S. plana	ADS 52	Absent	-
S. frondosa	ADS 112	Absent	-
S. subalpina-02	ADS 136	Present	7.26-7.33
S. intermedia-02	ADS 135	Present	7.23-7.29
S. aristata	ADS 8	Present	7.22-7.30
S. ciliaris	ADS 46	Present	7.24-7.32
S. repanda	ADS 134	Absent	-
S. involvens-02	ADS 155	Present	7.25-7.31
S. ketra-ayam	ADS 146	Present	7.23-7.30
S. velutina	ADS 141	Present	7.26-7.34
Selaginella sp. (Mt. Meja)	ADS 145	Absent	-
S. caudata	ADS 144	Absent	-

Note: Presence indicates detection of a chromatographic peak within ±0.2 min of the amentoflavone reference RT (≈7.27 min). Absence indicates that no peak meeting this criterion was detected under the applied analytical conditions

Intraspecific consistency across different localities

Evaluation of intraspecific consistency was conducted by comparing paired samples of the same *Selaginella* species collected from different localities and analyzed under identical extraction and chromatographic conditions. This comparison provides insight into whether putative amentoflavone occurrence represents a stable species-level characteristic or a variable feature potentially influenced by local environmental conditions. Two species represented by multiple samples—*Selaginella intermedia* and *Selaginella involvens*—were examined in detail.

For *S. intermedia*, contrasting results were observed between the two analyzed samples. Sample *S. intermedia*–01, collected from the Citalahab area in West Java, did not exhibit a detectable chromatographic peak within the RT window corresponding to the amentoflavone reference. In contrast, *S. intermedia*-02, collected from Gunung Bunder, showed a distinct peak within the defined RT tolerance (Table 2). The detected RT range for *S. intermedia*-02 was consistent with that of the reference standard and comparable to RT values observed in other species classified as positive for putative amentoflavone occurrence.

This divergence suggests that putative amentoflavone occurrence in *S. intermedia* may not be strictly invariant across populations. Several non-exclusive explanations may account for this pattern. Differences in microhabitat conditions, such as light exposure, moisture availability, or substrate characteristics, could influence secondary metabolite expression and lead to localized variation in detectable compound profiles. Alternatively, genetic differentiation among populations, even within the same nominal species, may contribute to differences in biflavonoid biosynthesis. Given the qualitative nature of the present analysis, it is not possible to distinguish between these possibilities, but the observed contrast highlights the importance of considering multiple populations when evaluating compound occurrence.

In contrast to *S. intermedia*, *S. involvens* exhibited a more consistent pattern across localities. Sample *S. involvens*–01, collected from Kledung in Central Java, did not show detectable RT correspondence with the amentoflavone standard, whereas *S. involvens*-02, collected from Batu Kahu Nature Reserve in Bali, consistently exhibited a peak within the reference RT window (Table 2). Although this also represents a presence–sabsence contrast, the RT signal detected in *S. involvens*-02 was reproducible across injections and fell within the same narrow RT range observed in other positive species.

Taken together, these paired comparisons indicate that putative amentoflavone occurrence can vary among populations of the same species. Such variation underscores that the absence of detection in a given sample does not necessarily imply species-wide absence of the compound. Instead, putative amentoflavone occurrence may reflect a combination of species-level biosynthetic capacity and population-level modulation influenced by ecological or physiological factors.

These findings reinforce the value of a presence–absence framework for exploratory screening while simultaneously highlighting its limitations. Intraspecific comparisons reveal that species-level generalizations regarding amentoflavone occurrence should be made cautiously and, where possible, supported by analyses of multiple populations and complementary analytical approaches.

Species lacking detectable putative amentoflavone

A subset of the analyzed *Selaginella* species did not exhibit any detectable chromatographic peak within the RT window corresponding to the amentoflavone reference standard. For these taxa, chromatograms recorded at 270 nm showed either a flat baseline or only minor background fluctuations in the RT region of approximately 7.27 ± 0.2 min. Based on the criteria defined in this study, these species were classified as lacking detectable putative amentoflavone under the applied analytical conditions (Table 2).

Species falling into this category included *Selaginella remotifolia*, *S. intermedia*-01, *S. involvens*-01, *S. wildenowii*, *S. mayeri*, *S. ornata*, *S. plana*, *S. frondosa*, *S. repanda*, *Selaginella* sp. (Mt. Meja), and *S. caudata*. The absence of RT-matched peaks was consistent across replicate injections, suggesting that the observed pattern reflects reproducible analytical outcomes rather than sporadic instrumental noise or transient chromatographic instability. It is important to emphasize that "absence" in this context refers strictly to the lack of detectable RT correspondence with the amentoflavone reference under the specific extraction and HPLC conditions employed in this study. This classification does not imply definitive absence of amentoflavone at the species level. Several factors may account for non-detection, including low compound concentration below the detection limit of UV-based HPLC, matrix interference, or the presence of structurally related biflavonoids with distinct chromatographic behavior.

The inclusion of species lacking detectable putative amentoflavone is analytically important, as it demonstrates that RT-based screening does not yield uniformly positive results across the genus. This heterogeneity reinforces the interpretation that putative amentoflavone occurrence in *Selaginella* is species- or population-specific rather than universal and provides essential context for interpreting positive detections reported elsewhere in this study.

Summary of occurrence patterns

The qualitative RT-based HPLC screening conducted in this study revealed a heterogeneous pattern of putative amentoflavone occurrence across the analyzed *Selaginella* species. Evaluation of chromatograms at 270 nm, anchored to the RT of the amentoflavone reference standard (Figure 1), demonstrated that detectable RT correspondence was present in a subset of species, while others consistently lacked signals within the defined RT window (Table 2).

Species classified as positive for putative amentoflavone occurrence exhibited a distinct chromatographic peak eluting at approximately 7.27 min, with minor interspecific variation remaining within the predefined tolerance range of ±0.2 min. The RT ranges

observed among positive species were narrowly clustered, indicating stable chromatographic behavior of the detected signal across different plant matrices. This consistency supports the use of RT-based screening as a reliable qualitative approach for exploratory assessment of compound occurrence.

In contrast, several species showed no detectable peak within the reference RT window despite being analyzed under identical extraction and chromatographic conditions. The absence of RT-matched peaks was reproducible across replicate injections and was not associated with analytical instability, as demonstrated by the consistent performance of the amentoflavone reference standard (Figure 1). These negative outcomes underscore that putative amentoflavone occurrence is not uniformly distributed across the genus.

Intraspecific comparisons further highlighted variability at the population level. Paired samples of *S. intermedia* and *S. involvens* collected from different localities showed contrasting presence–absence outcomes, indicating that detection of putative amentoflavone may vary within the same nominal species. Such variation suggests that species-level generalizations should be made cautiously when based on limited population sampling.

Taken together, the results presented above indicate that putative amentoflavone occurrence in *Selaginella* is characterized by species-specific and population-dependent patterns rather than universal presence. These findings establish a qualitative baseline for subsequent discussion of chemotaxonomic relevance, ecological modulation, and methodological limitations, and they provide a structured foundation for interpreting the broader implications of RT-based screening in phytochemical studies.

Discussion

Reliability and limitations of RT-based inference

The reliability of RT-based HPLC analysis as an exploratory tool for assessing putative compound occurrence depends on the stability of chromatographic conditions, the use of an appropriate reference standard, and the transparency with which analytical limitations are acknowledged. In the present study, RT-based inference was intentionally applied as a qualitative screening approach to evaluate the putative occurrence of amentoflavone across multiple *Selaginella* species, rather than as a definitive method for compound identification or quantification.

One of the principal strengths of RT-based inference lies in its reproducibility when chromatographic parameters are tightly controlled. The consistent elution of the amentoflavone reference standard at approximately 7.27 min across analytical runs, with RT variation confined within ±0.2 min, indicates stable system performance and supports the use of RT correspondence as a comparative benchmark. Similar tolerance ranges have been widely adopted in chromatographic fingerprinting and screening studies to accommodate minor instrumental fluctuations without compromising interpretability (Liang et al. 2004; Xie et al. 2006; Tistaert et al. 2011). Under such conditions, RT matching provides a practical and accessible means of surveying compound occurrence across multiple samples.

RT-based screening is particularly suitable for compounds such as amentoflavone, which exhibit strong UV absorbance and relatively stable chromatographic behavior on reversed-phase C18 columns (Lin et al. 2000; Stalikas 2007). When combined with an authentic reference standard analyzed under identical conditions, RT correspondence can serve as a reliable first-order indicator of putative presence, especially in comparative studies where the primary objective is to document presence–absence patterns rather than to establish structural identity.

Nevertheless, the limitations of RT-based inference must be explicitly recognized. Retention time alone does not provide definitive structural information, and different compounds with similar physicochemical properties may elute within overlapping RT windows. In plant extracts, which often contain complex mixtures of flavonoids and other phenolic compounds, co-elution or partial overlap cannot be fully excluded without complementary spectrometric evidence (Wolfender et al. 2003; Dunn et al. 2013). Consequently, RT correspondence should not be interpreted as conclusive proof of compound identity, and the use of the term “putative” is essential to reflect the inferential nature of the approach accurately.

Another limitation relates to detection sensitivity. UV-based HPLC may fail to detect compounds present at low concentrations, particularly when matrix effects suppress signal intensity or when compounds are unevenly distributed among tissues or developmental stages. Thus, absence of an RT-matched peak does not necessarily indicate true absence of amentoflavone at the species level, but rather absence of a detectable signal under the applied analytical conditions. This constraint is well recognized in phytochemical screening studies and underscores the importance of cautious interpretation of negative results (Stalikas 2007; Snyder et al. 2010).

Furthermore, RT-based inference does not capture quantitative variation in compound abundance. Differences in biosynthetic activity among species or populations may result in substantial variation in concentration without altering RT correspondence. By design, the present study did not incorporate peak area or height measurements, as the objective was to maintain a qualitative, presence–absence framework and to avoid biases associated with extraction efficiency or detector response. While this choice enhances methodological clarity, it also limits the scope of inference to qualitative patterns.

In light of these considerations, RT-based HPLC analysis should be viewed as a preliminary screening tool that occupies an intermediate position between broad, non-targeted surveys and highly specific, structure-confirming techniques such as LC–MS or NMR. Its value lies in its ability to generate reproducible, comparative data across multiple taxa under standardized conditions, thereby guiding the selection of species or populations for more detailed chemical investigation. When applied transparently and interpreted conservatively, RT-based inference provides a reliable exploratory foundation while

clearly delineating the boundaries beyond which additional analytical confirmation is required.

Chemotaxonomic relevance of amentoflavone distribution

The distribution of individual secondary metabolites has long been considered a potentially informative, albeit complementary, source of evidence in plant chemotaxonomy. Among flavonoids, biflavonoids have attracted particular attention because of their restricted occurrence and relative chemical stability, traits that may reflect underlying biosynthetic capacity conserved within certain evolutionary lineages (Markham 1988; Harborne 1994; Wollenweber et al. 1998). Within *Selaginella*, amentoflavone is frequently cited as a characteristic biflavonoid, yet its taxonomic relevance has rarely been evaluated from a comparative, interspecific perspective.

The presence–absence patterns documented in this study suggest that putative amentoflavone occurrence is not uniform across *Selaginella* species. Several taxa consistently exhibited RT correspondence with the amentoflavone reference standard, whereas others lacked detectable signals under identical analytical conditions (Table 2). From a chemotaxonomic standpoint, such heterogeneity indicates that amentoflavone should not be regarded as a universal chemical marker for the genus. Instead, its occurrence appears to be restricted to a subset of species, implying differential retention or expression of the biosynthetic pathway responsible for biflavonoid formation.

In classical chemotaxonomy, the taxonomic significance of a compound depends not only on its presence but also on its distributional pattern across related taxa (Harborne 1994; Wink and Waterman 1999). Compounds that are sporadically distributed may still hold taxonomic relevance if their occurrence correlates with specific lineages or morphological groups. However, the qualitative data presented here indicate that putative amentoflavone occurrence does not map straightforwardly onto geographic proximity or obvious ecological similarity among species. This observation suggests that the chemotaxonomic signal of amentoflavone, if present, is more likely linked to intrinsic biosynthetic traits rather than external environmental factors alone.

At the same time, the observed intraspecific variability, particularly in *S. intermedia* and *S. involvens*, cautions against overinterpreting presence–absence data as fixed species-level characters. Population-level modulation of secondary metabolite expression has been widely reported in vascular plants and is often influenced by microenvironmental conditions, physiological status, or genetic differentiation among populations (Gershenzon 1984; Wink 2003). In this context, the absence of detectable amentoflavone in one population does not necessarily negate its potential biosynthetic capacity within the species as a whole. Consequently, chemotaxonomic inference based on a single compound should be treated as suggestive rather than definitive.

Importantly, the chemotaxonomic relevance discussed here differs conceptually from pattern-based approaches that rely on entire metabolite profiles. The present analysis focuses exclusively on a single, biologically significant biflavonoid and evaluates its occurrence qualitatively across species. This compound-centric perspective avoids conflating broader chemical similarity with taxonomic affinity and instead highlights how individual metabolites may exhibit lineage-restricted or population-dependent distributions. Such information can complement, but not replace, chemotaxonomic insights derived from comprehensive metabolite profiling or molecular phylogenetic frameworks (Wink and Waterman 1999; Burleigh et al. 2009).

Within *Selaginella*, previous phytochemical studies have reported amentoflavone predominantly from species that are also known for diverse biflavonoid assemblages and notable bioactivities (Lin et al. 2000; Cao et al. 2010; Zhao et al. 2013; Yao et al. 2017). The selective occurrence observed in this study is consistent with these reports and suggests that amentoflavone may serve as an indicator of broader biflavonoid biosynthetic potential rather than as an isolated taxonomic marker. From this perspective, its chemotaxonomic value lies in flagging taxa of interest for deeper phytochemical investigation rather than in providing rigid taxonomic boundaries.

The distribution of putative amentoflavone across *Selaginella* species underscores both the potential and the limitations of single-compound chemotaxonomic inference. While presence–absence patterns reveal non-random chemical structuring within the genus, they must be interpreted in conjunction with population-level variation, methodological constraints, and independent lines of taxonomic evidence. When treated cautiously, amentoflavone distribution can contribute to a nuanced chemotaxonomic framework that integrates chemical, morphological, and molecular perspectives without overstating the taxonomic weight of a single metabolite.

Ecological and adaptive interpretation of occurrence patterns

Secondary metabolites such as flavonoids and biflavonoids are widely recognized as mediators of plant–environment interactions, contributing to physiological protection and ecological adaptation. In lycophytes and ferns, these compounds have been associated with tolerance to abiotic stresses, including high irradiance, temperature fluctuation, and oxidative pressure, as well as with defense against pathogens and herbivores (Close and McArthur 2002; Wink 2008; Agati et al. 2012). Within this broader framework, the heterogeneous patterns of putative amentoflavone occurrence observed across *Selaginella* species may reflect differences in ecological strategies and adaptive responses rather than purely taxonomic divergence.

Amentoflavone and related biflavonoids are known for their strong antioxidant properties, which can mitigate oxidative stress generated under challenging environmental conditions (Cao et al. 2010; Shim et al. 2018). In *Selaginella*, species that inhabit exposed habitats, rocky substrates, or environments characterized by fluctuating moisture availability may benefit from enhanced antioxidant capacity. The detection of putative amentoflavone in several species occupying such habitats suggests that its

biosynthesis could be associated with stress tolerance mechanisms, although the present qualitative data do not allow direct testing of this hypothesis.

Conversely, the absence of detectable putative amentoflavone in some species, including those collected from relatively shaded or mesic environments, may indicate reliance on alternative protective strategies or different suites of secondary metabolites. Plants often deploy diverse chemical defenses depending on ecological context, and the presence of one compound does not preclude functional redundancy with others (Gershenzon 1984; Wink 2003). In this sense, amentoflavone occurrence should be viewed as one component of a broader adaptive repertoire rather than as a singular determinant of ecological fitness.

Intraspecific variation observed in *S. intermedia* and *S. involvens* further supports the notion that local environmental conditions can modulate secondary metabolite expression. Differences in light intensity, substrate type, water availability, or nutrient status between collection sites may influence the activation of biosynthetic pathways responsible for biflavonoid production. Such plasticity has been documented in numerous vascular plants, where phenolic content can vary substantially across populations and microhabitats without underlying taxonomic differentiation (Close and McArthur 2002; Agati et al. 2012).

Adaptive interpretation of occurrence patterns must also consider the evolutionary history of *Selaginella*, a genus characterized by ancient lineages and remarkable ecological breadth. The retention or loss of specific secondary metabolites over evolutionary time may reflect historical selective pressures rather than current environmental conditions alone. From this perspective, putative amentoflavone occurrence could represent an inherited biosynthetic trait maintained in certain lineages where it confers adaptive advantages, while being reduced or absent in others where alternative strategies prevail.

The present study does not establish causal relationships between amentoflavone occurrence and ecological variables. The absence of quantitative data and environmental measurements precludes direct correlation analysis. Nonetheless, the qualitative patterns documented here provide a useful starting point for generating testable hypotheses regarding the ecological function of biflavonoids in *Selaginella*. Future studies integrating targeted chemical analyses with ecological measurements, experimental manipulation, or transcriptomic data could clarify the extent to which amentoflavone contributes to stress tolerance and adaptive differentiation within the genus.

The observed occurrence patterns are consistent with a view of amentoflavone as a potentially adaptive metabolite whose expression varies across species and populations in response to ecological and evolutionary factors. By situating these patterns within a broader ecological framework, this study highlights the value of compound-centric screening as an entry point for understanding functional diversity in early-diverging vascular plants, while acknowledging the need for complementary approaches to resolve adaptive significance fully.

Implications for phytochemical screening and future studies

The qualitative patterns of putative amentoflavone occurrence documented in this study have important implications for phytochemical screening strategies in *Selaginella* and other early-diverging vascular plants. The results demonstrate that RT-based HPLC analysis, when applied transparently and interpreted conservatively, can function as an efficient exploratory tool for surveying compound occurrence across multiple taxa. This approach is particularly relevant for species-rich genera with limited prior chemical characterization, where comprehensive structure-confirming analyses for all taxa may be impractical at the initial stage of investigation.

From a screening perspective, the presence–absence framework adopted here provides a pragmatic basis for prioritizing species or populations for downstream analysis. Species consistently exhibiting RT correspondence with amentoflavone under standardized conditions can be identified as candidates for more detailed phytochemical work, including quantitative profiling and structural confirmation using LC–MS or NMR. Conversely, taxa lacking detectable RT-matched signals may be provisionally deprioritized or redirected toward investigation of alternative secondary metabolites that could underlie their ecological or pharmacological attributes. In this sense, RT-based screening serves as a decision-support step rather than an analytical endpoint.

The findings also emphasize the importance of population-level sampling in phytochemical surveys. Intraspecific variation observed in *S. intermedia* and *S. involvens* indicates that chemical screening based on single populations may yield incomplete or potentially misleading conclusions. Future studies aimed at resolving compound distribution patterns should therefore incorporate multiple populations per species whenever feasible, particularly for taxa of medicinal or ecological relevance. Such designs would enable clearer differentiation between chemically stable traits and environmentally or genetically mediated variation.

Methodologically, this study highlights the need to distinguish exploratory screening from definitive compound identification clearly. By explicitly treating RT correspondence as evidence of putative occurrence, the analysis avoids overinterpretation while still revealing biologically meaningful patterns. Building on this foundation, future research can integrate complementary analytical techniques. For example, coupling RT-based HPLC screening with targeted LC–MS analysis would allow confirmation of compound identity and detection of structurally related biflavonoids that may co-elute or escape UV-based detection, an approach increasingly advocated in contemporary phytochemical research (Dunn et al. 2013; Wolfender et al. 2013).

Beyond analytical refinement, the occurrence patterns reported here open avenues for interdisciplinary investigation. Integrating phytochemical screening with ecological measurements, experimental stress assays, or gene expression analyses could clarify the functional significance of amentoflavone in *Selaginella*. Comparative studies under controlled environmental conditions may help

determine whether amentoflavone biosynthesis is constitutive or inducible, while integration with phylogenetic frameworks could shed light on the evolutionary history of biflavonoid pathways within the genus.

Finally, the implications of this work extend to applied research contexts. Given the pharmacological relevance of amentoflavone, identifying taxa with consistent putative occurrence may inform bioprospecting strategies and conservation prioritization. Species highlighted through preliminary screening can be evaluated further for sustainable utilization, while recognizing that phytochemical diversity itself constitutes an important component of biodiversity, warranting protection. The present findings underscore the value of RT-based HPLC screening as a foundational step in phytochemical research. When embedded within a transparent methodological framework and followed by targeted, hypothesis-driven analyses, such screening approaches can efficiently guide discovery while maintaining scientific rigor.

Methodological limitations and perspectives

While the RT-based HPLC approach employed in this study provides a useful exploratory framework for assessing putative amentoflavone occurrence, several methodological limitations must be acknowledged to ensure appropriate interpretation of the results. Recognizing these constraints is essential for situating the findings within their proper analytical context and for guiding future methodological refinement.

A primary limitation of this study is the reliance on retention time correspondence and UV detection as the sole basis for inferring compound occurrence. Although RT stability and reference standard comparison support reproducible screening, retention time alone does not provide definitive structural confirmation. Co-elution of structurally related biflavonoids or other phenolic compounds cannot be completely excluded, particularly in complex plant matrices. As such, the present results should not be interpreted as conclusive evidence of amentoflavone identity but rather as indicative of a putative occurrence that warrants further confirmation using spectrometric or spectroscopic techniques.

Another limitation relates to analytical sensitivity. UV-based detection may fail to identify compounds present at low concentrations, leading to false-negative outcomes in some species or populations. Differences in extraction efficiency, tissue composition, or matrix interference may further influence detectability. Consequently, the absence of RT-matched peaks should be interpreted cautiously and not equated with true biochemical absence. Incorporating more sensitive detection methods, such as tandem mass spectrometry, would enhance the ability to detect low-abundance compounds and refine presence–absence assessments.

The qualitative design of this study also constrains interpretation. By focusing on presence–absence patterns, the analysis does not capture quantitative variation in amentoflavone content among species or populations. Such variation may hold ecological or pharmacological significance but lies beyond the scope of the present screening-based framework. Future studies incorporating quantitative calibration and internal standards could address this limitation and provide deeper insight into metabolite abundance and variability.

From a sampling perspective, although multiple species and selected paired populations were included, coverage remains incomplete. Expanding geographic and population-level sampling would improve representativeness and allow more robust evaluation of intra- and interspecific variability. Additionally, integrating environmental metadata and experimental controls would help disentangle genetic versus ecological drivers of metabolite expression.

Looking forward, the methodological perspective emerging from this study emphasizes integration rather than replacement. RT-based HPLC screening is best viewed as a first-tier approach that efficiently narrows the field of candidate taxa and hypotheses. Subsequent analytical tiers, including LC–MS, NMR, and transcriptomic or enzymatic studies, are essential for confirming compound identity, elucidating biosynthetic pathways, and understanding functional roles. By combining exploratory screening with targeted analytical validation, future research can build a more comprehensive and reliable understanding of chemical diversity in *Selaginella* and other early-diverging plant lineages.

Importantly, the qualitative screening approach adopted here is intentionally inclusive and hypothesis-generating, designed to identify candidate taxa for further investigation; in contrast, more restrictive RT feature definition and alignment are appropriate for comparative fingerprinting or similarity-based analyses that prioritize feature homology over detection sensitivity.

In conclusion, this study presents a comparative, compound-centric assessment of the putative occurrence of amentoflavone across *Selaginella* species using RT-based HPLC screening. Analysis of 20 samples representing 17 species revealed RT correspondence consistent with amentoflavone in 9 species (≈53%), whereas 8 species (≈47%) consistently lacked detectable RT-matched peaks under standardized analytical conditions. Detected RT values among positive species were narrowly clustered around the reference peak (≈7.27 min), indicating stable chromatographic behavior across different plant matrices. Intraspecific comparisons further revealed population-level variability, with contrasting presence–absence outcomes observed among samples of the same species collected from different localities. These findings indicate that putative amentoflavone occurrence in *Selaginella* is species-specific and population-dependent rather than uniformly distributed across the genus. The results highlight the utility of RT-based HPLC as an exploratory, qualitative screening tool for surveying compound occurrence and prioritizing taxa for further investigation. However, interpretations are constrained by reliance on RT correspondence and UV detection alone, which do not provide structural confirmation or quantitative information. Accordingly, all detections are treated as putative, and absence of RT-matched peaks does not imply true biochemical absence. Future studies integrating structure-confirming and more sensitive techniques, such as LC–

MS/MS or NMR, alongside expanded population-level sampling, are required to validate compound identity and clarify the ecological and chemotaxonomic significance of amentoflavone in *Selaginella*.

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