



Article

Comparative Biological Activities of Cotyledon and True Leaf Stages in Diverse Native Plant Seedlings

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Highlights

What are the main findings?

- True leaves generally exhibit higher phenolic and flavonoid accumulation than cotyledons.
- Specific cotyledons exhibit stronger antibacterial activity than mature true leaves.

What are the implications of the main findings?

- Ontogenetic stages dictate distinct shifts between antioxidant and virulence inhibition.
- These findings enable targeted biomass harvesting for specific functional applications.

Abstract

The transition from cotyledons to true leaves involves substantial metabolic changes. However, the stage-specific bioactive properties of native ornamental plants have not been thoroughly investigated. This study compared total phenolic and flavonoid contents together with preliminary biological activities of the cotyledon and true leaf stages in *Lespedeza cyrtobotrya*, *Amorpha fruticosa*, *Flueggea suffruticosa*, *Ulmus parvifolia*, *Tripura divaricata*, and *Paulownia coreana* seedlings to optimize their potential for horticultural applications. Total phenolic and flavonoid contents (TPC and TFC) of ethanol extracts were evaluated, and radical scavenging capacities were assessed using ABTS and DPPH assays. Preliminary antibacterial activities were also investigated against *Staphylococcus aureus*, *Acinetobacter baumannii*, while potential quorum sensing (QS) inhibition was tested against *Chromobacterium violaceum*. The transition to the true leaf stage was consistently associated with increased TPC and TFC levels, suggesting increased antioxidant potential in *L. cyrtobotrya*, *A. fruticosa*, *F. suffruticosa*, and *U. parvifolia*. In contrast, cotyledons of *T. divaricata* and *P. coreana* exhibited preliminary antibacterial and antioxidant potential that surpassed those of the true leaves. As the true leaves matured, pigment-suppression activity was observed in selected extracts, suggesting that the true leaf stage may possess potential QS inhibitory activity. These stage-dependent bioactivities provide essential baseline data for optimizing harvest timing and highlight the potential of specific seedling stages for applications in urban horticulture.



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Keywords: antioxidant activity; antibacterial activity; cotyledon; quorum sensing; true leaf stage

1. Introduction

The rapid spread of multidrug-resistant bacteria and the increasing prevalence of diseases associated with oxidative stress represent major global health challenges [1]. Conventional single-target synthetic drugs have significant limitations, creating an urgent need for natural bioactive compounds with multi-target capabilities [2]. Plant secondary metabolites, particularly flavonoids and phenolic acids, are considered promising next-generation antibacterial and antioxidant agents because they can restore intracellular redox balance and disrupt microbial pathogenicity without readily inducing resistance [3,4]. In this context, the diverse flora of the Republic of Korea represents a valuable reservoir of natural bioactive resources for horticultural optimization and crop protection programs, owing to its wide variety of endemic plant species shaped by unique climatic and edaphic conditions [5,6]. Approximately 63% of the Republic of Korea's land area is covered by forests, supporting highly diverse vascular flora comprising 3975 native taxa (3307 species and 668 infraspecific taxa) classified into 183 families and 951 genera [6]. Many of these native woody and herbaceous plants are highly valuable for urban horticulture, landscape design, and medicinal gardens [7].

Despite the clear horticultural and secondary metabolite potential of these plant resources, phytochemical evaluations have largely focused on fully mature plant tissues, such as adult leaves and bark [8,9]. However, the biosynthesis of secondary metabolites is dynamically regulated throughout plant ontogeny [10]. The transition from cotyledons (seed leaves), which temporarily utilize stored embryonic nutrients to support early growth and defense, to autotrophic true leaves is accompanied by substantial changes in metabolic flux [11]. Young cotyledons often synthesize high concentrations of soluble defense compounds to compensate for their limited physical barriers, whereas mature true leaves allocate more energy toward the production of complex polyphenols and structural defense mechanisms [12]. Therefore, determining which developmental stage possesses greater biological value is highly species-dependent and remains insufficiently explored. Since QS-based strategies interfere with pathogen activity rather than eliminating the bacteria, they minimize the selective pressure for resistance, thereby avoiding the rapid evolution of pesticide resistance associated with conventional chemicals [13–15].

To address this gap, we selected six phylogenetically diverse native woody taxa of significant landscape and nursery value to evaluate and compare the baseline biological profiles of their cotyledon and true leaf stages. Previous studies have shown that these species biosynthesize potent specialized metabolites capable of exerting antimicrobial and antioxidant effects. In particular, disrupting bacterial quorum sensing (QS), the chemical communication system that regulates collective virulence and biofilm formation, has attracted attention as a quorum-quenching strategy that minimizes the selective pressure associated with conventional antibiotics [14]. While horticultural applications ultimately target specific crop pathogens (such as *Pseudomonas*, *Xanthomonas*, or *Erwinia*), preliminary screenings for antimicrobial and QS inhibition regularly leverage robust model strains to map baseline biological efficacy before micro-propagation or field scale-up. Therefore, assessing raw extracts against standard model bacteria establishes a fundamental screening benchmark for these underutilized horticultural species.

Therefore, this study aimed to spectrophotometrically estimate the total phenolic and flavonoid contents (TPC and TFC) and pharmacological activities of six native Korean

seedlings at the cotyledon and true leaf stages. We evaluated their radical scavenging capacities and investigated their defense-related biological activities by comparing direct bactericidal effects against major clinical pathogens (*Staphylococcus aureus* and *Acinetobacter baumannii*) and QS inhibitory activity using the *Chromobacterium violaceum* biosensor model. This stage-specific profiling provides a foundational framework for optimizing the harvest timing and application of highly efficient natural therapeutics within horticultural production systems.

2. Materials and Methods

2.1. Plant Materials and Cultivation Conditions

The cotyledon (C) and true leaf (T) stages of six native Korean plant species were investigated: *Lespedeza cyrtobotrya* Miq. (LCC and LCT, respectively), *Amorpha fruticosa* L. (AFC and AFT, respectively), *Flueggea suffruticosa* (Pall.) Baill. (FSC and FST, respectively), *Ulmus parvifolia* Jacq. (UPC and UPT, respectively), *Tripura divaricata* (Maxim.) P.D.Cantino (TDC and TDT, respectively), and *Paulownia coreana* Uyeki (PCC and PCT, respectively) [16]. These six specific species were selected to enhance the potential applications of forest resources. The selection criteria included species that are widely distributed throughout the forests of the Republic of Korea or occur across multiple regions for ease of seed collection and species that do not require specific pretreatment methods for breaking seed dormancy. Furthermore, these taxa are recognized for their robust environmental stress tolerance and resistance to seedling-stage diseases, suggesting a highly active and dynamically regulated secondary metabolism during early ontogeny. By evaluating species across diverse families, this selection allows for a comparative analysis of developmental phytochemistry across distinct taxonomic lineages relevant to woody plant propagation. The plants were cultivated and authenticated by Dr. C.-H. Choi at the Gyeonggi-do Forestry Environment Research Center, Osan, Republic of Korea. Seeds of each species were collected between mid-October and late October 2024 from the locations listed in Table 1 and Figure 1. Following collection (Figure 2), the seeds were thoroughly cleaned and stored in a specialized seed storage room maintained at 2 ± 1 °C to preserve viability until propagation. In early April 2025, prior to sowing, all seeds were soaked in distilled water for 24 h to promote uniform imbibition. The treated seeds were then sown in a commercial horticultural substrate (Nongwoo Bio Co., Ltd., Suwon, Republic of Korea) within a controlled greenhouse setting at the Osan experimental field. The germination rates of each species are presented in Table 1. Following germination, the seedlings were cultivated under greenhouse conditions for approximately 20 days to allow for the sufficient development of true leaves. Cotyledon and true leaf samples were separately collected by carefully removing the seedlings from the potting soil by hand to avoid any structural damage or stress-induced alterations to the delicate plant tissues. The harvested samples were immediately washed with distilled water to completely remove any adhering soil particles or substrate residues.

Table 1. Source of plant materials and germination rate.

Plant Species (Family)	Collection Location	Germination Rate (%)
<i>L. cyrtobotrya</i> (Fabaceae)	Joan-ri, Joan-myeon, Namyangju-si	45.3 ± 4.2
<i>A. fruticosa</i> (Fabaceae)	Tonghyeon-ri, Yeoncheon-eup, Yeoncheon-gun	40.7 ± 6.4
<i>F. suffruticosa</i> (Phyllanthaceae)	Jeokmok-ri, Buk-myeon, Gapyeong-gun	68.7 ± 4.2
<i>U. parvifolia</i> (Ulmaceae)	Sucheong-dong, Osan-si	44.7 ± 3.1
<i>T. divaricata</i> (Verbenaceae)	Maeryong-dong, Yeosu-si	52.7 ± 5.0
<i>P. coreana</i> (Paulowniaceae)	Choji-ri, Gilsang-myeon, Ganghwa-gun	24.0 ± 2.0

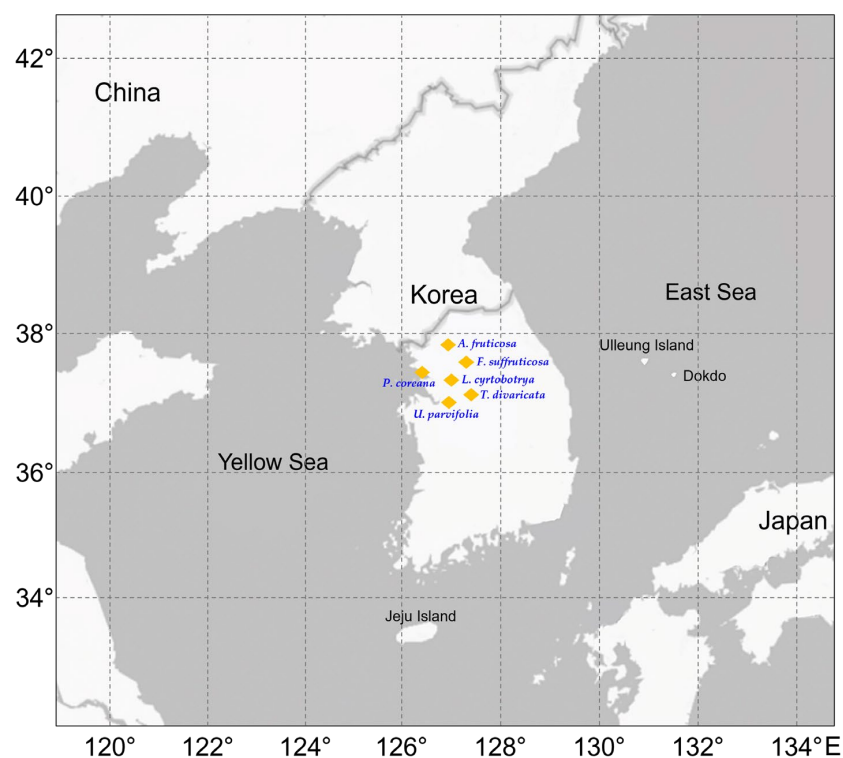


Figure 1. Collection locations of plant materials.

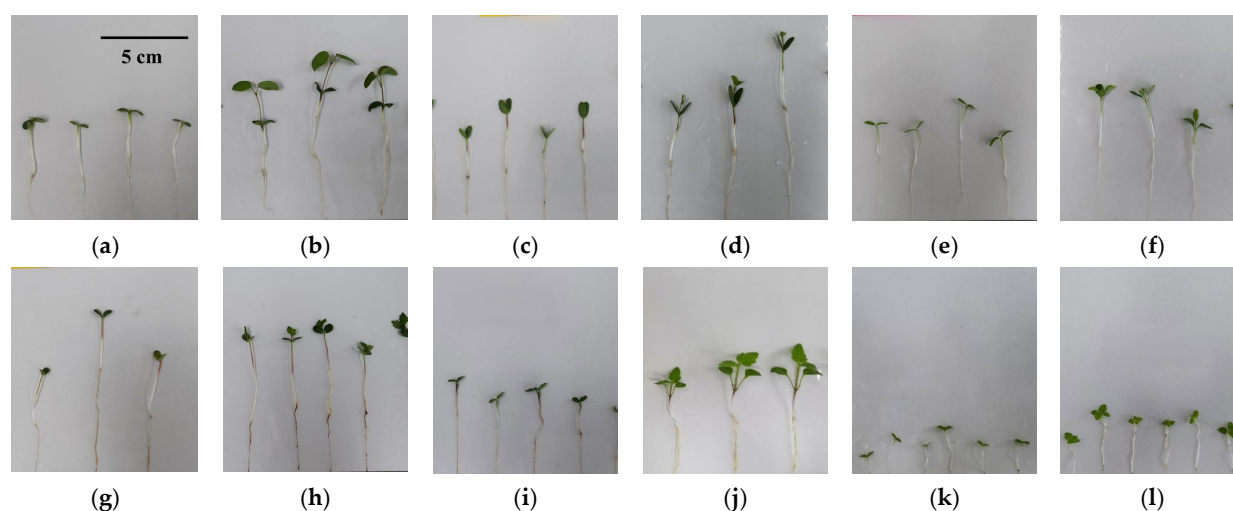


Figure 2. Representative images of seedlings: (a) LCC, (b) LCT, (c) AFC, (d) AFT, (e) FSC, (f) FST, (g) UPC, (h) UPT, (i) TDC, (j) TDT, (k) PCC, and (l) PCT. (LCC, *L. cyrtobotrya* cotyledon; LCT, *L. cyrtobotrya* true leaf; AFC, *A. fruticosa* cotyledon; AFT, *A. fruticosa* true leaf; FSC, *F. suffruticosa* cotyledon; FST, *F. suffruticosa* true leaf; UPC, *U. parvifolia* cotyledon; UPT, *U. parvifolia* true leaf; TDC, *T. divaricata* cotyledon; TDT, *T. divaricata* true leaf; PCC, *P. coreana* cotyledon; PCT, *P. coreana* true leaf).

To preserve the phytochemical profiles and minimize thermal degradation, the cleaned samples were subsequently dried in a forced-air drying oven set at 50 °C for 5 days until a constant dry weight was achieved. The thoroughly dried samples were finely ground and stored in airtight containers equipped with silica gel desiccants at room temperature until subsequent extraction and biochemical analyses.

2.2. Instruments and Chemicals

The absorbance of colorimetric assays was measured using a microplate reader (Epoch, BioTek Instruments, Winooski, VT, USA). Organic solvents were removed using a rotary

vacuum evaporator (OSB-2100, Eyela Tokyo Rikakikai Co., Ltd., Tokyo, Japan). Ethanol (EtOH) was purchased from Samchun Pure Chemical Co., Ltd. (Pyeongtaek, Republic of Korea). Dimethyl sulfoxide (DMSO) was obtained from J.T. Baker (Center Valley, PA, USA). Folin–Ciocalteu reagent, sodium carbonate, tannic acid, quercetin potassium persulfate, 2,2-azinobis(3-ethylbenzothiazoline-6-sulfonate) (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), and ascorbic acid (AA) were purchased from Sigma-Aldrich (St. Louis, MO, USA) [17].

2.3. Extraction of Samples

The dried samples were finely ground using a mixer to facilitate solvent penetration. Powdered material (0.5 g, dry weight) from each sample was extracted three times with 300 mL of EtOH at a constant temperature of 80 °C under reflux for 3 h per cycle. This specific reflux protocol was selected based on our previous studies on cotyledon and true leaf to maintain methodological consistency and maximize the baseline yield of robust, polar phenolic and flavonoid fractions [18]. The combined extracts were filtered to remove insoluble residues and concentrated under reduced pressure using a rotary evaporator.

2.4. TPC Assay

TPC was quantified using the Folin–Ciocalteu assay as previously described [19]. Sample extracts were serially diluted to concentrations ranging from 1.25 to 5.0 mg/mL. Briefly, 60 µL of each sample was dispensed into a 96-well plate, followed by the addition of 40 µL of 2 N Folin–Ciocalteu reagent and 100 µL of 7.5% sodium carbonate solution. The plate was shaken using a microplate shaker and incubated in the dark at room temperature for 30 min. Absorbance was then measured at 760 nm using a microplate reader. Tannic acid solutions ranging from 0.008 to 0.250 mg/mL were used to generate the calibration curve, and the results were expressed as milligrams of tannic acid equivalents per gram of extract (mg TAE/g).

2.5. TFC Assay

TFC was quantified according to a previously reported method [19]. Briefly, 100 µL of serially diluted extracts (0.125–2.0 mg/mL) was mixed with 100 µL of $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$ solution in a 96-well microtiter plate. The mixtures were prepared in triplicate, thoroughly mixed, and incubated in the dark for 10 min. Absorbance was measured at 430 nm using a microplate reader. Quercetin solutions (0.004–0.125 mg/mL) were used to construct the standard calibration curve, and the results were expressed as milligrams of quercetin equivalents per gram of extract (mg QE/g).

2.6. ABTS Radical Scavenging Assay

The ABTS radical scavenging activity of the sample extracts was evaluated using a modified version of a previously reported method [20,21]. The ABTS radical cation solution was prepared by mixing ABTS (7.4 mM) with potassium persulfate (2.6 mM) in deionized water (DW). While a 12–16 h incubation is traditionally utilized for ABTS activation [22], the mixture in this study was incubated in the dark at 4 °C for 24 h. The resulting solution was diluted with DW to achieve an absorbance of 1.00 ± 0.05 at 734 nm before use. Extract samples were serially diluted in DMSO/EtOH (1:1, *v/v*) to final concentrations ranging from 2.5 to 10 mg/mL. A 10 µL aliquot of each sample was added to a 96-well microplate, followed by 200 µL of the ABTS working solution. The mixtures were thoroughly mixed and incubated in the dark at room temperature for 30 min. Absorbance was measured at 734 nm using a microplate reader. AA dissolved in DW was used as a positive control. Radical scavenging activity was calculated based on the reduction in absorbance relative to

the control and expressed as a percentage. IC₅₀ values were estimated from concentration–response curves. All experiments were performed in triplicate.

2.7. DPPH Radical Scavenging Assay

The DPPH radical scavenging activity of the sample extracts was evaluated according to a modified version of a previously reported method [20,21]. A 2 mM stock solution of DPPH was prepared in EtOH and further diluted with EtOH to obtain an absorbance of 0.80 ± 0.05 at 514 nm. Extract samples were serially diluted in DMSO/EtOH (1:1, *v/v*) to final concentrations ranging from 2.5 to 10 mg/mL. A 10 µL aliquot of each sample was added to a 96-well microplate, followed by 200 µL of the DPPH working solution. The mixtures were thoroughly mixed and incubated in the dark at room temperature for 30 min. Absorbance was measured at 514 nm using a microplate reader. AA dissolved in DW was used as a positive control. Radical scavenging activity was calculated based on the reduction in absorbance relative to the control and expressed as a percentage. IC₅₀ values were estimated from concentration–response curves. All experiments were performed in triplicate.

2.8. Preparation of Microbial Inoculum

The test strains, *S. aureus* (ATCC BAA-1708, ATCC, Manassas, VA, USA) and *A. baumannii* (ATCC BAA-1605, ATCC, Manassas, VA, USA), were cultivated on Mueller–Hinton agar (MHA, Sigma-Aldrich), whereas *C. violaceum* (ATCC 12472, ATCC, Manassas, VA, USA) was maintained on Luria–Bertani (LB) agar (Sigma-Aldrich). All microbial cultures were incubated at 37 °C for 18–24 h. To prepare bacterial suspensions, isolated colonies were dispersed in sterile 0.85% NaCl solution according to the antimicrobial susceptibility testing guidelines established by the Clinical and Laboratory Standards Institute (CLSI). The turbidity of each suspension was adjusted to the 0.5 McFarland standard, corresponding to an approximate cell density of 1.5×10^8 CFU/mL. Plate inoculation was performed within 15 min after turbidity adjustment to maintain consistency across replicates.

2.9. Antimicrobial Activity by Agar-Well Diffusion Assay

The antibacterial activities of the extracts were evaluated using the agar-well diffusion assay. MHA plates were prepared by pouring 95 mL of medium into each 150 mm Petri dish to obtain a uniform agar depth of approximately 6 mm. MHA plates were uniformly inoculated with standardized bacterial suspensions immediately after preparation of the inoculum. Sterile cork borers were used to create 6 mm wells in the agar. Extracts were dissolved in 10% DMSO to obtain a stock concentration of 100 mg/mL, and 30 µL of each extract solution was added to each agar well. Streptomycin at 10 mg/mL was used as the positive control, while 10% DMSO was used as the negative control. The negative control was included to verify that the solvent did not contribute to the observed inhibition zones. The plates were incubated at 37 °C for 18–24 h. After incubation, inhibition zone diameters for the different treatments were measured in millimeters. The absence of a visible inhibition halo in the 10% DMSO negative control was recorded as 0 mm. The experimental design utilized three independent biological replicates, with each biological replicate evaluated using three technical replicates ($n = 9$), and the results were expressed as mean inhibition zone diameters.

2.10. QS Inhibitory Activity

Potential QS-related pigment-suppression activity was assessed using *C. violaceum* as the biosensor strain following a modified agar-well diffusion assay described previously [23]. LB agar plates were prepared using 95 mL LB agar per 150 mm Petri dish to

obtain an agar depth of approximately 6 mm. LB agar plates were then uniformly inoculated with a 0.5 McFarland suspension of the biosensor strain. Subsequently, 30 µL aliquots of the extracts (100 mg/mL) were added to the agar wells, and the plates were incubated at 30 °C for 24 h. Cinnamaldehyde (10 mg/mL, a known QS inhibitor) was used as the positive control, while 10% DMSO served as the negative control. Positive QS inhibition was identified by the presence of a hazy, pigment-free zone surrounding the well, indicating suppression of violacein production without bactericidal effects. In contrast, a completely transparent zone indicated microbial death. Because crude extract halos were often hazy, subjective, and not perfectly concentric, calculated QS inhibitory zone values were not derived by subtracting clear zones from pigment-free zones. To support interpretation, a parallel lawn-growth control was also performed using the same extract concentration to confirm whether pigment suppression occurred without complete inhibition of *C. violaceum* lawn growth. These observations were treated as preliminary qualitative screening data and were not used to quantitatively rank QS inhibitory potency. The qualitative screening assay was performed using three independent biological replicates, with each biological replicate evaluated using three technical replicates ($n = 9$).

2.11. Statistical Analysis

Statistical analyses and graphical presentations were performed using GraphPad Prism version 10.2.3 (GraphPad Software, Boston, MA, USA). Colorimetric assays were performed in triplicate, and QS inhibitory activity was measured in nine replicates. Prior to statistical testing, data normality and variance homogeneity were verified using Shapiro–Wilk and Levene’s tests. Multiple-group comparisons were performed using two-way ANOVA followed by Tukey’s post hoc test. The model was used to evaluate two distinct independent factors: ‘Plant Species’ (six levels) and ‘Developmental Stage’ (two levels: cotyledon vs. true leaf), alongside their interaction term, to determine whether ontogenetic shifts in secondary metabolism are dependent on specific taxonomic lineages.

3. Results and Discussion

3.1. TPC and TFC

Data analysis revealed a consistent trend in which TPC and TFC increased as all six plant species transitioned from the cotyledon stage to the true leaf stage (Figure 3). In all cases, true leaves exhibited higher phenolic and flavonoid concentrations than their corresponding cotyledons. Specifically, the TPC of *L. cyrtobotrya* increased from 58.23 mg TAE/g in cotyledons (LCC) to 93.91 mg TAE/g in true leaves (LCT). Similarly, the TFC of *U. parvifolia* increased markedly from 0.84 mg QE/g in cotyledons (UPC) to 3.72 mg QE/g in true leaves (UPT). Among all samples, the highest TPC was observed in the true leaves of *F. suffruticosa* (FST, 118.65 mg TAE/g), whereas the highest TFC was recorded in the true leaves of *A. fruticosa* (AFT, 4.42 mg QE/g).

Ontogenetic shifts in plant metabolism are driven by developmental requirements that enable adaptation to new ecological and physiological challenges [24–26]. The observed significant increase in TPC and TFC as seedlings transition from cotyledons to true leaves is consistent with the distinct functional roles of these organs. Cotyledons are formed during embryogenesis and primarily function as temporary storage structures and initial photosynthetic organs that support early seedling establishment [12]. Once autotrophic true leaves develop, they become the permanent photosynthetic organs and are fully exposed to environmental stressors such as UV radiation and atmospheric pathogens. This transition likely triggers the upregulation of secondary metabolite biosynthesis, particularly flavonoids and phenolics, to strengthen structural and chemical defense mechanisms [18]. While comprehensive phytochemical profiling (e.g., LC-MS/MS or GC-MS) was not performed in

the present study, related metabolomic profiles literature indicates that *U. parvifolia* bark and mature leaves dynamically accumulate complex flavonoids and terpene glycosides associated with long-term defense and tissue repair [27], whereas mature *A. fruticosa* leaves actively synthesize prenylated flavanones and rotenoids, which is consistent with the increased TPC and TFC observed in our true leaf samples [28].

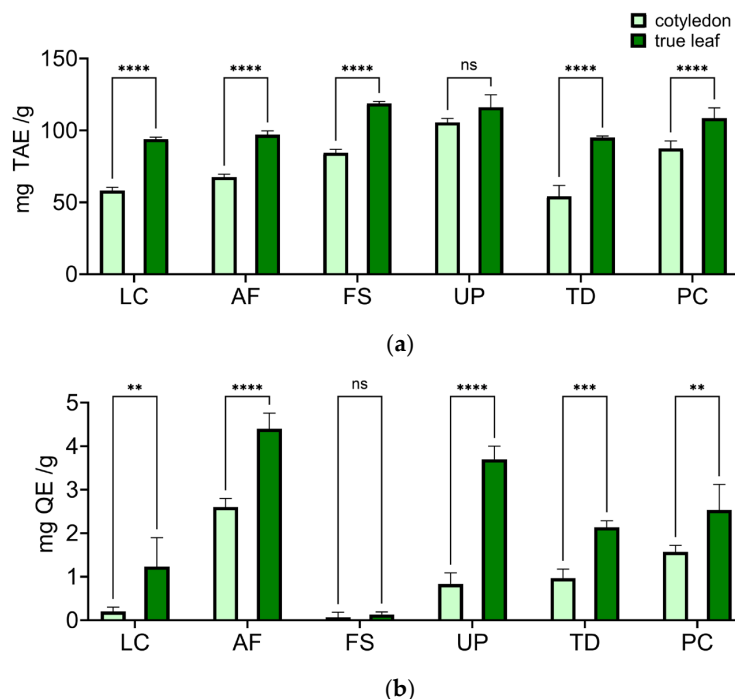


Figure 3. TPC (a) and TFC (b) of seedling extracts. Data are presented as mean $\pm$ standard deviation ($n = 3$). TPC: Linear range = 0.008–0.250 mg/mL, $R^2 > 0.9997$ (TAE). TFC: Linear range = 0.004–0.125 mg/mL, $R^2 > 0.9955$ (QE). Statistical significance between paired samples was determined by two-way ANOVA followed by Tukey's post hoc test. (ns, not significant; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$).

The developmental reprogramming observed in these seedlings aligns with the broader concept that plant defense is not static but is instead reshaped by ontogeny, environmental exposure, and tissue function [26,29]. Cotyledons serve as transient organs that support early seedling establishment, whereas true leaves become long-term photosynthetic structures exposed to stronger abiotic and biotic stress, including UV radiation, oxidative stress, and pathogen attack [30,31]. This functional transition plausibly explains why TPC and TFC increased in most species as seedlings matured, as phenylpropanoid metabolism is commonly induced during the establishment of autonomous photosynthesis and stress-responsive defense networks [32]. Similar stage-dependent enrichment of bioactive constituents has been reported in other seedling systems, including *Adenocaulon himalaicum*, where true leaves exhibited higher phytochemical levels and antioxidant capacity than cotyledons [18].

3.2. ABTS and DPPH Radical Scavenging Activity

The increase in phenolic metabolites generally resulted in enhanced antioxidant activity at the true leaf stage in most species (Figure 4). The ABTS and DPPH radical scavenging assays revealed clear ontogenetic variation in antioxidant capacity across the tested species. In *L. cyrtobotrya*, *A. fruticosa*, *F. suffruticosa*, and *U. parvifolia*, the IC_{50} values for both ABTS and DPPH assays were significantly lower in true leaves than in cotyledons, indicating stronger free radical scavenging activity. Notably, UPT exhibited particularly strong antiox-

idant activity in both assays (ABTS IC_{50} : 2.20 mg/mL; DPPH IC_{50} : 4.50 mg/mL). These results suggest a positive correlation between increased TPC and TFC and antioxidant potential in mature leaves, consistent with previous studies demonstrating the central role of polyphenols in radical scavenging systems. From an ontogenetic perspective, this pattern suggests a physiological strategy in which cotyledons primarily utilize stored reserves for early growth, whereas true leaves generate higher levels of reactive oxygen species due to active photosynthesis and therefore require increased flavonoid accumulation to mitigate oxidative stress.

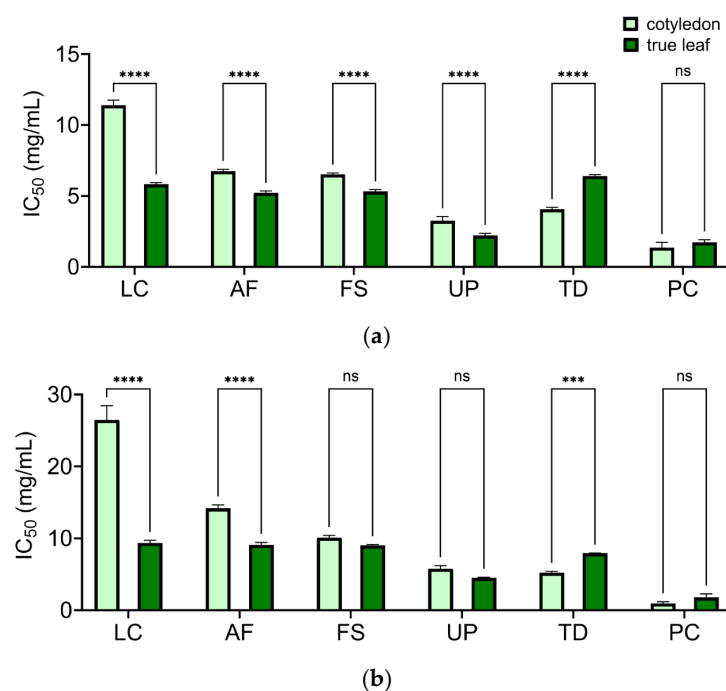


Figure 4. Antioxidant activity of seedling extracts determined by ABTS (a) and DPPH (b) radical scavenging assays, expressed as IC_{50} values (mg/mL). Data are presented as mean $\pm$ standard deviation ($n = 3$). Statistical significance between paired samples was determined by two-way ANOVA followed by Tukey's post hoc test. (ns, not significant; *** $p < 0.001$; **** $p < 0.0001$).

Interestingly, in TDC/TDT and PCC/PCT, antioxidant capacity was higher in the cotyledon stage than in the true leaf stage. TDC showed stronger radical scavenging activity than TDT in both the ABTS (4.06 mg/mL vs. 6.40 mg/mL) and DPPH (5.21 mg/mL vs. 7.91 mg/mL) assays. Similarly, PCC exhibited higher activity than its corresponding true leaves (PCT) in the ABTS assay (1.35 mg/mL vs. 1.73 mg/mL). Notably, PCC showed the lowest IC_{50} values among all samples, indicating particularly strong radical scavenging activity, although the activity was lower than that of the positive control, (AA; ABTS IC_{50} : 0.12 mg/mL; DPPH IC_{50} : 0.11 mg/mL). This result suggests that specific antioxidant metabolites or other early-stage metabolites, rather than general phenolics alone, may be transiently upregulated during the cotyledon stage. Early developmental stages can sometimes accumulate specific bioactive compounds as an acute defense strategy before structural protective systems are fully developed [33,34], highlighting the complexity of stage-dependent secondary metabolite regulation.

The close association between phenolic accumulation and antioxidant activity is also chemically reasonable, as polyphenols can directly donate electrons or hydrogen atoms, chelate pro-oxidant metal ions, and interrupt radical chain reactions [35]. However, the present results also indicate that antioxidant capacity is not determined solely by TPC [36], as demonstrated by the stronger cotyledon activity observed in *P. coreana* and *T. divaricata*

despite their lower or comparable TPC and TFC values. This discrepancy suggests that antioxidant potential depends not only on compound quantity but also on compound composition, with early-stage tissues potentially enriched in highly active low-abundance metabolites such as specialized metabolites that are not fully captured by broad colorimetric assays. This discrepancy implies that antioxidant potential depends heavily on compound composition; early-stage tissues are hypothesized to be enriched in highly active low-abundance metabolites, such as specific terpenoids, iridoids, or lipophilic fractions, which are not captured by broad colorimetric assays. Because no direct chemical characterization or individual component isolation was executed in this study, these mechanistic assumptions must be evaluated strictly as preliminary hypotheses. These findings strongly underscore the requirement for targeted metabolomic profiling in future work to identify individual active constituents at each distinct seedling stage [37].

3.3. Preliminary Antibacterial Susceptibility Screening

Antibacterial susceptibility screening was performed to evaluate the preliminary growth-inhibitory activity of cotyledon and true leaf extracts against the tested bacterial strains. The Gram-positive *S. aureus* and Gram-negative *A. baumannii* were selected as representative test organisms to compare the responses of different bacterial cell types to the extracts. Because the assay was based strictly on the agar-well diffusion methodology, these results must be interpreted explicitly as introductory qualitative screening data rather than measurements of precise antimicrobial potency. Agar diffusion methodologies present notable limitations, including an inability to determine exact quantitative efficacy endpoints and vulnerability to variations in extract viscosity, compound solubility, or agar matrix density, which can suppress or amplify individual inhibition zones. Therefore, the observed results avoid definitive confirmation of general antimicrobial properties and instead outline initial baseline patterns. Analysis of the inhibition zones (Figure 5) revealed stage-dependent differences in antibacterial activity depending on the plant species and growth stage.

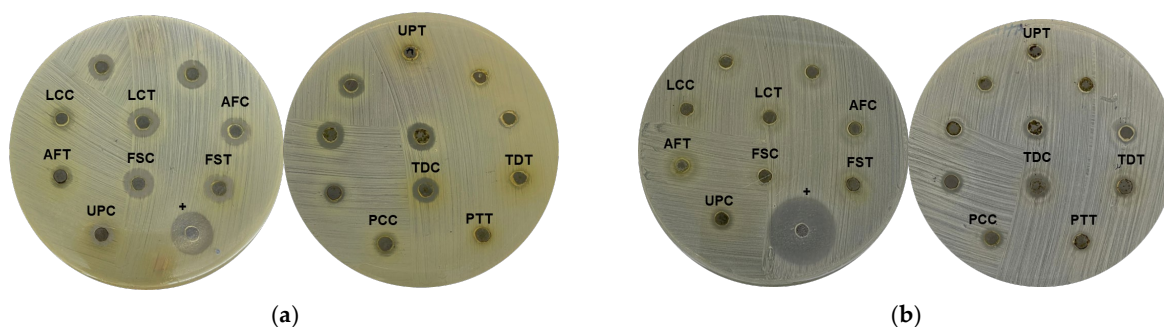


Figure 5. Preliminary antibacterial activity of seedling extracts against *S. aureus* (a) and *A. baumannii* (b), determined by agar-well diffusion assay. Agar plates inoculated with the test microorganisms were treated with 30 μ L (100 mg/mL) of different seedling extracts, and inhibition zones were measured after incubation. The (+) symbol indicates the positive control. Wells visible on the agar plates contain samples from a separate, independent experiment and are not relevant to this manuscript.

Against Gram-positive *S. aureus*, FSC exhibited the highest antibacterial potential, with a zone of inhibition of 13.11 mm. This was followed by LCT (11.69 mm), TDC (11.51 mm), and UPC (11.30 mm) (Figure 6a). Against Gram-negative *A. baumannii*, TDC showed the strongest antibacterial activity (12.27 mm), followed closely by PCC (11.20 mm) and UPC (11.14 mm) (Figure 6b). The negative control showed no inhibition (0 mm).

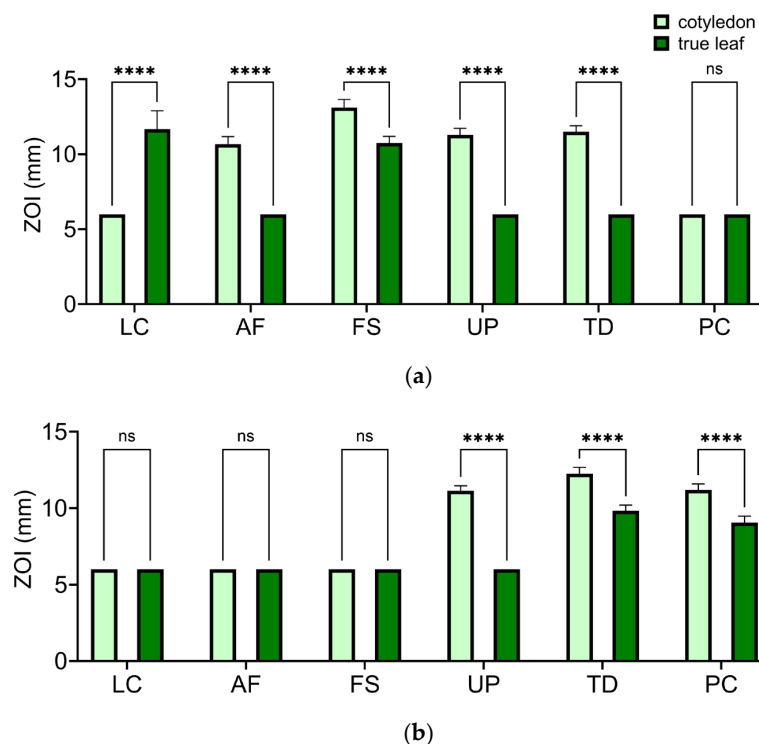


Figure 6. Preliminary antibacterial activity of seedling extracts against *S. aureus* (a) and *A. baumannii* (b). Agar plates inoculated with the test microorganisms were treated with 30 μ L (100 mg/mL) of different seedling extracts, and inhibition zones were measured after incubation. Data are presented as mean $\pm$ standard deviation ($n = 9$). Statistical significance between paired samples was determined by two-way ANOVA followed by Tukey's post hoc test. (ns, not significant; **** $p < 0.0001$).

Both UPC and TDC produced measurable inhibition zones against both tested bacterial strains, suggesting potential growth-inhibitory activity against *S. aureus* and *A. baumannii*. The reduced or absent inhibition zones observed in the corresponding true leaf extracts, UPT and TDT, may reflect stage-dependent differences in extractable bioactive compounds. This pronounced activity in UPC and TDC likely reflects an acute defense strategy essential for the survival of young seedlings, which lack fully developed protective structures such as a hardened cuticle [38], highlighting a strict stage-dependent reallocation of metabolic resources as these species transitioned to the true leaf stage. However, this interpretation should be made cautiously because using crude extract may result in poor solubility or limited diffusion of some compounds through the agar matrix, which may also have influenced the observed inhibition patterns. Furthermore, as the plates were incubated without specific humidity control, the possible influence of agar dehydration on compound diffusion over the 18–24 h period cannot be completely excluded. To accurately validate the actual potency and agricultural or biomedical utility of these species, further studies establishing minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) against an expanded panel of target pathogens are mandatory prerequisites. Therefore, further MIC and MBC determination, together with phytochemical profiling and activity-guided fractionation, is needed to better evaluate the antimicrobial potential and identify the compounds responsible for the observed activity.

3.4. Potential QS Inhibitory Activity

QS is a regulatory system in which bacteria secrete autoinducers, such as acyl-homoserine lactones, to monitor population density and coordinate collective behaviors, including biofilm formation and virulence activation, once a critical threshold is reached. Using the *C. violaceum* biosensor model, QS inhibitory activity was evaluated based on the

suppression of violacein pigment production without bacterial growth inhibition (Figure 7). The negative control yielded 0 mm zones of inhibition, confirming that the solvent did not contribute to bacterial growth inhibition or pigment suppression. In the parallel lawn-growth control, *C. violaceum* showed visible growth at the extract concentrations used, and no complete clearing of the bacterial lawn was observed around the pigment-suppression halos. This indicates that the observed zones were primarily associated with reduced violacein pigmentation rather than complete bactericidal inhibition. Most of the tested extracts showed visible pigment-suppression responses in the *C. violaceum* assay, although the response was more visually apparent in UPC and LCT. These observations are presented only as qualitative pigment-suppression patterns and should not be interpreted as a quantitative ranking of QS inhibitory potency. Although reduced violacein pigmentation may suggest possible interference with pigment production or QS-related processes, mild growth inhibition cannot be fully excluded using the agar-well diffusion assay alone. Therefore, the present findings should be considered preliminary evidence of potential QS-related activity rather than definitive confirmation of QS inhibitory activity.

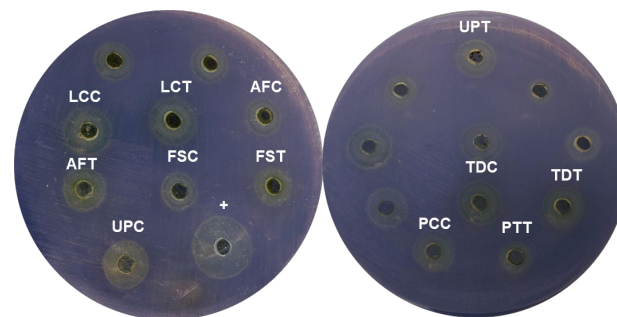


Figure 7. Quorum-sensing inhibitory activity of seedling extracts against *C. violaceum*. Agar plates inoculated with the test microorganism were treated with 30 μ L (100 mg/mL) of different seedling extracts, and inhibition zones were measured after incubation. The (+) symbol indicates positive control. Wells visible on the agar plates contain samples from a separate, independent experiment and are not relevant to this manuscript.

The antimicrobial and QS-related observations may indicate a stage-associated defense trade-off. Cotyledons of several species showed more evident direct antibacterial activity, which may reflect the presence of compounds involved in early-stage defense in vulnerable tissues that have not yet developed stronger structural protection [39] (Figure 8a). In contrast, the pigment-suppression zones observed in UPT and LCT true leaf extracts suggest that later developmental stages may contain metabolites capable of affecting violacein production or other QS-related processes (Figure 8b). This interpretation remains preliminary because the assay was based on crude extracts and qualitative agar-based screening.

Because QS interference has been proposed as a potential strategy for reducing coordinated pathogenic behavior, including biofilm formation, without relying solely on direct bacterial killing [13,18], these findings may provide useful baseline information for future studies on plant-derived compounds in nursery and horticultural systems. However, the present results should not be interpreted as definitive evidence of anti-virulence activity or as a quantitative comparison of QS inhibitory potency. Further validation using bacterial growth-curve analysis, spectrophotometric quantification of violacein production and/or inhibition, sub-inhibitory extract concentrations, and molecular validation assays against relevant biofilm-forming horticultural pathogens is necessary before specific crop-protection applications can be proposed. Overall, these results indicate that harvest timing should be aligned with the intended application, with cotyledons being more suitable

for direct antimicrobial screening, while true leaves are better suited for antioxidant and anti-QS applications.

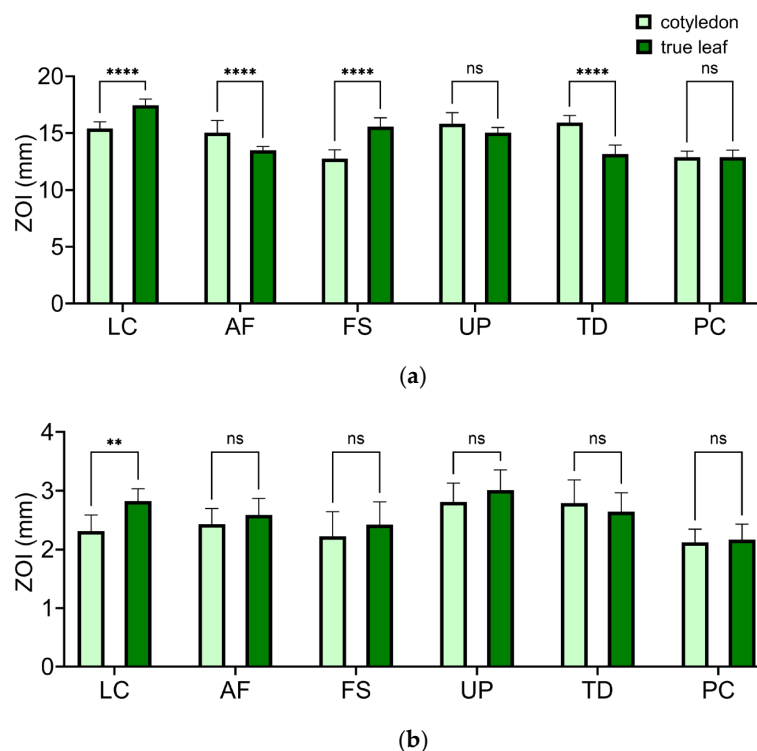


Figure 8. Potential QS inhibitory activity against *C. violaceum* determined by the agar-well diffusion. Clear zones indicating bacterial growth inhibition (a) and QS inhibitory activity (b). Agar plates inoculated with the test microorganism were treated with 30 μ L (100 mg/mL) of different seedling extracts, and inhibition zones were measured after incubation. Data are presented as mean $\pm$ standard deviation ($n = 9$). Statistical significance between paired samples was determined by two-way ANOVA followed by Tukey's post hoc test. (ns, not significant; ** $p < 0.01$; **** $p < 0.0001$).

4. Conclusions

This study provides a comparative evaluation of quantitative changes in TPC and TFC, along with corresponding shifts in free radical scavenging, preliminary antimicrobial, and QS inhibitory activities, between the cotyledon and true leaf stages of six phylogenetically diverse native Korean plant species. The transition from cotyledon to true leaf was generally associated with increased accumulation of total phenolics and flavonoids, leading to enhanced ABTS and DPPH radical scavenging capacity, as observed in *L. cyrtobotrya*, *A. fruticosa*, *F. suffruticosa*, and *U. parvifolia*. However, TDC and PCC exhibited distinct developmental profiles, showing strong direct bactericidal and antioxidant activities that exceeded those of their corresponding true leaves. As true leaves matured, a functional shift toward QS inhibition was observed, particularly in *U. parvifolia* and *L. cyrtobotrya*, suggesting enhanced attenuation of bacterial virulence without imposing strong selective pressure. Because this investigation represents a baseline screening of crude extracts without advanced phytochemical profiling, compound isolation, or toxicological valuation, drawing definitive conclusions regarding human clinical therapies or immediate agricultural field applications is premature. Nonetheless, these findings establish an essential ontogenetic framework demonstrating that early seedling development stages exhibit distinct, tissue-specific bioactivities, providing a foundational baseline for optimizing harvest timing and discovering plant-derived bioactive extracts for sustainable crop and post-harvest management frameworks.

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