RESEARCH PAPER

Comparative phytochemical profiling, antioxidant activity and HPLC fingerprinting of two cytotypes of *Verbena officinalis* L. and two morphotypes of *Verbena bonariensis* L.

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Abstract:

The verbena family has long attracted scientific interest because of its medicinal value, and among its members, *Verbena officinalis* L. and *Verbena bonariensis* L. are distinguished by their rich repertoire of phenolics, flavonoids, iridoids, and other bioactive secondary metabolites. Yet a question has remained sidelined: how much do cytotypic and morphotypic differences between closely related accessions actually shape their morphology, chemistry, and antioxidant behaviour? The present study set out to answer that question by comparing two cytotypes of *V. officinalis* (diploid and octoploid) and two morphotypes of *V. bonariensis* through a combination of morphometric measurements, spectrophotometric estimation of total phenolic and flavonoid contents, four antioxidant assays (DPPH radical scavenging, hydrogen peroxide scavenging, reducing power, and IC_{50} determination), and HPLC fingerprinting of methanolic extracts. Highly significant differences appeared among all evaluated accessions ($P < 0.001$). The octoploid cytotype of *V. officinalis* clearly led the group, showing superior vegetative growth, stronger reproductive performance, greater biomass accumulation, the highest total phenolic content (91.38 ± 1.31 mg GAE g^{-1} DW) and total flavonoid content (65.41 ± 1.04 mg QE g^{-1} DW), the strongest DPPH radical scavenging activity (92.7 ± 0.4%), and the lowest IC_{50} value (51.6 ± 1.1 µg mL^{-1}). HPLC chromatograms consistently revealed four signature compounds — rutin, quercetin, β-sitosterol, and cinnamic acid — across all accessions, but their peak intensities were visibly strongest in the octoploid extract, supporting the spectrophotometric trends. The two *V. bonariensis* morphotypes occupied an intermediate position, with Morphotype II consistently outperforming Morphotype I across both phytochemical parameters and antioxidant readouts. When the four accessions were ranked by overall biological performance, a clear hierarchy emerged: *V. officinalis* (8x) > *V. bonariensis* Morphotype II > *V. bonariensis* Morphotype I > V. officinalis (2x). Taken together, these data make a strong case that polyploidization exerts a pronounced enhancing effect on phytochemical accumulation and antioxidant capacity in *V. officinalis*. The octoploid cytotype acts as a particularly attractive genetic source for future phytopharmaceutical development, targeted breeding programmes, and the quality standardization of medicinal plant material.

Keywords: *Verbena officinalis*; *Verbena bonariensis*; polyploidy; antioxidant activity; phenolic compounds; HPLC fingerprinting; medicinal plants.

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.Introduction

Medicinal plants continue to occupy a central place in global healthcare because their secondary metabolites provide a renewable source of natural antioxidants capable of counteracting oxidative stress and the chronic degenerative pathologies associated with it [1–4]. The most pharmacologically relevant of these metabolites are phenolics and flavonoids, whose ability to donate hydrogen atoms or single electrons converts reactive oxygen species into stable products, thereby interrupting the chain reactions that drive oxidative damage to lipids, proteins and nucleic acids [5,6]. Because the phenolic and flavonoid profile of a given accession translates directly into its free-radical-scavenging capacity, systematic evaluation of these compounds—through complementary spectrophotometric assays (DPPH radical scavenging, hydrogen peroxide scavenging, reducing power and IC_{50} determination) and chromatographic fingerprinting—has become the operational standard for the quality assurance and pharmacopoeial standardisation of herbal materials [4,7].

Within this wider landscape, *Verbena officinalis* L. and *Verbena bonariensis* L. have attracted particular interest on account of their documented traditional applications against digestive and inflammatory complaints, an activity that is now mechanistically linked to their multiple phenolic, flavonoid and iridoid constituents [1,8,9]. The taxonomy of the Verbenaceae is, however, notoriously complex, and authentication of genuine botanical material is a prerequisite for any reproducible phytochemical or

pharmacological comparison; chromatographic profiling combined with DNA-based quality-control methods has emerged as the analytical framework of choice for differentiating genuine *Verbena* accessions from their adulterants [10,11].

A second source of intraspecific variability in the genus is polyploidization. Whole-genome duplication is a known driver of the "gigas effect" in medicinal plants, modifying gene dosage, enzyme activity and metabolic flux in ways that frequently translate into both enhanced biomass and elevated accumulation of pharmacologically active phenolics, flavonoids and glycosides [5,8,12]. At the practical level, that linkage means that ploidy is no longer a minor descriptor of a botanical accession but a stratifying variable of immediate relevance to germplasm selection, breeding programmes and the industrial production of standardised phytopharmaceuticals [7].

Most published investigations of the genus have nevertheless focused on individual species or single populations, and the joint influence of cytotypic variation, morphotypic differentiation, phytochemical composition and antioxidant capacity is still incompletely characterised [6,9]. In particular, the comparative performance of polyploid cytotypes of *V. officinalis* against contrasting morphotypes of *V. bonariensis*—evaluated simultaneously by morphometric descriptors, total phenolic and flavonoid contents, complementary antioxidant assays and HPLC fingerprinting of methanolic extracts—has not, to our knowledge, been reported previously [8,11,12].

To address this gap, the present study undertook a comparative evaluation of two cytotypes of *V. officinalis* (diploid 2x and octoploid 8x) and two morphotypes of *V. bonariensis* based on (i) morphological descriptors and biomass accumulation, (ii) total phenolic and flavonoid contents, (iii) antioxidant activity (DPPH radical scavenging, hydrogen peroxide scavenging, reducing power and IC_{50} determination) and (iv) HPLC fingerprinting of methanolic extracts for the marker compounds rutin, quercetin, β-sitosterol and cinnamic acid. The expected outcome—that the octoploid cytotype of *V. officinalis* would display superior vegetative growth, higher phenolic and flavonoid accumulation, stronger free-radical-scavenging activity and more intense HPLC marker peaks than the remaining accessions—provides a directly testable framework for selecting high-yielding, phytochemically enriched germplasm for future breeding, phytopharmaceutical development and the quality standardization of *Verbena*-based herbal materials [1,2,7,8].

Materials and methods

2.1 Plant Material and Experimental Design

Two cytotypes of Verbena officinalis L., namely diploid (2x) and octoploid (8x), were collected from Srinagar (Jammu & Kashmir), while two populations of *Verbena bonariensis* L. were collected from Srinagar (Jammu & Kashmir) and Padhar, Mandi (Himachal Pradesh). Plant specimens were identified using standard taxonomic floras, and voucher specimens were deposited in the Herbarium of the Department of Botany, Mata Gujri College, Fatehgarh Sahib (MGCH). The aerial part(s) of the collected plants were thoroughly washed, shade-dried at room temperature, pulverised into a fine powder, and kept in airtight containers until further phytochemical, antioxidant, and HPLC analyses.

2.2 Morphological Characterization

Morphological characterization was carried out on ten randomly selected healthy plants from each accession. Both vegetative and reproductive characters were recorded following standard plant taxonomic descriptors. The evaluated traits included plant height (cm), stem diameter (mm), number of primary branches, internode length (cm), leaf length (cm), leaf width (cm), leaf area (cm^2), petiole length (cm), spike length (cm), number of spikes/ plant, number of flowers/ spike, corolla diameter (mm), root length (cm), fresh biomass (g), and dry biomass (g). Leaf area was calculated using a digital leaf area meter. Fresh biomass was recorded immediately after harvesting, whereas dry biomass was determined after oven drying (60 °C) until constant weight (Table 1).

2.3 Preparation of Plant Extracts

Methanolic and aqueous extract(s) were prepared separately from the dried aerial plant material. For methanolic extraction, 10 g of powdered sample was extracted with 100 mL of analytical-grade methanol using a Soxhlet extraction system for 6–8 h. The extracts were passed through Whatman No. 1 filter paper and concentrated under reduced pressure employing a rotary vacuum evaporator at 40 °C. For aqueous extraction, 10 g of ground substance was mixed with 100 mL of distilled water and extracted by continuous stirring at 60 °C for 2 h. The extract(s) were filtered and concentrated using a lyophilizer (freeze dryer). All extracts were stored at 4 °C in amber-colored bottles until further phytochemical and antioxidant analyses.

2.4 Estimation of Total Phenolic Content

Total phenolic content (TPC) was determined using the Folin–Ciocalteu colorimetric method with slight modifications. Briefly, 200 µL of plant extract was mixed with 1.0 mL of diluted Folin–Ciocalteu reagent followed by the addition of 800 µL of 7.5% sodium carbonate solution. The reaction mixture was incubated in darkness for 30 min at room temperature, and absorbance was recorded at 765 nm

using a UV–Visible spectrophotometer. Gallic acid was used for preparing the standard calibration curve, and the results were expressed as milligrams of gallic acid equivalents per gram dry weight (mg GAE g^{-1} DW).

2.5 Estimation of Total Flavonoid Content

Total flavonoid content (TFC) was estimated using the aluminium chloride colorimetric method. Briefly, plant extract was mixed with aluminium chloride solution and potassium acetate, then incubated for 30 min at room temperature. Absorbance was measured at 415 nm. Quercetin served as the reference standard, and flavonoid content was expressed as milligrams of quercetin equivalents per gram dry weight (mg QE g^{-1} DW).

2.6 Antioxidant Assays

2.6.1 DPPH Radical Scavenging Activity

The free radical scavenging activity was determined using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. Briefly, 1 mL of plant extract was mixed with freshly prepared DPPH solution (0.1 mM in methanol) and incubated in darkness for 30 min. The decrease in absorbance was measured at 517 nm.

where:

- = Absorbance of the control
- = Absorbance of the sample

2.6.2 Hydrogen Peroxide Scavenging Assay

Hydrogen peroxide scavenging activity was recorded according to the method described by Ruch et al. [13]. Plant extracts were mixed with phosphate buffer containing hydrogen peroxide solution, and absorbance was recorded at 230 nm after incubation (Table 2).

2.6.3 Ferric Reducing Power Assay

Reducing power was estimated following the method of Oyaizu [14]. Plant extracts were mixed with phosphate buffer and potassium ferricyanide, incubated at 50 °C, followed by the addition of trichloroacetic acid and ferric chloride. Absorbance was measured at 700 nm, where increased absorbance indicated greater reducing power.

2.7 HPLC Fingerprinting

High-performance liquid chromatography (HPLC) analysis was performed using a reverse-phase HPLC system equipped with a quaternary pump, autosampler, UV–Visible detector, and C18 analytical column (250 × 4.6 mm, 5 µm particle size). Approximately 20 µL of filtered methanolic extract was injected into the chromatographic system. Separation was achieved using a gradient mobile phase consisting of solvent A (0.1% phosphoric acid in water) and solvent B (acetonitrile) at a flow rate of 1.0 mL min^{-1}. Detection was carried out at 280 nm. Commercial analytical standards of rutin, quercetin, β-sitosterol, and cinnamic acid were used for compound identification by comparing retention times and UV spectra. Peak areas were calculated using chromatographic software.

2.8 Statistical Analysis

All experiments were performed in triplicate (n = 3), while morphological observations were recorded from ten biological replicates. Experimental data were expressed as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was performed to determine significant differences among the four accessions. Mean separation was carried out using Duncan's Multiple Range Test (DMRT) at $P < 0.05$. The F-value, least significant difference (LSD), coefficient of variation (CV), and probability values were calculated for each parameter. Statistical analyses were performed using SPSS version 26.0 (IBM Corp., Armonk, NY, USA), and graphical representations were prepared using GraphPad Prism 10.0.

3. Results

3.1 Morphological Characterization

Significant morphological variation was observed among the two cytotypes of *Verbena officinalis* and the two populations of *V. bonariensis* for the vegetative and reproductive traits evaluated (Table 1). Overall, the octoploid (8x) cytotype of *V. officinalis* exhibited better performance for most morphological characters, although it did not record the highest values for every parameter. Plant height increased from 68.4 ± 3.2 cm in the diploid cytotype to 138.7 ± 5.6 cm in the octoploid cytotype, while stem diameter ranged from 3.2 ± 0.2 mm to 6.5 ± 0.3 mm. The octoploid cytotype also showed higher values for several vegetative traits, including the number of primary branches, internode length, leaf dimensions, and biomass accumulation. Leaf area was approximately 3.5-fold greater in the octoploid cytotype (44.6 ± 1.9 cm^2) than in the diploid cytotype (12.8 ± 0.9 cm^2).

Reproductive traits generally followed a similar pattern. The octoploid cytotype produced longer spikes (22.9 ± 1.1 cm), a greater number of spikes per plant (39.4 ± 2.1), more flowers per spike (86.4 ± 3.5), and a larger corolla diameter (6.6 ± 0.3 mm) than the remaining accessions. Root length and fresh/ or dry biomass were also comparatively higher in the octoploid plants. Among the two *V. bonariensis* morphotypes I and II, of which morphotype II consistently presented higher values than morphotypes I for most vegetative and reproductive characters. Collectively, these observations show that the octoploid cytotype of *V. officinalis* generally displayed greater vegetative vigour and reproductive development than the diploid cytotype. However, this trend was not uniform across all evaluated parameters. However, exceptions were observed for leaf width and root length, where *V. bonariensis*

Population II recorded values comparable to or higher than those of the octoploid cytotype.

Table 1: Comparative morphological traits of *Verbena officinalis* cytotypes and *Verbena bonariensis* populations.

Character	*V. officinalis* (2x)	*V. bonariensis* MT-I	*V. bonariensis* MT-II	*V. officinalis* (8x)
Plant height (cm)	68.4 ± 3.2^{d}	103.6 ± 4.8^{c}	118.5 ± 5.3^{b}	138.7 ± 5.6^{a}
Stem diameter (mm)	3.2 ± 0.2^{d}	4.6 ± 0.2^{c}	5.3 ± 0.3^{b}	6.5 ± 0.3^{a}
Primary branches	9.8 ± 0.7^{c}	13.4 ± 0.8^{b}	14.6 ± 0.6^{b}	17.3 ± 0.9^{a}
Internode length (cm)	3.6 ± 0.2^{c}	4.3 ± 0.2^{b}	4.6 ± 0.3^{b}	5.2 ± 0.3^{a}
Leaf length (cm)	5.8 ± 0.3^{d}	8.6 ± 0.4^{c}	9.7 ± 0.4^{b}	11.8 ± 0.5^{a}
Leaf width (cm)	2.1 ± 0.1^{d}	3.8 ± 0.2^{c}	4.3 ± 0.2^{b}	5.2 ± 0.2^{a}
Leaf area (cm²)	12.8 ± 0.9^{d}	26.4 ± 1.3^{c}	31.8 ± 1.5^{b}	44.6 ± 1.9^{a}
Petiole length (cm)	1.1 ± 0.1^{c}	1.6 ± 0.1^{b}	1.8 ± 0.1^{b}	2.2 ± 0.1^{a}
Spike length (cm)	9.7 ± 0.5^{d}	15.8 ± 0.7^{c}	18.4 ± 0.8^{b}	22.9 ± 1.1^{a}
Spikes/plant	24.8 ± 1.6^{c}	31.5 ± 1.8^{b}	34.8 ± 1.7^{b}	39.4 ± 2.1^{a}
Flowers/spike	58.2 ± 2.5^{c}	71.6 ± 2.8^{b}	76.8 ± 3.0^{b}	86.4 ± 3.5^{a}
Corolla diameter (mm)	4.2 ± 0.2^{d}	5.3 ± 0.2^{c}	5.8 ± 0.2^{b}	6.6 ± 0.3^{a}
Root length (cm)	15.4 ± 0.8^{c}	20.8 ± 1.0^{b}	22.4 ± 1.1^{b}	26.5 ± 1.2^{a}
Fresh biomass (g)	38.6 ± 2.3^{d}	72.8 ± 3.6^{c}	82.4 ± 3.8^{b}	106.5 ± 4.5^{a}
Dry biomass (g)	9.8 ± 0.6^{d}	17.4 ± 0.8^{c}	20.6 ± 0.9^{b}	27.8 ± 1.2^{a}

- Values are presented as mean ± standard error (SE).
- Data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's Honest Significant Difference (HSD) test at $P \leq 0.05$.
- Mean values within a row followed by different superscript letters (a–d) differ significantly, whereas values sharing the same letter are not significantly different at $P \leq 0.05$.

3.2 Total Phenolic and Flavonoid Contents

Total phenolic and flavonoid contents differed substantially across all evaluated accessions in both methanolic and aqueous extracts ($P<0.001$) (Tables 2 and 3), with methanolic extracts consistently yielding higher concentrations of both phytochemical groups than the corresponding aqueous extracts. In methanolic extracts, TPC ranged from 62.45 ± 1.22 to 91.38 ± 1.31 mg GAE g^{-1} DW, while TFC varied between 38.72 ± 0.95 and 65.41 ± 1.04 mg QE g^{-1} DW. The octoploid cytotype of *V. officinalis* recorded the highest values for both parameters, followed in descending order by *V. bonariensis* Morphotype I & II, and the diploid cytotype of *V. officinalis*. A comparable gradient was observed in aqueous extracts, where TPC ranged from 46.82 ± 1.05 to 72.94 ± 1.22 mg GAE g^{-1} DW, and TFC ranged from 28.45 ± 0.74 to 49.37 ± 0.92 mg QE g^{-1} DW, again with the octoploid cytotype leading the ranking. Duncan's Multiple Range Test confirmed statistically significant differences among all pairwise accession comparisons for both phytochemical parameters ($P < 0.05$), supporting the overall ranking established by the omnibus test.

The methanolic extract of the octoploid cytotype of *V. officinalis* thus contained the highest concentration of total phenolics and total flavonoids, with *V. bonariensis* (Morphotype II) occupying the second rank—consistent with reports that methanol is more efficient than water for extracting phenolic constituents from *Verbena* species [9,12]. The pronounced accumulation of phenolic metabolites in polyploid plants, observed here as a clear octoploid-over-diploid advantage, parallels the genome-doubling-driven enhancement of secondary metabolite biosynthesis documented in several other medicinal taxa [2–4], supporting the view that ploidy manipulation is a practical strategy for improving the phytochemical yield of *Verbena* germplasm.

3.3 Antioxidant Activity

The antioxidant activities of methanolic and aqueous extracts differed significantly among the two cytotypes of *Verbena officinalis* and the two morphotypes of *V. bonariensis* ($P < 0.001$) (Table 2). In both extraction systems, the octoploid (8x) cytotype of *V. officinalis* consistently manifested the strongest antioxidant potential, recording the highest DPPH radical scavenging activity (92.7 ± 0.4% and 86.9 ± 0.5%), hydrogen peroxide scavenging activity (90.8 ± 0.5% and 84.5 ± 0.4%), and reducing power (1.39 ± 0.03 and 1.22 ± 0.03) in the methanolic and aqueous extracts, respectively. In contrast, the diploid (2x) cytotype showed the lowest antioxidant activities in all assays. The two *V. bonariensis* morphotypes

exhibited intermediate antioxidant capacities, with Morphotype II consistently outperforming Morphotype I in both extract systems. The DPPH IC_{50} values exhibited an inverse relationship with radical scavenging activity, with the octoploid cytotype recording the lowest IC_{50} values (51.6 ± 1.1 and 63.4 ± 1.4 µg mL^{-1} for methanolic and aqueous extracts, respectively). In contrast, the diploid cytotype showed the highest IC_{50} values (96.3 ± 2.2 and 121.4 ± 2.4 µg mL^{-1}, respectively). Although both extracts followed a similar trend, the methanolic extracts consistently presented higher antioxidant activities and lower IC_{50} values than the corresponding aqueous extracts. These findings are consistent with previous studies demonstrating substantial antioxidant activity in phenolic- and flavonoid-rich extracts of *V. officinalis* and supporting an association between phytochemical accumulation and antioxidant capacity [7,9].

Table 2. Comparative phytochemical composition and antioxidant activities of methanolic and aqueous extracts of two cytotypes of *Verbena officinalis* and two morphotypes of *Verbena bonariensis*

Parameter	Extract	VO 2x	VB MT-I	VB MT-II	VO 8x	F	P	LSD (5%)	CV (%)
Total phenolics (mg GAE g^{-1} DW)	Methanol	62.45 ± 1.22^{d}	74.83 ± 1.08^{c}	79.46 ± 1.15^{b}	91.38 ± 1.31^{a}	326.47	<0.001	2.84	2.76
	Water	46.82 ± 1.05^{d}	58.31 ± 0.96^{c}	61.27 ± 1.01^{b}	72.94 ± 1.22^{a}	284.11	<0.001	2.63	3.08
Total flavonoids (mg QE g^{-1} DW)	Methanol	38.72 ± 0.95^{d}	49.63 ± 0.87^{c}	53.84 ± 0.92^{b}	65.41 ± 1.04^{a}	291.62	<0.001	2.15	2.94
	Water	28.45 ± 0.74^{d}	37.62 ± 0.81^{c}	40.11 ± 0.76^{b}	49.37 ± 0.92^{a}	255.47	<0.001	1.92	3.01
DPPH scavenging activity (%)	Methanol	72.8 ± 0.7^{d}	82.4 ± 0.5^{c}	86.1 ± 0.6^{b}	92.7 ± 0.4^{a}	348.91	<0.001	1.86	1.92
	Water	64.3 ± 0.8^{d}	74.8 ± 0.7^{c}	78.2 ± 0.6^{b}	86.9 ± 0.5^{a}	302.26	<0.001	2.24	2.34
DPPH IC_{50} (µg mL^{-1})	Methanol	96.3 ± 2.2^{a}	78.5 ± 1.8^{b}	69.2 ± 1.5^{c}	51.6 ± 1.1^{d}	287.15	<0.001	3.74	3.11
	Water	121.4 ± 2.4^{a}	98.2 ± 2.1^{b}	87.5 ± 1.9^{c}	63.4 ± 1.4^{d}	266.93	<0.001	4.15	3.43
H_2O_2 scavenging activity (%)	Methanol	69.5 ± 0.8^{d}	79.8 ± 0.6^{c}	83.7 ± 0.5^{b}	90.8 ± 0.5^{a}	310.54	<0.001	2.02	2.18
	Water	61.8 ± 0.7^{d}	72.3 ± 0.5^{c}	76.6 ± 0.5^{b}	84.5 ± 0.4^{a}	287.51	<0.001	2.11	2.48
Reducing power (A700)	Methanol	0.84 ± 0.03^{d}	1.05 ± 0.02^{c}	1.16 ± 0.03^{b}	1.39 ± 0.03^{a}	268.42	<0.001	0.06	2.65
	Water	0.69 ± 0.02^{d}	0.91 ± 0.03^{c}	1.01 ± 0.02^{b}	1.22 ± 0.03^{a}	249.38	<0.001	0.05	2.91

Values are presented as mean ± SD (n = 3). Different superscript letters (a–d) within the same row denote significant differences according to Duncan's Multiple Range Test (DMRT) at $P < 0.05$. F = ANOVA F-statistic; LSD = least significant difference; CV = coefficient of variation; GAE = gallic acid equivalents; QE = quercetin equivalents; DW = dry weight; DPPH = 2,2-diphenyl-1-picrylhydrazyl; IC_{50} = concentration required to scavenge 50% of DPPH radicals; A700 = absorbance at 700 nm; VO 2x = *Verbena officinalis* diploid; VO 8x = *V. officinalis* octoploid; VB MT-I = *V. bonariensis* Morphotype I; VB MT-II = *V. bonariensis* Morphotype II.

3.4 HPLC Fingerprinting

HPLC analysis of the methanolic extracts generated distinct chromatographic fingerprints for all four accessions (Figure X). Four marker compounds, namely rutin, quercetin, β-sitosterol, and cinnamic acid, were detected in every accession, exhibiting comparable retention times of approximately 2.97–2.99, 24.12–24.46, 41.45–41.88, and 47.95–48.36 min, respectively. Although all accessions displayed similar qualitative chromatographic profiles, substantial quantitative variation was apparent in peak intensity and peak area. The octoploid cytotype consistently presented the largest peaks for all four marker compounds, whereas the diploid cytotype recorded the lowest peak responses. Morphotype II of

V. bonariensis showed higher peak intensities than Morphotype I for each identified compound.

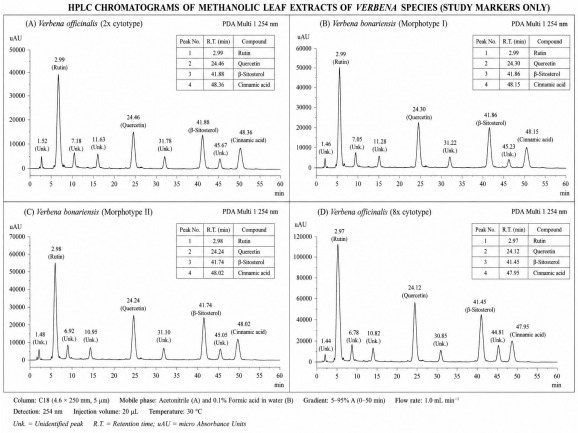

Figure 2. HPLC chromatographic fingerprints of methanolic leaf extracts of *Verbena* accessions. Representative HPLC chromatograms of (A) *Verbena officinalis* diploid (2x) cytotype, (B) *V. bonariensis* Morphotype I, (C) *V. bonariensis* Morphotype II, and (D) *V. officinalis* octoploid (8x) cytotype. Four marker compounds were identified based on their retention times: rutin (~2.97–2.99 min), quercetin (~24.12–24.46 min), β-sitosterol (~41.45–41.88 min), and cinnamic acid (~47.95–48.36 min). Additional unidentified peaks (Unk.) indicate the presence of other phytochemical constituents. Chromatographic separation was performed on a C18 column (250 × 4.6 mm, 5 µm) using acetonitrile and 0.1% formic acid in water as the mobile phase, with PDA detection at 254 nm and a flow rate of 1.0 mL min^{-1}.

Several additional unidentified peaks were observed throughout the chromatograms, particularly at retention times of 1.4–1.6, 6.7–7.2, 10.8–11.6, 30.8–31.8, and 44.8–45.7 min, indicating the presence of additional phytochemical constituents. The abundance of these unidentified compounds followed the same trend as the identified marker compounds, with the octoploid cytotype exhibiting the greatest peak abundance, followed by *V. bonariensis* Morphotype II, Morphotype I, and the diploid cytotype.

HPLC fingerprinting confirmed the presence of rutin, quercetin, β-sitosterol, and cinnamic acid in all accessions. These compounds have previously been identified as phytochemical constituents or markers associated with Verbena species and are considered important contributors to their biochemical activities [15,16].

Discussion

4.1 Morphological Variation and the Influence of Polyploidy

The present study revealed substantial morphological variation among the evaluated accessions, with the octoploid (8x) cytotype of *Verbena officinalis* consistently displaying superior vegetative and reproductive performance compared with the diploid (2x) cytotype and both *V. bonariensis* morphotypes. Greater plant height, stem diameter, branching index, leaf size, root length, spike length, flower production and biomass accumulation in the octoploid accession signify enhanced plant vigour directly attributable to genome duplication, in line with the "gigas effect" widely reported in polyploid medicinal plants [5,12]. The greater performance of *V. bonariensis* Morphotype II relative to Morphotype I further indicates considerable intraspecific variation, plausibly reflecting genetic diversity and adaptation to contrasting ecological conditions, a finding consistent with reported polyploid-driven morphological enhancement in Verbena officinalis microshoot cultures and soil-grown plants [8,12]. Because increased leaf area and biomass provide a larger photosynthetic surface for the biosynthesis and accumulation of secondary metabolites, the improved growth characteristics of the octoploid cytotype offer a plausible mechanistic basis for its correspondingly higher phenolic and flavonoid contents and stronger antioxidant activity documented in the present study, reinforcing its value as an elite germplasm resource for phytopharmaceutical development and the quality standardization of *Verbena*-based herbal material [7,8].

4.2 Phytochemical Accumulation in Different Cytotypes and Morphotypes

The present study showed significant variation in total phenolic and flavonoid contents across the evaluated *Verbena* accessions, with the octoploid (8x) cytotype of *V. officinalis* consistently recording the highest concentrations in both methanolic and aqueous extracts. In contrast, methanolic extracts yielded substantially higher levels of both metabolite groups than the corresponding aqueous extracts—confirming the greater efficiency of methanol for solubilising polyphenolic constituents of *Verbena* through its intermediate polarity [3,9]. The pronounced phenolic and flavonoid accumulation in the octoploid cytotype is plausibly linked to polyploidisation-driven upregulation of the phenylpropanoid pathway, in which increased gene dosage enhances transcription of key biosynthetic enzymes and elevates phenolic output, a pattern described across several medicinal plant genera [5,8,12]. The superior phytochemical profile of *V. bonariensis* Morphotype II relative to Morphotype I further indicates that intraspecific genetic variation and ecological adaptation contribute additively to metabolite accumulation [8,9].

Because phenolics and flavonoids constitute principal bioactive constituents contributing to the documented therapeutic activity of *Verbena*, the consistently

higher concentrations observed in the octoploid *V. officinalis* substantiate its greater pharmaceutical potential and identify it as a priority germplasm for future phytopharmaceutical development and quality standardisation of *Verbena*-based herbal material [1,7].

4.3 Antioxidant Potential in Relation to Phenolic and Flavonoid Content

The antioxidant assays showed a clear relationship between phytochemical accumulation and antioxidant capacity among the evaluated Verbena accessions. The octoploid cytotype exhibited the highest DPPH and hydrogen peroxide scavenging activities, greatest reducing power, and lowest IC_{50} values in both methanolic and aqueous extracts. In contrast, the diploid cytotype consistently recorded the weakest antioxidant activity. These data indicate that the enhanced antioxidant capacity of the octoploid cytotype is closely associated with its higher phenolic and flavonoid contents. Phenolic compounds and flavonoids are well known for their ability to donate hydrogen atoms or electrons, thereby neutralising reactive oxygen species and preventing oxidative damage to biomolecules [16,17]. The lower IC_{50} values observed in the octoploid cytotype further confirm its greater free radical scavenging efficiency, as lower IC_{50} values indicate stronger antioxidant potential. The consistently higher antioxidant activity of methanolic extracts compared with aqueous extracts also supports the higher recovery of antioxidant phytochemicals in methanol. Similar positive correlations between total phenolic content and antioxidant activity have been widely reported for Verbena officinalis and other medicinal plants [15,18,19]. Collectively, these results suggest that the superior antioxidant activity of the octoploid cytotype mainly results from enhanced accumulation of phenolic metabolites promoted by genome duplication.

4.4 HPLC Fingerprinting and Phytochemical Diversity

HPLC fingerprinting confirmed the presence of four characteristic marker compounds—rutin, quercetin, β-sitosterol, and cinnamic acid—in all *Verbena* accessions, showing a common qualitative phytochemical composition. However, substantial quantitative differences in peak intensity and peak area were observed among the evaluated cytotypes and morphotypes, with the octoploid *V. officinalis* consistently exhibiting the highest accumulation of all identified compounds. Similar HPLC profiles have previously been reported for *Verbena officinalis*, in which flavonoids and phenolic acids constitute the principal bioactive constituents responsible for its antioxidant and pharmacological activities [15,16].

The occurrence of several unidentified peaks further suggests the presence of additional secondary metabolites, including iridoid glycosides and phenylpropanoid derivatives that have been widely documented in *Verbena* species [15]. The greater abundance of both identified and unidentified compounds in the octoploid cytotype supports the hypothesis that polyploidization increases overall metabolic capacity rather than increasing only a few individual metabolites. Similar observations have been reported in other polyploid medicinal plants, where chromosome duplication resulted in greater phytochemical diversity and improved medicinal quality [20,21]. Therefore, HPLC fingerprinting not only confirmed the authenticity of the investigated *Verbena* accessions but also indicated that the octoploid cytotype possesses superior phytochemical richness, making it an encouraging genetic resource for phytopharmaceutical development, quality control, and future breeding programmes.

4.5 Medicinal Significance and Future Perspectives

The present investigation shows that both cytotypic and morphotypic variations markedly influence the morphological characteristics, phytochemical composition, antioxidant potential, and HPLC profiles of *Verbena* species. Among the evaluated accessions, the octoploid (8x) cytotype of *Verbena officinalis* consistently presented superior vegetative growth, higher concentrations of phenolics and flavonoids, stronger antioxidant activity, and greater accumulation of major HPLC marker compounds than the diploid cytotype and the two *V. bonariensis* morphotypes. These characteristics jointly indicate that the octoploid cytotype possesses enhanced medicinal value and may represent a superior source of bioactive phytochemicals for pharmaceutical and nutraceutical applications. Phenolic compounds, flavonoids, and phenylpropanoid derivatives are recognized as principal constituents contributing to the antioxidant, anti-inflammatory, antimicrobial, and other therapeutic properties of *Verbena* species [15,16]. Therefore, the greater accumulation of these metabolites in the octoploid cytotype suggests its potential as elite germplasm for the production of standardized herbal formulations and high-quality phytopharmaceuticals. Furthermore, the reproducible HPLC fingerprints generated in the present study provide valuable chemical markers for the authentication and quality control of *Verbena*-based herbal products, thereby supporting their safe and consistent medicinal use. Although the present study demonstrates the superiority of the octoploid cytotype under the investigated conditions, additional research is required to characterize other bioactive constituents using advanced metabolomic approaches such as LC–

MS/MS and GC–MS. Further investigations should also evaluate the antimicrobial, anti-inflammatory, antidiabetic, and anticancer activities of the identified cytotypes and morphotypes through *in vitro* and *in vivo* studies. Moreover, transcriptomic and metabolomic analyses could provide greater insight into the molecular mechanisms underlying enhanced secondary metabolite biosynthesis in polyploid *Verbena*. Such studies would facilitate the development of improved cultivars and strengthen the commercial utilization of *Verbena* as an important medicinal plant.

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