

Quantitative Phytochemical Profiling and DPPH Radical Scavenging Activity of Polyherbal Formulations Prepared from Selected Medicinal Plants

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Abstract

Polyherbal formulations from medicinal plants are gaining attention for their synergistic phytochemical profiles and antioxidant properties. This study quantitatively profiled phytochemicals and assessed in vitro antioxidant activity in four polyherbal formulations (F1–F4), derived from methanolic extracts of selected parts of *Andrographis paniculata*, *Aegle marmelos*, *Caesalpinia crista*, and *Syzygium cumini*. Analyses covered total phenolic content (TPC), total flavonoid content (TFC), alkaloids, tannins, steroids, proteins, amino acids, and reducing sugars via established biochemical assays. Antioxidant capacity was determined using the DPPH radical scavenging assay, reported as IC₅₀ values. Formulation F4 displayed the highest TPC ($\approx 3763 \mu\text{g/mL}$), TFC ($\approx 2258 \mu\text{g/mL}$), tannin ($\approx 2803 \mu\text{g/mL}$), steroid ($\approx 6855 \mu\text{g/mL}$), and protein ($\approx 9005 \mu\text{g/mL}$) levels. In contrast, F3 had elevated amino acid ($\approx 4057 \mu\text{g/mL}$) and reducing sugar ($\approx 3145 \mu\text{g/mL}$) contents. All formulations showed concentration-dependent DPPH scavenging, with IC₅₀ values ranking as follows: F1 ($1.92 \pm 0.10 \mu\text{g/mL}$) < F2 ($2.72 \pm 0.11 \mu\text{g/mL}$) < F3 ($3.06 \pm 0.01 \mu\text{g/mL}$) < F4 ($5.87 \pm 0.21 \mu\text{g/mL}$), highlighting F1's superior antioxidant potency despite F4's greater phytochemical yields. These results affirm the rich phytochemical diversity and potent radical-scavenging ability of polyherbal blends, underscoring their therapeutic promise. Differences in antioxidant efficacy imply that phytochemical synergy, beyond mere abundance, drives bioactivity. The data offer a foundation for standardising polyherbal antioxidants and warranting advanced pharmacological studies.

Keywords: Polyherbal formulation, Phytochemical profiling, DPPH radical scavenging activity, Antioxidant potential, Medicinal plants

Introduction

Polyherbal formulations, commonly used in traditional systems like Ayurveda and Traditional Chinese Medicine, harness the power of multiple herbs to deliver superior therapeutic benefits (Rana et al., 2023). This strategy relies on synergism, where the combined effects of diverse plant compounds produce more robust and holistic pharmacological results than single herbs alone, expanding treatment options and potentially reducing side effects (Shalini and Ilango, 2021). Rising global demand for natural remedies arises from concerns over synthetic drug safety and the need for sustainable, efficacious alternatives (Rana et al., 2023).

Oxidative stress defined as an imbalance between reactive oxygen species production and the body's antioxidant capacity underpins many chronic conditions, such as diabetes, cardiovascular disease, neurodegeneration, and cancers (Rana et al., 2023). Natural antioxidants that scavenge free radicals and curb oxidative harm are thus a major focus of research (Rana et al., 2023). Plants abound in bioactive molecules like polyphenols, flavonoids, and tannins, which boast strong antioxidant prowess and bolster plasma antioxidant levels to prevent disease and promote wellbeing (Shalini and Ilango, 2021; Aher et al., 2023).

Validating polyherbal formulations scientifically demands a thorough process, starting with detailed phytochemical profiling to detect and measure active ingredients (Vasanth and Purushotham, 2020). Such quantification reveals the chemical variety and potency of therapeutic agents (Vasanth and Purushotham, 2020). In vitro tests like the DPPH radical scavenging assay then gauge free radical neutralisation, with lower IC₅₀ values signalling greater potency (Askar et al., 2025). For instance, one polyherbal juice showed 68.45% DPPH activity, while formulations A and B achieved 55.04% and 58.85%, respectively (Askar et al., 2025).

This study examines the quantitative phytochemical composition and in vitro DPPH antioxidant activity of a novel polyherbal formulation from carefully selected plants. Our findings aim to substantiate traditional applications, highlight synergistic contributions to antioxidant efficacy, and pave the way for evidence-based natural therapeutics.

Materials and Methods

Collection of Plant Samples

Leaves of the selected species were gathered from urban and agricultural areas in Visnagar, Mehsana District, between June and October 2024. Botanical specimens were authenticated by experts in the Department of Botany, Sheth M. N. Science College, Patan.

Processing of Plant Samples

Collected leaves were washed repeatedly with tap water to eliminate surface dust, then shade-dried at ambient temperature for three weeks, avoiding direct sunlight to preserve phytochemical integrity. The dried material was pulverised using a laboratory grinder and stored in airtight plastic zip-lock bags.

Preparation of Hot Water Extract

For hot aqueous extraction, 5 g of finely powdered dried leaves were mixed with 200 mL distilled water in a beaker. The suspension was stirred continuously on a hot plate at 30–40 °C for 20 min. The resulting extract was filtered through Whatman No. 1 filter paper, and the filtrate was reserved for phytochemical screening. Unused portions were stored at 4 °C.

Preparation of Soxhlet Extracts

Soxhlet extraction was employed to obtain solvent-based crude extracts. Approximately 10 g of powdered plant material was loaded into a thimble and extracted sequentially with 130 mL each of acetone, chloroform, and methanol. Extraction continued for 24 h per solvent or until the siphon chamber ran clear. Extracts were transferred to beakers, concentrated on a hot plate at 30–40 °C to evaporate the solvent, and the residues stored at 4 °C for subsequent phytochemical analysis.

Quantitative Phytochemical Screening

Total Phenolic Content (TPC)

Total phenolic content was quantified using the Folin–Ciocalteu colorimetric method (Singleton et al., 1999; Davey et al., 2003). An aliquot of 0.5 mL extract (1 mg/mL) was mixed with 2.5 mL Folin–Ciocalteu reagent (1:10 dilution in distilled water) and 2 mL 7.5% sodium carbonate solution. The mixture was incubated in darkness at room temperature for 90 min, then absorbance measured at 765 nm using a UV–Vis spectrophotometer. A standard curve was prepared with gallic acid.

Total Flavonoid Content (TFC)

Flavonoid content was determined by an aluminium chloride colorimetric assay (Marinova et al., 2005). To 1 mL of extract (1 mg/mL), 0.3 mL of 5% sodium nitrate (NaNO_3), 0.3 mL of 10% aluminium chloride (AlCl_3), and 2 mL of 1 M sodium hydroxide (NaOH) were added. The mixture was incubated at room temperature in the dark for 30 min, and the final volume was adjusted to 10 mL with distilled water. The absorbance was measured at 510 nm against a quercetin standard curve (20–100 $\mu\text{g/mL}$).

Total Alkaloid Content

Total alkaloids were determined spectrophotometrically using bromocresol green (BCG) (Fazel & Hamidreza, 2007). Briefly, powdered plant extract was extracted with methanol in a Soxhlet apparatus for 24 h. The extract was evaporated to dryness under vacuum at 45°C , dissolved in 2 N HCl, filtered, and washed with chloroform ($3 \times 10 \text{ mL}$) to remove interferences. The aqueous layer was neutralized with 0.1 N NaOH, then mixed with 5 mL phosphate buffer (pH 4.7) and 5 mL BCG solution (0.01%, w/v in water). The yellow BCG-alkaloid complex was extracted with chloroform (1 + 2 + 3 + 4 mL), collected in a 10 mL volumetric flask, and diluted to volume with chloroform. Absorbance was measured at 470 nm (UV-Vis spectrophotometer) against a reagent blank. Quantification used an atropine calibration curve.

Total Tannin Content

Tannin content was quantified using a modified Folin–Ciocalteu colorimetric method (Singleton et al., 1999). A 1 mL extract sample (1 mg/mL) was diluted to 4 mL with distilled water, mixed with 0.5 mL Folin–Ciocalteu reagent, and 1 mL of 35% sodium carbonate (Na_2CO_3) solution. The mixture was incubated at room temperature in the dark for 30 min. Absorbance was measured at 760 nm using a UV–Vis spectrophotometer against a gallic acid standard curve (20–100 $\mu\text{g/mL}$).

Total Steroid Content

Steroid content was determined calorimetrically using the Liebermann–Burchard reaction (Amankwa et al., 2019). To 1 mL of extract (1 mg/mL) in acetic anhydride, 2 mL of concentrated sulphuric acid was carefully added along the wall of the test tube. Development of a green colour indicated the presence of steroids. The mixture was incubated at room temperature for an appropriate period (e.g., 10–30 min), and absorbance was measured at 660 nm using a UV–Vis spectrophotometer against a cholesterol standard curve (20–100 $\mu\text{g/mL}$).

Total Protein Content

Protein concentration was determined by the Lowry method (Lowry et al., 1951). To 0.1 mL extract (1 mg/mL, diluted if necessary) or BSA standards (20–100 µg/mL), 1 mL alkaline copper reagent (50 parts 2% Na₂CO₃ in 0.1 N NaOH + 1 part 1% CuSO₄·5H₂O + 1 part 1% sodium tartrate) was added, mixed, and incubated at room temperature for 10 min. Then, 0.1 mL Folin-Ciocalteu reagent (diluted 1:1 with water) was added rapidly with vortexing, followed by 30 min incubation. Absorbance was measured at 750 nm against a reagent blank.

Total Amino Acid Content

Amino acids were quantified using the ninhydrin method (Moore & Stein, 1948). A 1 mL extract aliquot (1 mg/mL) was mixed with 1 mL 2% ninhydrin in 0.1 M citrate buffer (pH 5.0) and heated in a boiling water bath for 15 min. After cooling, 5 mL distilled water was added, and absorbance measured at 570 nm against glycine standards (10–50 µg/mL).

Total Reducing Sugar Content

Reducing sugars were assayed using the 3,5-dinitrosalicylic acid (DNS) method (Miller, 1959). To 1 mL extract (1 mg/mL), 1 mL DNS reagent was added, mixed, and heated in a boiling water bath for 5 min. After cooling, 10 mL distilled water was added, and absorbance measured at 540 nm against glucose standards (10–100 µg/mL). Concentrations were expressed as mg glucose equivalents/g extract.

DPPH Radical Scavenging Activity

DPPH radical scavenging activity was evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay (Brand-Williams et al., 1995) to measure the antioxidant capacity of hot water and Soxhlet extracts. DPPH solution (0.1 mM in methanol) was prepared fresh daily.

Extracts were dissolved in methanol to yield concentrations of 20, 40, 60, 80, and 100 µg/mL. To 1 mL of each dilution, 1 mL DPPH solution was added and vortexed thoroughly. The mixtures were incubated in darkness at room temperature for 30 min. Absorbance was measured at 517 nm using a UV–Vis spectrophotometer, with methanol as blank.

Ascorbic acid (20–100 µg/mL) served as positive control, and 1 mL methanol + 1 mL DPPH as negative control. All assays were performed in triplicate. Percentage scavenging activity was calculated as:

$$\text{Scavenging activity (\%)} = [(\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}) / \text{Abs}_{\text{control}}] \times 100$$

IC₅₀ values (concentration inhibiting 50% DPPH radicals) were determined from dose-response curves using linear regression. Lower IC₅₀ indicates higher antioxidant potency.

Results and Discussion

The quantitative composition of the four polyherbal formulations prepared from selected medicinal plant parts revealed moderate variation in total phytomass and proportional contribution of individual constituents. The compositional dataset is presented in Table 1, while the proportional distribution of plant materials is illustrated through the stacked bar representation in Figure 1.

Table 1. Quantitative composition of constituent plant materials used in the preparation of polyherbal formulations F1–F4.

Polyherbal Formulation						
No.	Plant	Part	F1	F2	F3	F4
1	<i>Andrographis paniculata</i> (Burm f.) Nees	Leaf	3gm	2gm	4gm	6gm
2	<i>Aegle marmelos</i> (L.) Correa	Leaf	3gm	2gm	4gm	2gm
3	<i>Aegle marmelos</i> (L.) Correa	Fruit	3gm	2gm	4gm	2gm
4	<i>Caesalpinia crista</i> L.	Leaf	3gm	4gm	2gm	1gm
5	<i>Caesalpinia crista</i> L.	Seed	3gm	2gm	4gm	1gm
6	<i>Syzygium cumini</i> (L.) Skeels.	Leaf	3gm	4gm	2gm	2gm
7	<i>Syzygium cumini</i> (L.) Skeels.	Seed	3gm	4gm	2gm	6gm
		Total	21 gm	20 gm	22 gm	20gm

Formulation F3 exhibited the highest cumulative mass, followed by F1, F2, and F4, indicating intentional variation in phytomaterial blending among the formulations. Visual inspection of the stacked representation further demonstrated relatively greater contributions of *Syzygium cumini* seed and leaf fractions in F3 and F4, whereas *Aegle marmelos* components remained comparatively uniform across formulations. Such compositional variability is expected to directly influence the concentration and extractability of secondary metabolites, thereby shaping the quantitative phytochemical profile and antioxidant performance observed in subsequent analyses.

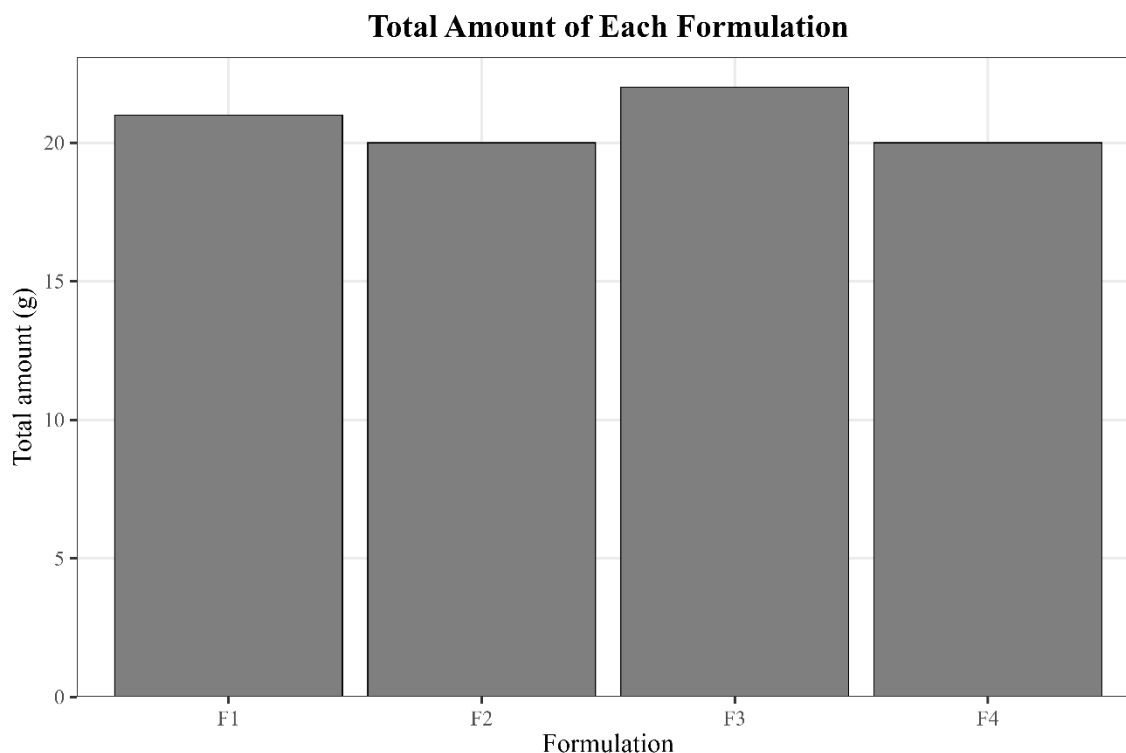


Figure 1. Comparative total quantity of plant constituents in polyherbal formulations F1–F4.

The methanolic extracts of the formulations showed pronounced variation in total phenolic and flavonoid contents, as summarized in Table 2 and graphically depicted in Figure 2. Total phenolic content ranged from approximately 2184 to 3763 $\mu\text{g/mL}$, with formulation F4 displaying the highest phenolic abundance, whereas formulation F1 contained the lowest level. Total flavonoid content followed a narrower distribution, yet comparatively elevated values were again associated with F3 and F4. Because phenolic and flavonoid compounds are well established as primary hydrogen-donating antioxidants capable of stabilizing free radicals through resonance mechanisms, the enrichment of these constituents in later formulations suggested the likelihood of enhanced antioxidant potential. However, quantitative abundance alone does not necessarily predict functional efficiency, a relationship that became evident upon comparison with DPPH scavenging outcome

Table 2. Quantitative phytochemical composition of methanolic extracts of polyherbal formulations F1–F4, including total phenolics (TPC), total flavonoids (TFC), alkaloids, tannins, steroids, protein, amino acids, and reducing sugars (mean $\pm$ SD).

Polyherbal Formulation										
No.	Sample	Extract	TPC ($\mu\text{g/ml}$)	TFC ($\mu\text{g/ml}$)	Alkaloid ($\mu\text{g/ml}$)	Tannin ($\mu\text{g/m}$)	Steroid ($\mu\text{g/m}$)	Protein ($\mu\text{g/m}$)	Amino acid	R. Sugar
1	Formu-1	Methanol	2288.75 $\pm$ 0.7	1832.17 $\pm$ 0.8	2456.14 $\pm$ 0.8	1463.75 $\pm$ 0.7	2757.50 $\pm$ 1.4	5447.56 $\pm$ 2.2	3410.56 $\pm$ 1.0	2304.86 $\pm$ 0.8
2	Formu-2	Methanol	3064.17 $\pm$ 0.8	2120.92 $\pm$ 1.1	3171.38 $\pm$ 1.0	2135.83 $\pm$ 0.8	4992.50 $\pm$ 1.4	8122.00 $\pm$ 1.9	3348.89 $\pm$ 0.4	2591.52 $\pm$ 1.0
3	Formu-3	Methanol	2184.17 $\pm$ 1.1	2206.33 $\pm$ 0.8	3815.19 $\pm$ 1.3	1851.67 $\pm$ 0.8	5245.83 $\pm$ 1.7	9265.33 $\pm$ 1.9	4057.22 $\pm$ 1.4	3144.86 $\pm$ 0.8
4	Formu-4	Methanol	3763.33 $\pm$ 0.8	2258.00 $\pm$ 0.7	4146.14 $\pm$ 0.8	2802.50 $\pm$ 0.7	6855.00 $\pm$ 1.4	9005.33 $\pm$ 1.9	3345.56 $\pm$ 1.0	1733.90 $\pm$ 1.3

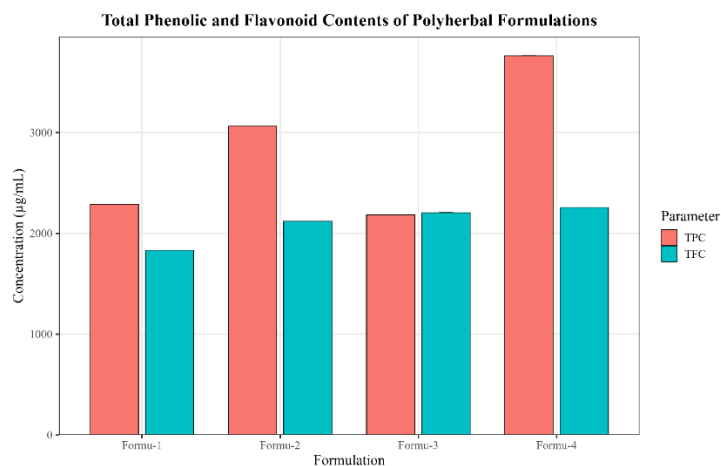


Figure 2. Total phenolic content (TPC) and total flavonoid content (TFC) of polyherbal formulations F1–F4

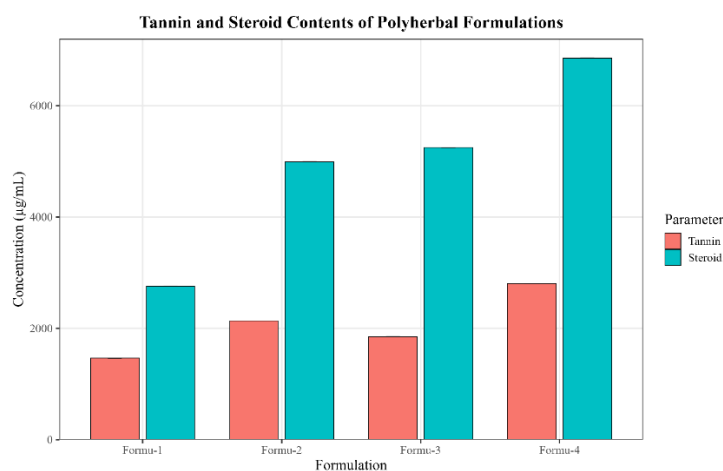


Figure 3. Tannin and steroid contents of polyherbal formulations F1–F4.

Alkaloid levels increased progressively from F1 to F4, with F4 showing the maximum accumulation. Tannin and steroid contents followed a similar pattern, again peaking in F4. These metabolite classes are widely reported to contribute to antioxidant defence through radical quenching, transition-metal chelation, and stabilization of oxidative intermediates. The consistent enrichment of all three phytochemical groups in F4 therefore indicated a markedly higher overall bioactive reservoir relative to the other formulations.

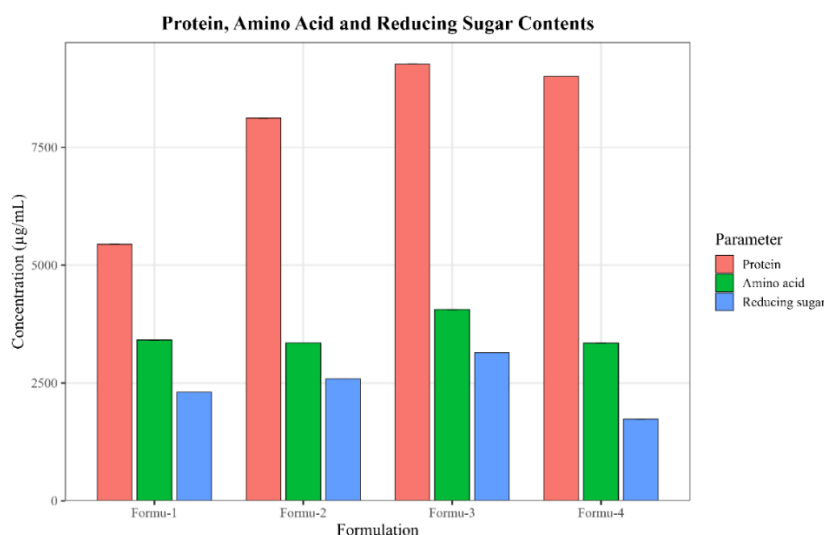


Figure 4. Protein, amino acid, and reducing sugar contents of polyherbal formulations F1–F4.

Primary metabolite profiling revealed notable variation in protein, amino acid, and reducing sugar concentrations (Figure 4). Formulation F3 exhibited the highest protein and amino-acid levels, whereas reducing sugars were lowest in F4. Reduced sugar concentration may be

advantageous in antioxidant systems because elevated sugars can promote non-enzymatic glycation and secondary oxidative reactions. The integrated contribution of secondary and primary metabolites was further visualized through cumulative bioactive loading (Figure 5), which demonstrated a clear increasing trend from F1 through F4, confirming progressive phytochemical enrichment across formulations.

Table 3. DPPH radical scavenging (IC₅₀) activity of polyherbal formulations F1–F4.

Formulation	IC ₅₀ (µg/mL)
F1	1.92 ± 0.10
F2	2.72 ± 0.11
F3	3.06 ± 0.01
F4	5.87 ± 0.21

This inverse relationship between cumulative phytochemical load and antioxidant potency provides important mechanistic insight. Antioxidant performance in polyherbal systems is governed not solely by total metabolite concentration but by the qualitative composition, structural redox properties, and synergistic interactions among constituents. Formulation F1, although comparatively lower in total phenolics and alkaloids, may contain a more favourable proportion of highly active phenolic structures capable of rapid hydrogen donation and radical stabilization.

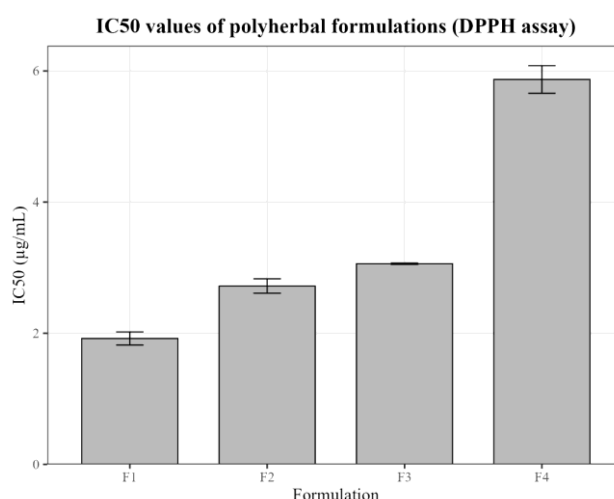


Figure 6. Concentration-dependent DPPH radical scavenging activity of polyherbal formulations F1–F4.

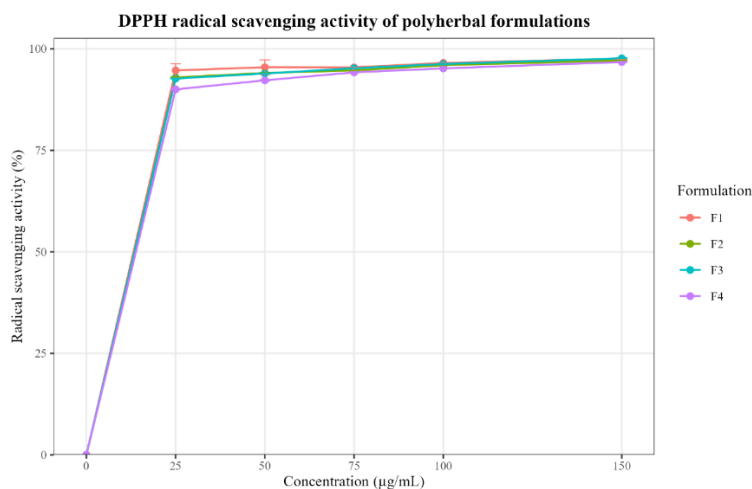


Figure 7. IC₅₀ values of polyherbal formulations F1–F4 obtained from the DPPH radical scavenging assay.

In contrast, excessive accumulation of diverse phytochemicals in F4 may introduce steric hindrance, reduced radical accessibility, or antagonistic intermolecular interactions that diminish effective scavenging efficiency. Similar phenomena have been reported in complex botanical mixtures where optimal antioxidant activity arises from balanced phytochemical synergy rather than maximal concentration.

Collectively, the findings demonstrate that all polyherbal formulations possess strong antioxidant capacity, yet functional potency is maximized in formulation F1, whereas formulation F4 represents the richest overall phytochemical reservoir. This distinction suggests different potential applications: F1 may be more suitable for rapid antioxidant therapeutic intervention, while F4 may serve as a concentrated nutraceutical or phytochemical source for long-term biological support. The study therefore highlights the critical importance of compositional optimization in polyherbal design and confirms that synergistic phytochemical balance, rather than absolute abundance, governs effective antioxidant activity.

The quantitative phytochemical profiling of methanolic extracts revealed substantial variation in both secondary and primary metabolites among the polyherbal formulations (F1–F4). Formulation F4 exhibited the highest levels of total phenolic content (TPC), flavonoids (TFC), alkaloids, tannins, and steroids, indicating a greater accumulation of bioactive secondary metabolites. Phenolic compounds and flavonoids are widely recognized for their antioxidant potential due to their ability to donate hydrogen atoms or electrons and stabilize free radicals (Shahidi & Ambigaipalan, 2015). Similarly, alkaloids and tannins contribute to

oxidative stress mitigation through metal chelation and inhibition of radical propagation (Panche et al., 2016). The progressive increase in these metabolites from F1 to F4 suggests enhanced phytochemical enrichment, which is typically associated with improved biological activity.

In contrast, primary metabolites demonstrated a different pattern. Formulation F3 showed the highest protein and amino acid content, reflecting its nutritional richness and potential role in cellular repair and metabolic regulation. However, reducing sugars were lowest in F4, which may be advantageous, as higher sugar concentrations can facilitate glycation reactions and promote oxidative stress (Singh et al., 2001). This balance between primary and secondary metabolites highlights the complexity of polyherbal systems.

Interestingly, despite possessing lower total phytochemical content, F1 exhibited the strongest antioxidant activity, as indicated by the lowest IC_{50} value in the DPPH assay. This inverse relationship suggests that antioxidant efficacy is not solely dependent on total metabolite concentration but also on the structural characteristics and synergistic interactions of individual compounds. Certain phenolics with specific hydroxyl configurations may exhibit superior radical scavenging capacity even at lower concentrations (Rice-Evans et al., 1996). Therefore, the superior activity of F1 may be attributed to the presence of more potent or synergistically active constituents.

Conclusion

This study establishes that polyherbal formulations from chosen medicinal plant materials harbour abundant phytochemicals and robust antioxidant capacity, validated through detailed profiling and DPPH scavenging assays. Notable differences emerged across formulations in phenolics, flavonoids, alkaloids, tannins, steroids, proteins, amino acids, and reducing sugars, underscoring how plant composition dictates bioactive yields. F4 stood out with the greatest overall phytochemical content, positioning it as a prime candidate for nutraceutical development. Yet, DPPH results showed F1 delivering the lowest IC_{50} and thus superior radical quenching, revealing that polyherbal efficacy stems primarily from synergistic balance and qualitative traits rather than sheer metabolite volume. This counterintuitive pattern stresses the need for precise blending in therapeutic design. In essence, thoughtfully crafted polyherbal blends offer potent natural antioxidants for phytomedicines, supplements, and health promotion. Next steps should include molecular identification, advanced

mechanistic tests, and animal trials to confirm benefits and enable real-world applications in functional foods or clinics.

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Conflict of Interest:

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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