COMPREHENSIVE EVALUATION OF THE ANTIOXIDANT PROFILE OF MULTI-COMPONENT HERBAL EXTRACTS

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RESEARCH ARTICLE

Abstract

Multi-component ethanolic plant extract mixtures derived from Artemisia absinthium L., Viola tricolor L., and Vaccinium myrtillus L. were assessed for their phytochemical composition and antioxidant potential. Plant materials were collected from unpolluted habitats in Bihor County, Romania, and extracts were obtained via Soxhlet extraction and concentrated under reduced pressure. Four mixtures (M1–M4) were evaluated for total phenolic content, total flavonoid content, and DPPH radical scavenging activity. Among the mixtures, M2, containing Vaccinium myrtillus L. and Viola tricolor L., exhibited the highest levels of total phenolics (154.82 ± 1.01 mg GAE/g dry extract) and flavonoids (30.20 ± 0.10 mg QE/g dry extract), as well as the greatest antioxidant activity (85.21 ± 0.39 % DPPH inhibition). These findings indicate a strong correlation between polyphenolic and flavonoid content and radical scavenging capacity, suggesting synergistic interactions within the mixtures. The results support the potential application of multi-component plant extract formulations as natural antioxidants in functional foods, nutraceuticals, and phytopharmaceuticals, warranting further mechanistic and bioavailability studies.

Keywords: *Artemisia absinthium L.*, *Viola tricolor L.*, *Vaccinium myrtillus L.*, phenolic compounds
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INTRODUCTION

Polyphenols are a diverse group of bioactive compounds widely recognized for their potent antioxidant properties, capable of neutralizing reactive oxygen species (ROS) and reactive nitrogen species (RNS), thereby protecting cells and tissues from oxidative damage and associated pathological conditions. These compounds, including flavonoids, phenolic acids, and anthocyanins, contribute not only to antioxidant defense but also to a broad range of pharmacological effects, such as anti-inflammatory, antimicrobial, and cytoprotective activities. The biological potential of plant-derived polyphenols is closely linked to their abundance, chemical diversity, and synergistic interactions within complex herbal matrices (Ciupei et al., 2024; Hassanpour and Doroudi, 2023; Sun et al., 2024).

Herbal extracts derived from *Artemisia absinthium L.* (*Absinthii herba*), *Viola tricolor L.* (*Violae tricoloris herba*), and *Vaccinium myrtillus L.* (*Myrtilli fructus*) exhibit a wide spectrum of therapeutic effects, combining common activities with species-specific properties. *Artemisia absinthium L.* is characterized by the presence of sesquiterpene lactones, flavonoids, and phenolic acids, which collectively underlie its digestive stimulant, antioxidant, and anthelmintic actions (Kosakowska et al., 2025; Liu et al., 2023; Saunoriūtė et al., 2023). *Viola tricolor L.* contains flavonoids, cyclotides, and phenolic acids, conferring antioxidant, anti-inflammatory, and cytotoxic properties (Batiha et al., 2023; Godara, 2022; Supty et al., 2025). *Vaccinium myrtillus L.* is particularly rich in anthocyanins, flavonoids, and chlorogenic acid, which support its potent antioxidant, antidiabetic, hypolipidemic, and vasoprotective effects (Chan and Tomlinson, 2020; Pires et al., 2020; Scalzo et al., 2015). Most of these bioactivities have been confirmed through in vitro and preclinical models, highlighting the pharmacological relevance of these species.

The combination of these three herbal extracts into multi-component formulations offers the potential for additive or synergistic effects, enhancing their antioxidant, anti-inflammatory, and antimicrobial activities while preserving their species-specific therapeutic actions (Karole et al, 2019; Palla et al., 2021). Understanding the phytochemical composition and bioactivity of such complex extracts is essential for their potential application as natural functional ingredients or as complementary therapeutic agents. Therefore, the present study aims to provide a comprehensive evaluation of the phytochemical profiles and antioxidant potential of multi-component extracts derived from *Absinthii herba*, *Violae tricoloris herba*, and *Myrtilli fructus*, contributing to the scientific basis for their use in functional foods, nutraceuticals, or phytopharmaceutical development.

MATERIAL AND METHOD

PLANT MATERIAL

The aerial parts of *Artemisia absinthium L.* and *Viola tricolor L.*, as well as the fully ripened fruits of *Vaccinium myrtillus L.*, were collected from unpolluted natural habitats in Bihor County, Romania. *Artemisia absinthium L.* was harvested in Podgoria (47.057441, 22.015630) in July 2022, collecting basal leaves and flowering tips up to 4 mm in diameter. *Viola tricolor L.* was gathered in Sititelec, Husasău de Tinca (46.886105, 21.900203) in June 2022, focusing on flowering aerial parts. *Vaccinium myrtillus L.* fruits were manually collected in Ponoară, Bratca (46.891488, 22.682169) in July 2022 at full ripeness. Plant material was carefully selected under optimal conditions to ensure health and integrity. Voucher specimens were deposited in the Herbarium of the Faculty of Medicine and Pharmacy, University of Oradea, under the codes UOP 05.708 for *Artemisia absinthium L.*, UOP 05.709 for *Viola tricolor L.*, and UOP 05.710 for *Vaccinium myrtillus L.*

REAGENTS AND EQUIPMENT

All chemicals used in this study were of analytical grade. The reagents included ethanol (p.a., Chimreactiv, Romania), gallic acid (Silver Chemicals, Romania), quercetin (Silver Chemicals, Romania), 2,2-diphenyl-1-picrylhydrazyl (DPPH, Merck, Germany), Folin–Ciocalteu reagent (Merck, Germany), sodium carbonate 20% (freshly prepared, Chimreactiv, Romania), sodium nitrite 5% (freshly prepared, Chimreactiv, Romania), aluminum chloride 10% (Merck, Germany), sodium hydroxide 1 M (Chimreactiv, Romania), and distilled water. Extracts were concentrated using a Hei-VAP Advantage rotary evaporator (Heidolph, Germany), and spectrophotometric analyses were performed using a PG Instruments T70+ UV-VIS spectrophotometer (UK).

METHODS

Extraction and Preparation of Extract Mixtures: The aerial parts of *Artemisia absinthium L.*, *Viola tricolor L.*, and the fruits of *Vaccinium myrtillus L.*, previously air-dried in a well-ventilated room at room temperature, were finely ground separately to enhance the extraction of bioactive compounds. For each plant, 20 g of the dried material were subjected to Soxhlet extraction using ethanol as solvent. The extraction was carried out for 4 hours for *Artemisia absinthium L.* and *Viola tricolor L.*, and 3 hours for *Vaccinium myrtillus L.*, ensuring complete recovery of the active constituents. The resulting extracts were concentrated under reduced pressure using a rotary evaporator and subsequently re-dissolved in ethanol to a defined volume for further analysis. To investigate the potential additive or synergistic effects, the individual extracts were combined according to the ratios indicated in Table 1 to obtain multi-component formulations. Each mixture was homogenized to ensure uniform distribution of bioactive compounds prior to further analyses.

Table 1

Composition of Extract Mixtures Studied

Mixtures	Constituent Species
M_1	*Artemisia absinthium L. + Vaccinium myrtillus L.* – 1:1
M_2	*Vaccinium myrtillus L. + Viola tricolor L.* – 1:1
M_3	*Artemisia absinthium L. + Viola tricolor L .* – 1:1
M_4	*Artemisia absinthium L. + Viola tricolor L. + Vaccinium myrtillus L.* – 1:1:1

Total Phenolic Content: The total phenolic content of the prepared extract mixtures (M1–M4) was determined using the Folin–Ciocalteu assay. For each mixture, 0.3 mL of the solution was combined with 0.45 mL of Folin–Ciocalteu reagent and 1.2 mL of distilled water, and the reaction was allowed to proceed in the dark for 10 minutes. Subsequently, 1.5 mL of freshly prepared 20% sodium carbonate solution was added, and the samples were incubated at room temperature for 60 minutes. Absorbance was recorded at 765 nm, and a blank containing ethanol was included for reference. All determinations were performed in triplicate. The total phenolic content was calculated using a gallic acid calibration curve (10–50 mg/mL), and results were expressed as milligrams of gallic acid equivalents per milliliter of extract mixture (Elferjane et al., 2024, Gonçalves et al., 2012; Hbika et al., 2022).

Total Flavonoid Content: The flavonoid content of the extracts and extract mixtures was determined using the aluminum chloride colorimetric method. Briefly, 1 mL of each extract was mixed with 4 mL of distilled water and 0.3 mL of 5% sodium nitrite, and allowed to react in the dark for 5 minutes. Then, 0.3 mL of 10% aluminum chloride was added and the mixture incubated for 6 minutes. Subsequently, 2 mL of 1 M sodium hydroxide was added, and the final volume was adjusted to 10 mL with distilled water. Absorbance was measured at 510 nm against a blank prepared with ethanol (Altiok et al., 2022; Msaada et al., 2015). All measurements were performed in triplicate, and flavonoid content was quantified using a quercetin calibration curve (2–10 mg/mL).

DPPH Radical Scavenging Assay: The antioxidant activity of the mixtures of extracts was evaluated using the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging method. Each mixture of extracts (0.5 mL) was combined with 3 mL of 0.1 mM DPPH solution and incubated in the dark at room temperature for 30 minutes. A blank containing ethanol instead of the extract mixture was prepared. Absorbance was measured at 517 nm, and the percentage of radical scavenging was calculated relative to the blank. All measurements were performed in triplicate to ensure reproducibility (Ciulca et al., 2021).

RESULTS AND DISCUSSIONS

The total phenolic content of the extract mixtures was assessed using the Folin–Ciocalteu method. Quantification was based on a calibration curve prepared with gallic acid ($Y = 0.007X + 0.0671$, $R^2 = 0.9918$), which confirmed a linear relationship between absorbance and concentration within the tested range. The results obtained for the different extract mixtures are presented in Table 2. Evaluation of the data indicates that the total phenolic content varied significantly among the mixtures. Mixture M2 exhibited the highest phenolic concentration, with a mean value of 154.82 ± 1.01 mg GAE/g dry extract, followed by M3 at 121.89 ± 0.96 mg GAE/g, M4 at 108.89 ± 0.53 mg GAE/g, and M1 displaying the lowest value of 85.74 ± 0.64 mg GAE/g. These findings suggest that the combination of plant constituents in M2 may contribute to additive or synergistic effects on phenolic content.

Table 2

Determination of total phenolic content in the extract mixtures

Sample	Sample absorbance measured at 765 nm	Sample concentration (mg GAE/g dry extract)	Mean concentration (mg GAE/g dry extract) ± SD
M_1	0,608	85,85	85,74 ± 0,64
	0,603	85,06	
	0,611	86,33	
M_2	0,989	154,94	154,82 ± 1,01
	0,994	155,78	
	0,982	153,76	
M_3	0,905	122,14	121,89 ± 0,96
	0,896	120,83	
	0,909	122,72	
M_4	0,886	108,32	108,89 ± 0,53
	0,891	108,98	
	0,894	109,37	

The quantification of total flavonoids in the analyzed extract mixtures was performed through the aluminum chloride colorimetric assay, a well-established method for assessing flavonoid concentration. The calibration curve obtained for quercetin standard solutions demonstrated good linearity within the tested concentration range, as expressed by the regression equation $Y = 0.033X + 0.1418$ ($R^2 = 0.9873$). The results of the spectrophotometric determinations, expressed as milligrams of quercetin equivalents per gram of dry extract (mg QE/g), are presented in Table 3. Distinct differences were observed among the mixtures regarding their flavonoid content. The M2 mixture exhibited the highest value (30.20 ± 0.10 mg QE/g dry extract), suggesting an enhanced accumulation of flavonoid compounds, potentially due to synergistic interactions between *Vaccinium myrtillus L.* and *Viola tricolor L*. Intermediate values were recorded for M3 (24.11 ± 0.09 mg QE/g) and M4 (18.13 ± 0.07 mg QE/g), while M1 showed the lowest flavonoid concentration (16.44 ± 0.08 mg QE/g).

Table 3

Total flavonoid content of the analyzed extract mixtures

Sample	Sample absorbance measured at 510 nm	Sample concentration (mg QE/1 g dry extract)	Mean concentration (mg QE/g dry extract) ± SD
M_1	0,628	16,37	16,44 ± 0,08
	0,630	16,43	
	0,633	16,53	
M_2	0,986	30,09	30,2 ± 0,10
	0,992	30,31	
	0,989	30,20	
M_3	0,925	24,21	24,11 ± 0,09
	0,921	24,09	
	0,919	24,03	
M_4	0,786	18,06	18,13 ± 0,07
	0,789	18,15	
	0,791	18,21	

The antioxidant capacity of the extract mixtures was determined by the DPPH assay, with the percentage of inhibition calculated from the absorbance values of the samples and the blank. The obtained results are presented in Table 4. The highest radical scavenging activity was observed for mixture M2 (85.21 ± 0.39%), followed by M3 (79.98 ± 0.18%) and M4 (78.28 ± 0.28%). The lowest activity was recorded for M1 (66.31 ± 0.28%). These results suggest that the combination of *Vaccinium myrtillus L.* and *Viola tricolor L.* (M2) shows superior antioxidant potential compared to the other formulations.

Table 4

DPPH radical scavenging activity of the analyzed extract mixtures

Sample	Blank absorbance	Sample absorbance	Inhibition %	Mean inhibition % ± SD
M_1	0,895	0,304	66,03	66,31 ± 0,28
		0,299	66,59	
		0,301	66,33	
M_2	0,895	0,132	85,25	85,21 ± 0,39
		0,129	85,58	
		0,136	84,80	
M_3	0,895	0,178	80,11	79,98 ± 0,18
		0,181	79,77	
		0,176	80,06	
M_4	0,895	0,192	78,54	78,28 ± 0,28
		0,194	78,32	
		0,197	77,98	

The mean values obtained for total phenolic content, total flavonoid content, and DPPH radical scavenging activity in the analyzed extract mixtures were systematized and represented graphically in Figure 1. Mixture M2 consistently exhibited the highest levels of phenolic compounds (154.82 ± 1.01 mg GAE/g dry extract), flavonoids (30.2 ± 0.10 mg QE/g dry extract), and antioxidant activity (85.21 ± 0.39%). These results suggest a strong correlation between the total phenolic and flavonoid contents and the radical scavenging activity, indicating that the synergistic combination of *Vaccinium myrtillus L.* and *Viola tricolor L.* contributes significantly to the overall antioxidant potential.

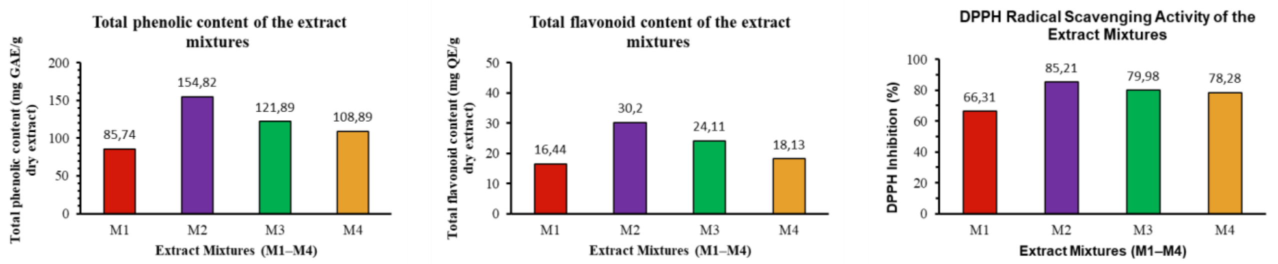

Figure 1. **Comparative evaluation of total phenolic and flavonoid contents and antioxidant activity (DPPH) of the extract mixtures**

CONCLUSIONS

The present study provides a comprehensive assessment of the phytochemical profiles and antioxidant properties of selected plant extract mixtures. Quantitative analyses revealed that mixture M2 consistently exhibited the highest concentrations of total phenolic compounds (154.82 ± 1.01 mg GAE/g dry extract) and flavonoids (30.2 ± 0.10 mg QE/g dry extract), which corresponded with markedly superior DPPH radical scavenging activity (85.21 ± 0.39 %). This strong correlation between polyphenolic and flavonoid contents and antioxidant performance underscores the critical role of these bioactive compounds in modulating free radical neutralization.

The results further suggest that synergistic interactions among the constituent extracts, particularly between *Vaccinium myrtillus L.* and *Viola tricolor L.*, potentiate the overall bioactivity of the mixtures beyond the additive effects of individual components. Such findings emphasize the potential of multi-component phytochemical formulations as promising natural functional ingredients with enhanced therapeutic efficacy. The data support the rationale for the development of standardized, synergistic plant-based preparations and warrant further investigation, including in vivo and mechanistic studies, to elucidate their pharmacokinetics, bioavailability, and safety profiles for potential clinical applications.

REFERENCES

Altiok, E., Kacmaz, S. and Altiok, D., 2022. Extraction of Bioactive Compounds in Wild Bilberry (Vaccinium Myrtillus L.) in The Eastern Black Sea Region With Different Techniques. Türk Doğa ve Fen Dergisi, 11(2), pp.130–136.

Batiha, G.E.-S., Olatunde, A., El-Mleeh, A., Hetta, H.F., Al-Rejaie, S., Alghamdi, S., Zahoor, M., Magdy Beshbishy, A., Murata, T., Zaragoza-Bastida, A. and Rivero-Perez, N., 2020. Bioactive Compounds, Pharmacological Actions, and Pharmacokinetics of Wormwood (Artemisia absinthium). Antibiotics, 9(6), p.353.

Chan, S.W. and Tomlinson, B., 2020. Effects of Bilberry Supplementation on Metabolic and Cardiovascular Disease Risk. Molecules, 25(7), p.1653.

Ciupei, D., Colişar, A., Leopold, L., Stănilă, A. and Diaconeasa, Z.M., 2024. Polyphenols: From Classification to Therapeutic Potential and Bioavailability. Foods, 13(24), p.4131.

Elferjane, M., Milutinović, V., Jovanović Krivokuća, M., Taherzadeh, M., Pietrzak, W., Marinković, A. and Jovanović, A., 2024. Vaccinium myrtillus L. Leaf Waste as a Source of Biologically Potent Compounds: Optimization of Polyphenol Extractions, Chemical Profile, and Biological Properties of the Extracts. Pharmaceutics, 16(6), p.740.

Godara, A., 2022. Preliminary Phytochemical Screening and Determination of Total Phenols and Flavonoids in Aerial Parts of Viola Tricolor. ECS Transactions, 107(1), pp.8219–8227.

Gonçalves, A.F.K., Friedrich, R.B., Boligon, A.A., Piana, M., Beck, R.C.R. and Athayde, M.L., 2012. Anti-oxidant capacity, total phenolic contents and HPLC determination of rutin in Viola tricolor (L) flowers. Free Radicals and Antioxidants, 2(4), pp.32–37.

Hassanpour, S.H. and Doroudi, A., 2023. Review of the antioxidant potential of flavonoids as a subgroup of polyphenols and partial substitute for synthetic antioxidants. Avicenna Journal of Phytomedicine, [online] 13(4).

Hbika, A., Daoudi, N.E., Bouyanzer, A., Bouhrim, M., Mohti, H., Loukili, E.H., Mechchate, H., Al-Salahi, R., Nasr, F.A., Bnouham, M. and Zaid, A., 2022. Artemisia absinthium L. Aqueous and Ethyl Acetate Extracts: Antioxidant Effect and Potential Activity In Vitro and In Vivo against Pancreatic α-Amylase and Intestinal α-Glucosidase. Pharmaceutics, 14(3), p.481.

Karole, S., Shrivastava, S., Thomas, S., Soni, B., Khan, S., Dubey, J., Dubey, S.P., Khan, N. and Jain, D.K., 2019. Polyherbal Formulation Concept for Synergic Action: A Review. Journal of Drug Delivery and Therapeutics, 9(1-s), pp.453–466.

Kosakowska, O., Węglarz, Z., Żuchowska, A., Styczyńska, S., Zaraś, E. and Bączek, K., 2025. Intraspecific Variability of Wormwood (Artemisia absinthium L.) Occurring in Poland in Respect of Developmental and Chemical Traits. Molecules, 30(14), p.2915.

Liu, Z., Li, X., Jin, Y., Nan, T., Zhao, Y., Huang, L. and Yuan, Y., 2023. New Evidence for Artemisia absinthium as an Alternative to Classical Antibiotics: Chemical Analysis of Phenolic Compounds, Screening for Antimicrobial Activity. International Journal of Molecular Sciences, 24(15), p.12044.

Msaada, K., Salem, N., Bachrouch, O., Bousselmi, S., Tammar, S., Alfaify, A., Al Sane, K., Ben Ammar, W., Azeiz, S., Haj Brahim, A., Hammami, M., Selmi, S., Limam, F. and Marzouk, B., 2015. Chemical Composition and Antioxidant and Antimicrobial Activities of Wormwood (Artemisia absinthium L.) Essential Oils and Phenolics. Journal of Chemistry, 2015, pp.1–12.

Palla, A.H., Amin, F., Fatima, B., Shafiq, A., Rehman, N.U., Haq, I.U. and Gilani, A.-H., 2021. Systematic Review of Polyherbal Combinations Used in Metabolic Syndrome. Frontiers in Pharmacology, 12, p.752926.

Pires, T.C.S.P., Caleja, C., Santos-Buelga, C., Barros, L. and Ferreira, I.C.F.R., 2020. Vaccinium myrtillus L. Fruits as a Novel Source of Phenolic Compounds with Health Benefits and Industrial Applications - A Review. Current Pharmaceutical Design, 26(16), pp.1917–1928.

Saunoriūtė, S., Ragažinskienė, O., Ivanauskas, L., Marksa, M., Laužikė, K. and Raudonė, L., 2023. Phenolic Diversity and Antioxidant Activity of Artemisia abrotanum L. and Artemisia absinthium L. during Vegetation Stages. Separations, 10(10), p.545.

Scalzo, J., Stevenson, D. and Hedderley, D., 2015. Polyphenol compounds and other quality traits in blueberry cultivars. Journal of Berry Research, 5(3), pp.117–130

Sun, S., Liu, Z., Lin, M., Gao, N. and Wang, X., 2024. Polyphenols in health and food processing: antibacterial, anti-inflammatory, and antioxidant insights. Frontiers in Nutrition, 11, p.1456730.

Supty, I.Z., Choudhury, T. and Parvin, M., 2025. Viola tricolor L.: A systematic review on ethnopharmacology, phytochemistry and pharmacology of an important traditional medicinal plant. Journal of Pharmacognosy and Phytochemistry, 14(4), pp.501–508