

Article

Phytochemical Characterization and In Vitro Antioxidant Activity of the Extract and Fractions from *Bidens subalternans* Leaves

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Abstract

The species *Bidens subalternans* DC., belonging to the Asteraceae family, is a native plant of Brazil. The objective of this study was to perform preliminary phytochemical characterization and evaluate the antioxidant activity and acute toxicity of the ethanol extract (EE) and leaf fractions of *B. subalternans*. Phytochemical screening revealed the presence of steroids, saponins, alkaloids, coumarins, flavonoids and tannins. Analysis of the EE and the dichloromethane fraction (DCMF) by gas chromatography–mass spectrometry (GC-MS) suggested the presence of phytol, ethyl esters, fatty acids, terpenoid compounds, stigmasterol, untriacontane, ketones, and loliolide. The presence of chlorogenic acid, cinnamic acid derivatives, coumaric acid derivatives, caffeic acid derivatives, and flavonoids was putatively assigned using high-performance liquid chromatography coupled with a diode-array detector (HPLC-DAD). The ethyl acetate fraction (EAF) and DCMF showed higher levels of total phenolic compounds and flavonoids compared to the EE and hydroethanol fraction (HEF) (EAF > DCMF > EE > HEF). In antioxidant activity assays, the EAF exhibited the greatest potential, followed by the DCMF, EE, and HEF. The results showed no mortality of *Artemia salina* larvae, indicating an absence of acute toxicity under the tested conditions. This study contributes to the preliminary chemical characterization of *B. subalternans*, toxicity and antioxidant activity in vitro, highlighting it as a potential source of natural antioxidants.

Keywords: *Bidens subalternans*; phenolic compounds; *Artemia salina*; antioxidant activity



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1. Introduction

Brazilian plant biodiversity is remarkably rich, offering a vast resource for the discovery of novel drug prototypes and therapeutic agents. However, many species remain underutilized or are viewed exclusively from a negative economic perspective, as is the case with weeds. These plants are generally defined as species that grow spontaneously in areas of human interest and interfere adversely with agricultural production systems [1], competing for nutrients, light, and water, thereby reducing crop productivity. Nevertheless, weeds

may also offer significant benefits, particularly in medicinal applications, representing valuable reservoirs of bioactive compounds with therapeutic potential [2].

The field of weed ecology and management is of great importance to the Brazilian economy and agricultural sector. Due to their competitive behavior and allelopathic potential, weeds can cause substantial damage to crop production, negatively affecting agricultural productivity and profitability [3].

The genus *Bidens* has considerable ecological, pharmacological, and agronomic relevance and has been extensively studied [4]; however, integrated information regarding the antioxidant potential and the safety/toxicity of the species *Bidens subalternans* remains limited. *B. subalternans* is an herbaceous species belonging to the Asteraceae family, popularly known in Brazil as “carrapicho-de-pontas,” “fura-capá,” “picão-do-campo,” and “picão-preto” [5]. This species is characterized by a slightly quadrangular stem; petiolate leaves arranged oppositely at the base and alternately toward the apex; yellow capitulum-type inflorescences; and dry fruits classified as achenes [5].

B. subalternans occurs predominantly in the Central-West, Southeast, and South regions of Brazil, frequently found in annual and perennial crop fields and fruit orchards, where it is considered a problematic weed. Its presence in cultivated areas is undesirable not only due to competition with agricultural crops but also because it acts as a host for pests and bears fruits with spiny appendages (parts with spines, trichomes, or sharp areoles) that easily adhere to animal bodies, facilitating its dispersal [5,6]. Species such as *B. pilosa* and *B. subalternans* are invasive weeds capable of reducing the productivity of crops such as common beans by up to 48%. This aggressiveness is further intensified by their resistance to herbicides that inhibit the enzyme acetolactate synthase (ALS) [4].

B. subalternans has been reported to contain maslinic acid, oleanolic acid, stigmasterol, and stigmasterol-3-O- β -D-glucoside [7,8], which demonstrated anti-inflammatory activity in the carrageenan-induced paw edema model [7]. The 70% hydroethanol extract exhibited antibacterial activity against *Escherichia coli*, *Acinetobacter baumannii*, and *Enterobacter aerogenes* [9].

Therefore, this study aimed to investigate the preliminary phytochemical profile of the ethanol extract and fractions of *B. subalternans* leaves using GC-MS and HPLC-DAD, and to evaluate their antioxidant activity and preliminary toxicity using in vitro assays.

2. Materials and Methods

2.1. Reagents and Solvents

For this study, the following reagents were used: 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,6-di-*tert*-butyl-4-methylphenol (BHT), ascorbic acid (AA), 2,4,6-tripyridyl-s-triazine (TPTZ), quercetin, dimethyl sulfoxide (DMSO), sodium acetate, gallic acid, HPLC-grade methanol, benzoic acid, caffeic acid, cinnamic acid, chlorogenic acid, catechin, kaempferol, and rutin (Sigma-Aldrich, St. Louis, MO, USA); C₉–C₃₀ *n*-alkane standard (Supelco, Bellefonte, PA, USA); formic acid, ferric chloride (FeCl₃), aluminum chloride (AlCl₃), bismuth subnitrate, potassium iodide, and iron(III) chloride hexahydrate (Vetec, Duque de Caxias, RJ, Brazil); acetic anhydride (Dinâmica Química Contemporânea, Indaiatuba, SP, Brazil); ethanol, hydrochloric acid, and sulfuric acid (H₂SO₄) (Alphatec, Macaé, RJ, Brazil); sodium hydroxide (NaOH), methanol, ethyl acetate, sodium carbonate, and glacial acetic acid (CRQ Produtos Químicos, Diadema, SP, Brazil); synthetic sea salt Marinemix (hW-Wiegandt Brasil, São Paulo, SP, Brazil); dichloromethane (ISOFAR Indústria e Comércio de Produtos Químicos Ltda., Duque de Caxias, RJ, Brazil); acetic acid (Neon Reagentes Analíticos, Suzano, SP, Brazil); and Folin–Ciocalteu reagent (Êxodo Científica, Sumaré, SP, Brazil).

2.2. Plant Material and General Characterization

The study was conducted between March 2024 and July 2025 in the Pharmacognosy and Natural Products Laboratory and the Phytochemistry Laboratory at the Federal University of São João Del-Rei (UFSJ), Divinópolis Campus, Minas Gerais, Brazil. Leaves of *Bidens subalternans* DC. (Asteraceae) were collected in February 2019 from a grassland area with Cerrado *sensu stricto* vegetation at Parque do Gafanhoto, Divinópolis, Minas Gerais, Brazil (20°06'38.459" S, 44°50'47.184" W).

The voucher specimen was identified by Andréia Fonseca Silva (researcher in botany) and deposited in the PAMG Herbarium of the Agricultural Research Company of Minas Gerais (EPAMIG, Belo Horizonte, Minas Gerais, Brazil) under accession number 58636. Plant collection and the associated research project were duly authorized by SISBIO (n° 30006) and SisGen (n° A6704FE), in accordance with Brazilian Law No. 13,123/2015.

2.3. Preparation and Partitioning of the Ethanol Extract (EE)

Fresh plant material (155.42 g) was dried in a forced-air circulation oven (Edutec FDC-1000 Serials, Sapucaia do Sul, RS, Brazil) at 40 °C for 7 days, yielding 27.8 g of dry biomass, which was subsequently ground using a knife mill (Marconi MA 048, Piracicaba, SP, Brazil). Extraction was performed by turbo-extraction (Ultra-Turrax Marconi MA-102 Plus, Piracicaba, SP, Brazil) using 70% (*v/v*) ethanol at a solvent-to-plant ratio of 9:1 (180 mL of solvent/20 g of plant material). After filtration, the ethanol extract (EE) was concentrated using a rotary evaporator (Ika RV 10 digital V-C, Staufen, Germany) at a temperature of 40 °C and 115 rpm, and further dried in an oven at 40 °C until constant weight was achieved (8.22 g). An aliquot of the EE (6 g) redissolved in 100 mL of 70% (*v/v*) ethanol was subjected to liquid–liquid partitioning using solvents of increasing polarity, namely dichloromethane and ethyl acetate (2 × 50 mL of each solvent). The solvents were concentrated and the fractions were dried, yielding the dichloromethane (DCMF, 0.953 g), ethyl acetate (EAF, 0.397 g), and hydroethanol (HEF, 4.507 g) fractions. The dried samples were stored under refrigeration (−20 °C) in airtight, light-protected amber vials until the phytochemical and biological analyses were performed between March 2024 and July 2025.

2.4. Phytochemical Characterization

2.4.1. Phytochemical Screening

The EE and the fractions were subjected to qualitative colorimetric and precipitation tests (2 mg/mL solutions in 92.8% ethanol) to screen for classes of secondary metabolites [10,11], following the methodologies described below: flavonoids (addition of H₂SO₄, resulting in yellow, orange, or red coloration); saponins (vigorous shaking with distilled water for 1 min, assessing foam persistence for 15 min); tannins (addition of 5% FeCl₃, yielding a green precipitate for condensed tannins and a blue precipitate for hydrolysable tannins); alkaloids (reaction with bismuth subnitrate and potassium iodide solutions—Dragendorff's reagent, resulting in precipitate formation or turbidity); coumarins and anthraquinones (addition of 1 mol/L NaOH, resulting in yellow coloration for coumarins and violet for anthraquinones); and steroids and triterpenoids (reaction with acetic anhydride and analytical-grade H₂SO₄—Liebermann–Burchard test, resulting in a green or blue upper phase for steroids and a pink-to-violet interfacial halo for triterpenoids).

2.4.2. Gas Chromatography–Mass Spectrometry (GC-MS) Analysis

The EE and the DCMF were analyzed using a Shimadzu GCMS-QP2020 NX (Kyoto, Japan) system equipped with a Restek RTx-5MS column (Bellefonte, PA, USA) (30 m, 0.25 mm i.d., 0.25 µm film thickness). The samples were weighed and dissolved in ethyl acetate to produce a 10 mg/mL solution. This solution was placed in 2 mL vials, and 1 µL

of each sample was injected in split mode (1:10) at 250 °C, using helium as the carrier gas (120.0 kPa) and electron ionization (EI) mode at 70 eV. The temperature program started at 40 °C (1 min), increasing at 7 °C/min to 280 °C (held for 15 min). The interface and ion source temperatures were set at 250 °C. Data acquisition (m/z 40–450; 5 scans/min) began after the solvent delay (10 min) (Supplementary Material Figure S1). The samples were injected only once. Compounds were putatively assigned by calculating Linear Retention Indices relative to *n*-alkanes (C₉–C₃₀), and by comparing the mass fragmentation profile of each constituent with that of reference compounds from the NIST 17 (USA) electron impact mass spectral library, focusing on matches with a fragmentation spectrum (similarity $\geq 90\%$). Relative content was determined via area normalization.

2.4.3. High-Performance Liquid Chromatography Coupled to a Diode-Array Detector (HPLC-DAD) Analysis

The EE and the fractions (2 mg/mL in methanol, filtered through a 0.45 µm membrane) were analyzed using a Shimadzu UFLC Prominence (Kyoto, Japan) system coupled to a diode-array detector (DAD), employing a Phenomenex Luna C₁₈ column (Torrance, CA, USA) (150 mm × 4.6 mm × 5 µm, 100 Å). The mobile phase consisted of (A) water acidified with 0.1% formic acid and (B) methanol, at a flow rate of 0.5 mL/min and with gradient elution: 40–70% of B (0–5 min), 70% of B (5–19 min), and 70–40% of B (19–22 min). The injection volume was 30 µL for EE and EAF, whereas 80 µL was applied for DCMF and HEF solely to optimize the signal-to-noise ratio (S/N) for minor constituents at 254 nm. Consequently, HPLC-DAD analyses were conducted strictly for qualitative profiling (chromatographic fingerprinting), and inter-sample quantitative comparisons were supported exclusively by spectrophotometric assays. Detection was performed at 254 nm (190–800 nm scan), and the compounds were putatively assigned by comparing retention times and UV spectra [12] with authentic standards (benzoic acid, caffeic acid, cinnamic acid, chlorogenic acid, gallic acid, catechin, kaempferol, quercetin, and rutin).

2.5. Determination of Total Phenolic and Flavonoid Contents

2.5.1. Determination of Total Phenolic Content (TPC)

The total phenolic content of the samples (EE 0.5 mg/mL, DCMF 0.1 mg/mL, EAF 0.1 mg/mL, and HEF 0.5 mg/mL) was determined using the Folin–Ciocalteu method [13], with some modifications. Each test tube contained 250 µL of the sample, 2250 µL of Folin–Ciocalteu reagent (previously diluted 1:4 *v/v* with distilled water), and 250 µL of a 4% saturated sodium carbonate solution; after mixing, the tubes were incubated for 30 min at room temperature. For the blank, 250 µL of sample and 2500 µL of water were pipetted. Absorbance was measured using a spectrophotometer (QUIMIS Q798U, Diadema, SP, Brazil) at a wavelength of 750 nm. Gallic acid was used as the reference compound to construct the calibration curve at concentrations ranging from 5 to 30 µg/mL ($R^2 = 0.9984$). Determinations were performed in technical triplicates (using three replicate tubes derived from a single extract/fraction or gallic acid preparation). The concentration (C) was calculated by interpolating net sample absorbances ($\Delta\text{abs}_{750} = \text{Abs}_{\text{sample}} - \text{Abs}_{\text{blank}}$) into the linear regression equation ($y = 0.0191x - 0.0064$) derived from the gallic acid calibration curve. The total phenolic content/reducing capacity was expressed in µg of gallic acid equivalents per mg of extract or fraction (µg GAE/mg) calculated by the following equation $\text{TPC} (\mu\text{g GAE/mg}) = (C \times V_T \times D)/m$, where C = concentration obtained (in µg/mL) by equation; V_T = total volume obtained from the sample (1 mL), D = dilution factor (sample not diluted before being added to the reagents, D = 1), and m = dry mass of the sample initially weighed (0.1 or 0.5 mg).

2.5.2. Determination of Total Flavonoid Content (TFC)

Total flavonoid content was determined using the extract (0.2 mg/mL) and the DCMF (0.2 mg/mL), EAF (0.2 mg/mL), and HEF (1 mg/mL). The total flavonoid content was estimated using the aluminum chloride method, in accordance with the Brazilian Pharmacopoeia [14]. Aliquots of 100 μ L of the EE or fractions, 1900 μ L of ethanol, and 500 μ L of 5% (*w/v*) AlCl_3 solution were pipetted. For the blank, 100 μ L of sample and 2400 μ L of ethanol were pipetted. After incubation for 30 min, absorbance was measured at 425 nm using a spectrophotometer (QUIMIS Q798U, Diadema, SP, Brazil). Quercetin was used as the reference compound to generate the calibration curve at concentrations ranging from 5 to 50 μ g/mL ($R^2 = 0.9989$). Assays were performed in technical triplicates (using three replicate tubes derived from a single extract/fraction or quercetin preparation). The concentration (C) was calculated by interpolating net sample absorbances ($\Delta\text{abs}_{425} = \text{Abs}_{\text{sample}} - \text{Abs}_{\text{blank}}$) into the linear regression equation ($y = 0.0271x - 0.0481$) derived from the quercetin calibration curve. The total flavonoid content was expressed as μ g of quercetin equivalents per mg of extract or fraction (μ g QE/mg) calculated by the following equation $\text{TFC } (\mu\text{g QE/mg}) = (C \times V_T \times D)/m$, where C = concentration obtained (in μ g/mL) by equation; V_T = total volume obtained from the sample (1 mL), D = dilution factor (sample not diluted before being added to the reagents, D = 1), and m = dry mass of the sample initially weighed (0.2 or 1 mg).

2.6. Biological Activity Assays

2.6.1. Evaluation of Antioxidant Activity by the 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) Method

The assay was performed in 96-well microplates [15,16] by pipetting 100 μ L of the samples or 2,6-di-*tert*-butyl-4-methylphenol (BHT) standard (solubilized in ethanol) and 200 μ L of an ethanolic DPPH solution (0.002% *w/v*), resulting in final concentrations of 1, 10, 100, 250, and 500 μ g/mL in the wells. Ethanol (300 μ L) was used for background signal correction. For blanks, 100 μ L of the samples or standard and 200 μ L of ethanol were pipetted. For the DPPH control, 200 μ L of ethanolic DPPH solution and 100 μ L of ethanol were pipetted. After 30 min at room temperature (25 $^{\circ}$ C) and protected from light, absorbance was measured at 520 nm in a spectrophotometer (Power Wave XS2/US, Biotec, Winooski, VT, USA). The percentage of inhibition was calculated by the following equation [17]: % DPPH inhibition = $[1 - (A_a/A_b)] \times 100$, where A_a = absorbance of the sample or standard after subtracting the blank absorbance and A_b = absorbance of the DPPH solution (Supplementary Material Table S6). Effective concentration values (EC_{50}), along with their parameter uncertainties expressed as 95% profile likelihood confidence intervals (95% CIs), were determined using a non-linear regression dose–response model [log(inhibitor) vs. response–variable slope (four parameters) in GraphPad Prism 7.0. For the ethyl acetate fraction (EAF), the Bottom parameter was constrained to 0 (Bottom = 0) due to rapid radical scavenging observed between 1 and 10 μ g/mL. The EC_{50} values represent the relative curve midpoint (halfway between the fitted Bottom and Top values of the non-linear dose–response curve) rather than absolute 50% inhibition. Assays were performed in technical triplicates ($n = 3$ replicate wells derived from a single extract/fraction or BHT preparation), and complete non-linear regression fitting parameters, degrees of freedom, and goodness-of-fit statistics ($R^2 > 0.998$) are fully detailed in Supplementary Material Table S7.

2.6.2. Evaluation of Antioxidant Activity by the Ferric Reducing Antioxidant Power (FRAP) Method

The ferric reducing antioxidant power (FRAP) assay was performed according to Urrea-Victoria et al. [18] and Nwachukwu et al. [19], with minor modifications. Aliquots

of 60 µL of the samples or BHT at concentration of 0.05 mg/mL (in ethanol) and 240 µL of the FRAP reagent (0.3 M acetate buffer, pH 3.6; 10 mM TPTZ in 40 mM HCl; and 20 mM FeCl₃·6H₂O) were added to microplate wells. For the blank, 240 µL of FRAP reagent and 60 µL of ethanol were pipetted. A calibration curve was constructed using ascorbic acid as the reference standard across a final concentration range of 1 to 15 µg/mL ($R^2 = 0.9962$, linear regression equation: $y = 0.158x + 0.0081$, as detailed in Supplementary Material Figure S3 and Tables S8–S10). After incubation (25 °C/30 min in the dark), absorbance was measured at 595 nm in a spectrophotometer (Power Wave XS2/US, Biotec, Winooski, VT, USA). FRAP reducing capacity was quantitatively calculated by interpolating net sample absorbances ($\Delta\text{Abs}_{595} = \text{Abs}_{\text{sample}} - \text{Abs}_{\text{blank}}$) into this validated linear regression equation. Results were expressed as micrograms of ascorbic acid equivalents per milligram of extract or fraction (µg AAE/mg) calculated by the equation $\text{FRAP } \mu\text{g AAE/mg} = (C \times V_T \times D)/m$, where C = concentration obtained (µg/mL) by the equation; V_T = total sample volume (1 mL); D = dilution factor ($D = 1$); and m = initial dry mass (0.05 mg). Assays were performed in technical triplicates ($n = 3$ replicate wells derived from a single extract/fraction or BHT preparation). Raw absorbance values, statistical parameters, and step-by-step calculation memories are fully disclosed in Supplementary Material Tables S8–S10.

2.6.3. Lethality Assessment Against *Artemia salina*

A. salina cysts were incubated in artificial seawater (36 g/L) under aeration and constant illumination for 48 h [20]. The extract and fractions were initially solubilized in 1% dimethyl sulfoxide (DMSO) and diluted in seawater to concentrations of 1000, 500, 250, and 125 µg/mL. Ten nauplii (stage II) were transferred to tubes containing 5 mL of the test solutions or corresponding controls. Assays were performed in technical triplicates ($n = 3$ replicate tubes derived from a single extract/fraction preparation). Three independent experiments were conducted on different days. After 24 h, the number of surviving nauplii was recorded.

2.7. Statistical Analyses

Data were expressed as mean $\pm$ standard deviation (SD) for chemical and antioxidant analyses, and as mean $\pm$ standard error (SE) for the toxicity assay. Spectrophotometric quantifications (total phenolics and flavonoids) and antioxidant assays (DPPH and FRAP) were performed in technical triplicates ($n = 3$ analytical tubes or wells per sample) from a single extract/fraction or controls preparation. Conversely, the *Artemia salina* lethality bioassay was conducted as three independent biological experiments performed on separate days ($n = 90$ nauplii total per concentration group). Before statistical analysis, the Shapiro–Wilk test and Kolmogorov–Smirnov test were applied to evaluate the normality of the data, and the Brown–Forsythe test or Bartlett’s test was applied to determine the homogeneity of variance. The data for each group were analyzed through one-way ANOVA, and post hoc Dunnett’s test was used to compare significant differences between experimental groups with controls), with $p < 0.05$ significance levels. The EC₅₀ values for antioxidant activity of DPPH assay were determined using nonlinear regression analysis for dose–response inhibition [$\log(\text{inhibitor})$ vs. response–variable slope (four parameters)] using the GRAPHPAD PRISM 7.0 software (San Diego, CA, USA). For the ethyl acetate fraction (EAF), the Bottom parameter was constrained to 0 due to rapid radical clearance observed between 1 and 10 µg/mL. All EC₅₀ values are reported alongside their 95% profile-likelihood confidence intervals (95% CI). It should be noted that the reported EC₅₀ values represent the relative curve midpoint (halfway between the fitted Bottom and Top values of the non-linear dose–response curve) rather than absolute 50% inhibition.

3. Results

3.1. Phytochemical Screening

The results obtained from the phytochemical screening of the EE and the DCMF, EAF, and HEF suggested the presence of steroids, saponins, alkaloids, and coumarins in the EE. Furthermore, coumarins and flavonoids were found in the DCMF, EAF, and HEF, alongside tannins in the DCMF and saponins in the HEF.

3.2. Gas Chromatography–Mass Spectrometry (GC-MS) Analysis

GC-MS analyses of the ethanol extract and the dichloromethane fraction (DCMF) of *B. subalternans* leaves revealed distinct chemical profiles among the samples. Peaks detected within the first 10 min of the run, as well as the peak with RT $\approx$ 33.401 corresponding to the phthalate DEHP, were excluded, as these are contaminants originating from the ethyl acetate solvent used to solubilize the samples for injection (Supplementary Material Figure S1A). In the EE, 14 major peaks were observed, with 10 compounds putatively assigned using the NIST 17 library and literature retention indices (Table 1); notable compounds included phytol (22.62%), ethyl linolenate (13.94%), ethyl palmitate (6.92%), and palmitic acid (6.44%), as well as neophytadiene, phytadiene, supraene, linoleic and linolenic acids, and stigmaterol.

Table 1. Composition and relative percentages of compounds detected by GC-MS in the ethanol extract (EE) of *Bidens subalternans* leaves.

Peak	RT	Compound	RI	LRI	%
1	20.024	UID	1602	-	2.39
2	23.900	Neophytadiene	1840	1836 (a)	5.54
3	24.560	Phytadiene	1883	1861 (b)	1.51
4	25.736	Palmitic acid	1962	1959 (a)	6.44
5	26.210	Ethyl palmitate	1995	1994 (a)	6.92
6	27.910	Phytol	2117	2128 (a)	22.62
7	28.284	Linolenic acid	2145	2134 (a)	6.17
8	28.563	Linoleic acid	2166	2156 (b)	3.61
9	28.665	Ethyl linolenate	2173	2177 (b)	13.94
10	36.508	Supraene	2835	2834 (b)	1.98
11	44.935	Stigmaterol	-	3170 (b)	7.82
12	46.589	UID	-	-	3.40
13	49.135	UID	-	-	2.11
14	49.280	UID	-	-	2.93

RT: retention time (min); RI: retention index of the compound on the RTx-5MS column; LRI: literature retention index on the DB-5MS column (a), and HP-5MS (b); UID: unidentified.

Conversely, DCMF displayed 14 chromatographic peaks, five of which corresponded to putatively annotated compounds (Table 2). The major constituent was the aliphatic hydrocarbon untriacontane, accounting for 66.91% of the fraction's relative composition, followed by loliolide (6.29%), 2-decanone (4.24%), palmitic acid (3.43%), and 2-nonanone (1.18%).

Table 2. Composition and relative percentages of compounds detected by GC-MS in the dichloromethane fraction (DCMF) of *Bidens subalternans* leaves.

Peak	RT	Compound	RI	LRI	%
1	10.422	2-Nonanone	1120	1094 (a)	1.18
2	11.485	2-Decanone	1168	1190 (a)	4.24
3	20.017	UID	1602	-	1.33
4	20.291	UID	1618	-	1.46
5	20.567	UID	1635	-	1.78
6	20.728	UID	1644	-	1.27
7	21.480	UID	1688	-	2.80
8	23.019	Loliolide	1783	1784 (b)	6.29
9	25.725	Palmitic acid	1962	1959 (a)	3.43
10	26.474	UID	2014	-	1.65
11	26.664	UID	2027	-	3.21
12	29.702	UID	2253	-	2.00
13	30.938	UID	2452	-	1.06
14	38.843	Untriacontane	3100	3100 (a)	66.91

RT: retention time (min); RI: retention index of the compound on the RTx-5MS column; LRI: literature retention index on the DB-5MS column (a) and HP-5MS column (b); UID: unidentified.

Comparison of the chemical profiles revealed that palmitic acid was the only putatively assigned compound in both the extract and the DCMF. Overall, GC-MS analysis showed the presence of fatty acids, fatty acid esters, terpenoids, and sterol in the EE, whereas the DCMF consisted of a hydrocarbon, ketones, a fatty acid, and a monoterpene lactone. Finally, the putative assignment of a sterol in the EE corroborates data previously obtained during phytochemical screening.

3.3. Analysis by High-Performance Liquid Chromatography Coupled with a Diode-Array Detector (HPLC-DAD)

The chromatograms obtained by HPLC-DAD are shown in Figure 1, with detection at 254 nm. In the chromatograms at 254 nm, 18 peaks were detected for EE, 16 peaks for DCMF, 10 peaks for EAF, and 6 peaks for HEF (Figure 1).

High-performance liquid chromatography analysis at 254 nm revealed complex chromatographic profiles for the extract and fractions (Table 3). Definitive identification (MSI Level 1) was established for chlorogenic acid (Peak 4 in EE and DCMF, RT = 3.71 min; $\lambda_{\max} \approx 298$ and 327 nm), which exhibited strict retention time and UV spectral alignment with the authentic reference standard injected under identical analytical conditions (RT = 3.72 min; $\lambda_{\max} = 254, 274, 337$, and 354 nm). Conversely, other detected constituents were putatively assigned (MSI Level 2/3) to metabolic classes based on diagnostic UV-Vis absorption bands within the 225–400 nm range: cinnamic acid derivatives ($\lambda_{\max} \approx 283$ –297 and 327 nm), coumaric acid derivatives ($\lambda_{\max} \approx 287$ –298 and 307–317 nm), caffeic acid derivatives ($\lambda_{\max} \approx 299$ and 330 nm), flavonols ($\lambda_{\max} \approx 269$ and 347 nm), flavones ($\lambda_{\max} \approx 268$ and 331 nm), and flavanones ($\lambda_{\max} \approx 289$ and 329 nm) [12,21]. This HPLC-DAD analysis of the fractions indicated the presence of different classes of flavonoids, corroborating the phytochemical screening performed on the DCMF, EAF, and HEF.

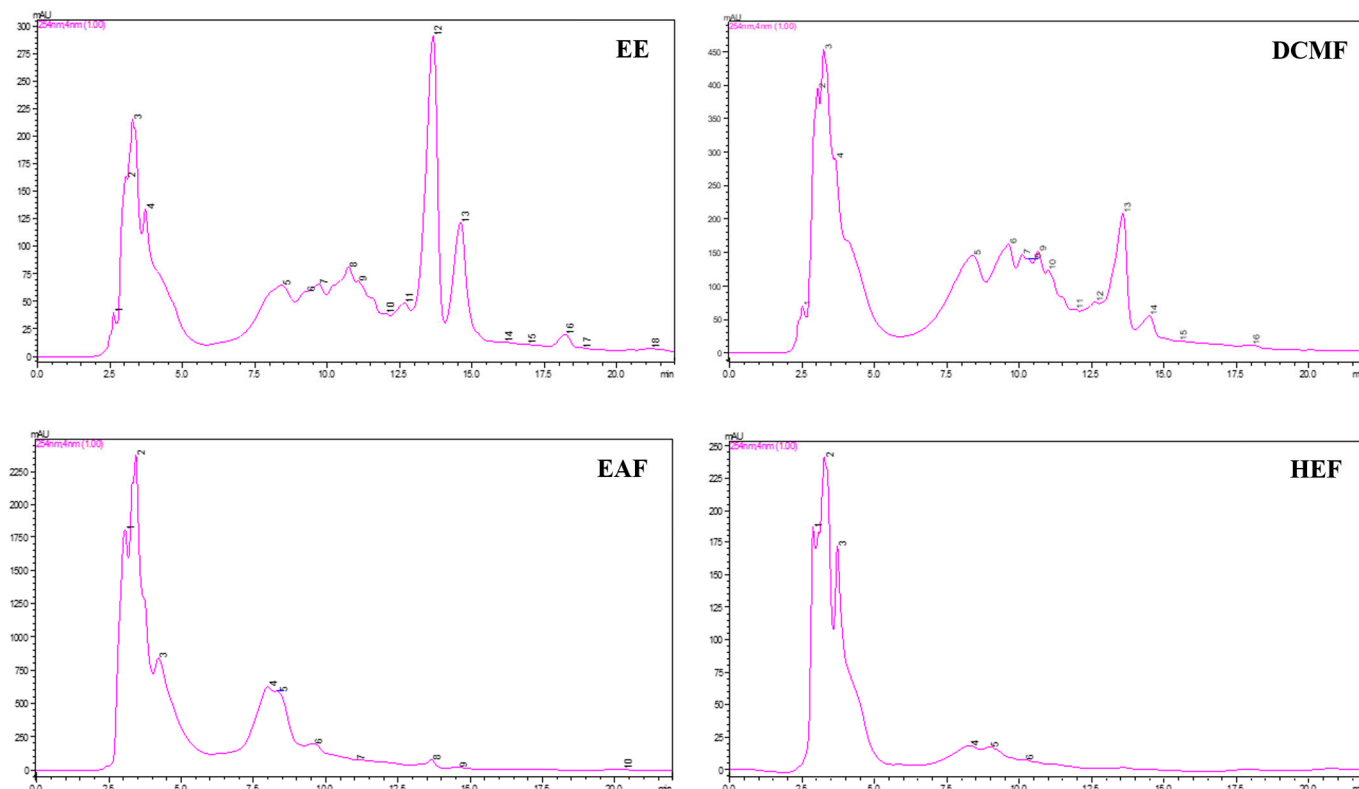


Figure 1. Chromatographic profile of the extract and fractions of *Bidens subalternans* by HPLC-DAD at 254 nm. EE: ethanol extract, DCMF: dichloromethane fraction, EAF: ethyl acetate fraction, HEF: hydroethanol fraction.

Table 3. Chromatographic parameters (RT, $\lambda_{\max}$), level of identification, and structural assignment rationale for phenolic compounds of the extract and fractions of *Bidens subalternans* by HPLC-DAD at 254 nm.

Peak	RT	$\lambda_{\max}$ (225–400 nm)	Putative/Definitive Assignment	Molecular Formula	Chemical/Analytical Rationale	Identification Level
1 (EE)	2.61	285	Simple phenolic derivative	ND	Isolated UV band at 285 nm corresponding to simple aromatic ring electronic transition.	Level 3
4 (EE/ DCMF)	3.71/3.66	298/327	Chlorogenic acid	$C_{16}H_{18}O_9$	Definitive characterization. Strict alignment of RT and UV profile with authentic standard (RT = 3.72 min).	Level 1
12 (EE)	13.66	229/239/ 255/269/347	Flavonol glycoside (quercetin/ kaempferol derivative)	$C_{21}H_{20}O_{12}$	Bipolar absorption (Band II at 269 nm, Band I at 347 nm); earlier elution than free aglycone.	Level 2
5 (DCMF)	8.41	268/296/331	Flavone derivative	$C_{15}H_{10}O_2$ (core)	Diagnostic UV spectrum of flavone subclass (Band II at 268 nm, Band I at 331 nm).	Level 2
1,2,7 (EAF)	3.08/3.45/ 11.03	230/289/ 308/329	Flavanone derivative	$C_{15}H_{12}O_5$	Intense Band II at 289 nm with shoulder/Band I at 329 nm, characteristic of C_2 – C_3 saturated flavanones.	Level 2

EE: ethanol extract, DCMF: dichloromethane fraction, EAF: ethyl acetate fraction; RT: retention time (min).

3.4. Determination of Total Phenolic Compounds and Flavonoids

The total phenolic and total flavonoid contents determined for the EE and DCMF, EAF, and HEF of *B. subalternans* leaves are shown in Table 4.

Table 4. Results for Total Phenolic and Flavonoid Content.

Sample	Phenols ($\mu\text{g GAE/mg}$)	Flavonoids ($\mu\text{g QE/mg}$)
EE	62.834 ± 0.018	38.825 ± 0.114
DCMF	166.003 ± 0.067	42.577 ± 0.053
EAF	268.970 ± 0.116	57.706 ± 0.038
HEF	33.585 ± 0.027	10.852 ± 0.046

EE: ethanol extract, DCMF: dichloromethane fraction, EAF: ethyl acetate fraction, HEF: hydroethanol fraction; $\mu\text{g GAE/mg}$: micrograms of gallic acid equivalents per milligram of extract or fraction; $\mu\text{g QE/mg}$: micrograms of quercetin equivalents per milligram of extract or fraction. Results are expressed as mean $\pm$ standard deviation ($n = 3$ technical replicate tubes).

Regarding total phenolic compounds, the EAF and DCMF showed the highest levels, with the EAF standing out, while the EE and HEF exhibited the lowest values.

Regarding total flavonoids, the highest levels were observed in the EAF and DCMF, followed by the EE, whereas the HEF showed the lowest value (Table 4).

3.5. Antioxidant Activity

The antioxidant activity results obtained by the DPPH radical method for the EE and fractions of *B. subalternans* are presented in Table 5. Antioxidant effects were observed at all tested concentrations (1, 10, 100, 250, and 500 $\mu\text{g/mL}$); notably, at concentrations of 250 and 500 $\mu\text{g/mL}$, the extract and fractions demonstrated high potential, with inhibition percentages ranging from 83.34% to 97.51%. Additionally, the DCMF and EAF at concentrations of 10, 100, and 250 $\mu\text{g/mL}$ were statistically more effective in stabilizing free radicals than the synthetic standard 2,6-di-*tert*-butyl-4-methylphenol (BHT) ($p < 0.05$), also exhibiting higher antioxidant activities expressed by lower EC_{50} values ($p < 0.05$) (Supplementary Material, Table S7).

Table 5. Evaluation of the antioxidant activity of the ethanol extract and fractions of *Bidens subalternans* using the DPPH method.

Sample	% DPPH Inhibition					EC_{50} ($\mu\text{g/mL}$) [95% CI]
	1 $\mu\text{g/mL}$	10 $\mu\text{g/mL}$	100 $\mu\text{g/mL}$	250 $\mu\text{g/mL}$	500 $\mu\text{g/mL}$	
EE	9.25 ± 0.22^a	25.86 ± 0.50	92.66 ± 0.44^a	94.44 ± 0.77	97.51 ± 0.69	21.08 (18.95–23.17) ^a
DCMF	13.73 ± 1.53^a	51.61 ± 1.83^a	94.94 ± 0.29^a	95.76 ± 0.29^a	96.46 ± 0.11	10.77 (10.34–11.30) ^a
EAF	19.38 ± 2.28	92.46 ± 0.47^a	94.33 ± 0.11^a	95.02 ± 0.39^a	95.20 ± 0.22^a	1.86 (1.67–2.01) ^{a*}
HEF	9.34 ± 0.64^a	12.57 ± 0.46^a	39.72 ± 1.16^a	83.34 ± 1.94^a	94.27 ± 0.46^a	130.50 (123.4–138.8) ^a
BHT (Standard)	20.504 ± 0.51	28.507 ± 0.55	85.505 ± 0.58	92.501 ± 0.56	98.501 ± 0.52	37.03 (32.43–42.16)

EE: ethanol extract, DCMF: dichloromethane fraction, EAF: ethyl acetate fraction, HEF: hydroethanol fraction, BHT: 2,6-di-*tert*-butyl-4-methylphenol, EC_{50} : effective concentration representing the relative curve midpoint (halfway between fitted Bottom and Top) derived from the non-linear regression curve. Percentages of DPPH inhibition are expressed as mean $\pm$ standard deviation ($n = 3$ technical replicate wells). EC_{50} values and their 95% profile likelihood confidence intervals (95% CIs) were determined using GraphPad Prism 7.0. ^a $p < 0.05$ compared to BHT. * For EAF, EC_{50} was calculated constraining Bottom = 0 due to rapid radical scavenging between 1 and 10 $\mu\text{g/mL}$.

The ferric reducing antioxidant power (FRAP) of *B. subalternans* samples was quantitatively determined using an ascorbic acid calibration curve (1–15 µg/mL, $R^2 = 0.9962$; Supplementary Material, Figure S3) and expressed in ascorbic acid equivalents (Table 6). The ethyl acetate fraction (EAF) and dichloromethane fraction (DCMF) exhibited the highest ferric reducing capacities (148.13 µg AAE/mg and 91.13 µg AAE/mg, respectively), displaying values comparable to the synthetic standard BHT (208.72 µg AAE/mg). The ethanol extract (EE) and hydroethanol fraction (HEF) displayed lower reducing capacities (42.52 µg AAE/mg and 25.64 µg AAE/mg, respectively).

Table 6. Ferric reducing antioxidant power (FRAP) of the ethanol extract and fractions of *Bidens subalternans* leaves expressed in Ascorbic Acid Equivalents.

Sample	FRAP Reducing Capacity (µg AAE/mg Extract)
EE	42.52 ± 1.43 ^a
DCMF	91.13 ± 3.38 ^a
EAF	148.13 ± 0.57 ^a
HEF	25.64 ± 0.15 ^a
BHT (Standard)	208.72 ± 4.65

EE: ethanol extract, DCMF: dichloromethane fraction, EAF: ethyl acetate fraction, HEF: hydroethanol fraction, BHT: 2,6-di-*tert*-butyl-4-methylphenol. µg AAE/mg: micrograms of ascorbic acid equivalents per milligram of dry extract or fraction. Results are expressed as mean ± standard deviation ($n = 3$ technical replicate wells). ^a $p < 0.05$ compared to BHT.

3.6. *Artemia salina* Lethality Assay

To evaluate the toxicity of *B. subalternans* leaf samples, the *A. salina* lethality test was employed. Neither the ethanol extract nor the tested fractions induced mortality in the nauplii at any of the concentrations evaluated (125, 250, 500, and 1000 µg/mL), as shown in Figure 2. Based on the criteria established by [22], which defines the lethality limits for bioactive substances, the extract and all fractions of *B. subalternans* were not considered toxic under the experimental conditions analyzed.

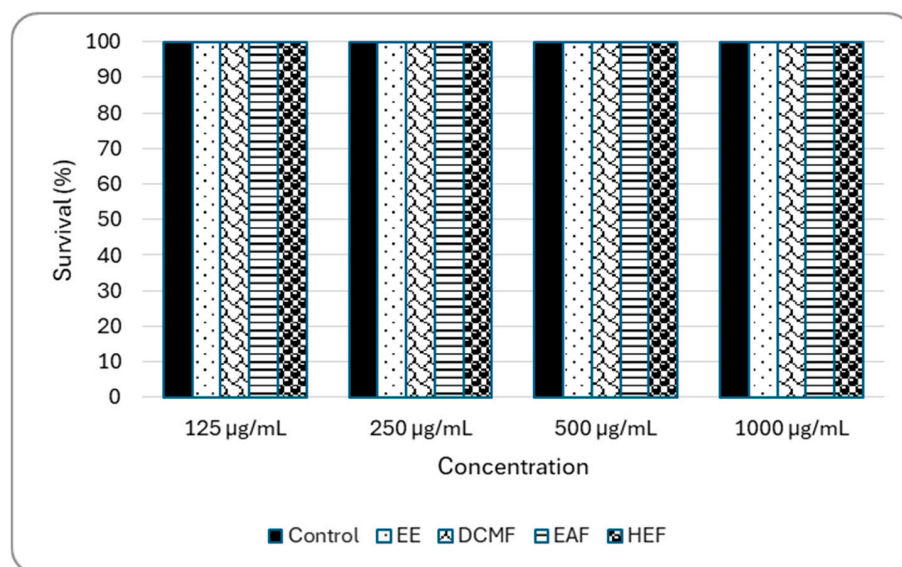


Figure 2. *Artemia salina* nauplii survival percentage when treated with the extract and fractions of *Bidens subalternans* leaves. EE: ethanol extract, DCMF: dichloromethane fraction, EAF: ethyl acetate fraction, HEF: hydroethanol fraction. Results are means ± standard error ($n = 3$ replicate tubes with 10 nauplii each, for three independent experiments performed on different days).

4. Discussion

4.1. Phytochemical Screening

Valdés et al. [23] detected the presence of flavonoids, phenols, tannins, and terpenoids in the ethanol extract of *B. odorata*. Using Soxhlet extraction, Ruiz-Reyes et al. [24] reported the presence of alkaloids, reducing sugars, phenols, flavonoids, tannins, and saponins in the ethanol extract of *B. pilosa* leaves. Phiri et al. [25] confirmed the presence of alkaloids, phenols, tannins, flavonoids, phytosteroids, terpenoids, glycosides, and saponins in the ethanol extract of *B. pilosa* leaves. Furthermore, Souza et al. [26] evaluated the aqueous extract from the aerial parts of *B. pilosa* and detected flavonoids, condensed tannins, and saponins. Dávila et al. [27] verified the presence of anthocyanins, alkaloids, lactones, flavonoids, cardenolides, tannins, reducing sugars, and phenols in the ethanol extract of *B. pilosa* leaves.

Ajanaku et al. [28] performed a phytochemical analysis on the methanol extract of *B. pilosa* leaves, as well as on its dichloromethane and ethyl acetate fractions. In the methanol extract, they detected carbohydrates, tannins, flavonoids, alkaloids, quinones, glycosides, cardiac glycosides, and terpenoids. The dichloromethane fraction revealed tannins, alkaloids, cardiac glycosides, terpenoids, triterpenoids, phenols, coumarins, and steroids, while the ethyl acetate fraction contained tannins, alkaloids, cardiac glycosides, terpenoids, triterpenoids, phenols, and steroids [28].

The chemical composition of plant extracts can vary significantly among species of the same genus due to biological and methodological factors. Interspecific differences and the plant part used (leaves, roots, stems, seeds) directly influence the secondary metabolite profile of the analyzed material. Furthermore, the extraction method and conditions, as well as the nature of the solvent, are crucial determinants of the final phytochemical profile. Solvent polarity strongly influences the solubility and extraction efficiency of different compound classes, yielding extracts and fractions with distinct compositions [29,30].

4.2. Gas Chromatography–Mass Spectrometry (GC-MS) Analysis

Although specific phytochemical studies on *B. subalternans* are scarce, the putatively assigned constituents show strong agreement with the profile described for the genus *Bidens*. Stigmasterol has previously been identified in the species *B. subalternans* [7,8]. The detection of phytol, palmitic acid, ethyl linolenate, and stigmasterol is corroborated by studies on the species *B. bipinnata*, *B. biternata*, and *B. pilosa*, in which the use of GC-MS enabled the characterization of these same bioactive metabolites [31].

The major hydrocarbon in the DCMF, putatively assigned as the long-chain alkane untriacontane (C₃₁), is characteristic of the genus's cuticular waxes, which frequently exhibit a predominance of odd-chain alkanes [32]. Furthermore, the presence of loliolide and terpene derivatives underscores the chemical complexity of the genus, indicating that *B. subalternans* shares significant phytochemical markers with other widely documented *Bidens* species [30,32].

To the best of our knowledge, these compounds are reported here for the first time in *B. subalternans* leaves.

4.3. Analysis by High-Performance Liquid Chromatography Coupled with a Diode-Array Detector (HPLC-DAD)

Phytochemical studies on *B. subalternans* are scarce; however, the data obtained in this study corroborate reports on other species of the genus. Analysis of the ethyl acetate extract from the aerial parts of *B. gardneri* by liquid chromatography coupled with a diode-array detector and mass spectrometry (LC-DAD-MS) allowed the identification of eleven phenolic compounds, including chlorogenic acid derivatives and flavonoids, such as flavanones,

flavones derived from apigenin and luteolin, chalcone and a coumaric acid derivative [21]. Similar results were observed for *B. pilosa*, with phytochemical analyses revealing a profile rich in caffeic and chlorogenic acid derivatives, as well as a wide diversity of flavonoids, including flavonols, flavones, and flavanones [31,33]. This pattern reinforces the genus's tendency to synthesize phenolic compounds with results similar to those observed in the present study.

More recent studies further confirm that extracts obtained using solvents of intermediate polarity from *Bidens* species tend to concentrate a wider variety of phenolics detectable by HPLC-DAD and exhibit profiles consistent with high antioxidant activity [34], and the chromatographic complexity observed in the analyzed *B. subalternans* samples corroborates the literature data.

In general, HPLC-DAD analysis showed that the extract and fractions of *B. subalternans* have a complex chromatographic profile, characterized predominantly by the presence of phenolic compounds, with a predominance of cinnamic, caffeic, chlorogenic and coumaric acid derivatives, as well as different subclasses of flavonoids. Variations in the number of peaks across the fractions reflect the influence of solvent polarity during fractionation, indicating a differential distribution of these metabolites. The results provide a preliminary phytochemical characterization of the species, expand current knowledge of its phenolic profile, and establish a solid foundation for future studies involving the isolation, structural identification, and biological activity assessment of these compounds.

4.4. Determination of Total Phenolic Compounds and Flavonoids

The quantitative profile observed for total phenolic compounds and flavonoids is directly associated with the affinity of these substances for solvents of intermediate polarity, which favored their concentration in the EAF and DCMF during the partitioning process. In general, flavonoids constitute an important group of secondary metabolites found primarily in leaves, flowers, and roots, widely recognized for their antimicrobial, antioxidant, cytotoxic, and antitumor properties [35].

The Folin–Ciocalteu reagent is used to quantify total phenolic compounds; however, it operates via a single-electron transfer (SET) redox mechanism and reacts with any non-phenolic reducing species present in the plant matrix (such as ascorbic acid, reducing sugars, and aromatic amines)—rather than selectively binding to phenolic hydroxyl groups—which is why the results are appropriately interpreted as total reducing capacity, expressed in gallic acid equivalents [36].

Compared with the literature data, the total phenolic content obtained for *B. subalternans* is higher than those reported for other species of the genus. Regarding the overall reducing capacity evaluated via the Folin–Ciocalteu method, Compaore et al. [37] recorded lower levels for *B. engleri*, with values of approximately 10 µg GAE/mg in the hexane fraction, 80 µg GAE/mg in the dichloromethane fraction, and 150 µg GAE/mg in the ethyl acetate fraction. Low concentrations were also observed for the ethanolic extracts of *B. pilosa*, which showed values between 27.035 and 48.609 µg GAE/mg in the study by Ruiz-Reyes et al. [24] and 16.99 ± 0.02 µg GAE/mg in the research by Phiri et al. [25]. Similarly, in *B. frondosa* leaves, levels ranged from 37.25 to 82.56 µg GAE/mg across extracts and fractions [38], whereas the *B. tripartita* herb extract showed 32.70 ± 2.47 µg GAE/mg [39].

For total flavonoids, in the *B. engleri* fractions, values were approximately 30 µg QE/mg for the dichloromethane fraction and 15 µg QE/mg for the ethyl acetate fraction [36]. For the ethanolic extracts of *B. pilosa*, contents were between 12.554 and 17.665 µg QE/mg [24] and 9.01 ± 0.28 µg QE/mg [25] were reported, whereas *B. tripartita* flowers exhibited a low value of 0.649 µg QE/mg [40].

The Folin–Ciocalteu assay quantifies overall electron-donating capacity; however, the data on total phenolic compounds corroborate the qualitative analysis performed via HPLC-DAD on the ethanol extract and intermediate-polarity fractions (EAF and DCMF), which indicated the presence of phenolic acid derivatives and flavonoids. Concurrently, the quantification of total flavonoids complements and validates the findings of the initial phytochemical screening, which had already indicated the presence of this class in the DCMF, EAF, and HEF. Taken together, the results demonstrate that *B. subalternans* stands out within the *Bidens* genus due to the accumulation of high levels of these substances in its intermediate-polarity fractions. This chemical profile suggests biological potential, solidifying the relevance of *B. subalternans* as a promising source for future pharmacological and macromolecular isolation studies.

4.5. Antioxidant Activity

The DPPH and FRAP assays involve distinct chemical mechanisms of antioxidant action in each methodology. The DPPH assay relies on the antioxidant's ability to donate protons or hydrogen atoms to stabilize the purple-colored free radical, converting it into the yellow, stable DPPH-H form; efficiency is measured by the decrease in absorbance [16,41–43]. In contrast, the FRAP method evaluates antioxidant potential via an electron transfer mechanism based on the sample's ability to reduce ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}) within the TPTZ (2,4,6-tripyridyl-s-triazine) complex, resulting in a color change quantified at 595 nm [18].

The antioxidant activity observed in the intermediate-polarity fractions (EAF and DCMF) arises from the presence of phenolic compounds and flavonoids, as these non-enzymatic antioxidants act directly to scavenge reactive species through their redox properties [44,45]. Structurally, compounds such as derivatives of caffeic, cinnamic, and coumaric acids—suggested by HPLC-DAD analysis—donate electrons or hydrogen atoms to interrupt oxidative chain reactions, with the resulting radical stabilized by resonance within the aromatic ring; furthermore, these phenolic acids chelate pro-oxidant metal ions, protecting biomolecules against severe damage [45].

When compared with the literature on the genus *Bidens*, radical-scavenging capacity profiles vary considerably. In the DPPH assay, Kwiecinski et al. [46] reported EC_{50} values for *B. pilosa* L. leaves of $98.0 \pm 1.0 \mu\text{g/mL}$ (crude extract), $248.0 \pm 1.8 \mu\text{g/mL}$ (chloroform fraction), and $32.3 \pm 6.7 \mu\text{g/mL}$ (ethyl acetate fraction). Other studies on ethanolic extracts of *B. pilosa* leaves recorded EC_{50} values of $239.33 \mu\text{g/mL}$ [24] and $35.24 \mu\text{g/mL}$ [25]. For the species *B. frondosa*, EC_{50} values of approximately 25, 50, 11.96, and $238.18 \mu\text{g/mL}$ were reported for the methanolic extract, chloroform fraction, ethyl acetate fraction, and hydro-methanolic fraction, respectively [38]. Regarding *B. tripartita* herb, the ethanolic leaf extract exhibited an EC_{50} of 1.59 mg/mL [39]. Considering these data, the ethanol extract and fractions of *B. subalternans* corroborate the antioxidant activity described in the literature for the DPPH assay.

The FRAP assay evaluates electron-transfer capacity based on the reduction of Fe^{3+} –TPTZ to the intense blue Fe^{2+} –TPTZ complex at acidic pH [18,19]. By quantifying reducing capacity through an ascorbic acid standard curve ($1\text{--}15 \mu\text{g/mL}$), the intermediate-polarity fractions (EAF and DCMF) demonstrated superior electron-donating capacity compared to the ethanol extract and HEF. This activity strongly aligns with the presence of hydroxycinnamic derivatives and flavanones putatively assigned in EAF and DCMF by HPLC-DAD, as phenolic hydroxyl groups directly contribute to single-electron transfer mechanisms [19,43]. Furthermore, adopting an ascorbic acid calibration curve addresses methodological variations in FRAP protocols reported in the literature [19,47], providing a standardized, mathematically robust measure of ferric reducing capacity without

reliance on arbitrary percentage calculations or extrapolated EC_{50} values. Regarding the FRAP assay, the literature indicates that the dichloromethane and ethyl acetate fractions of *B. engleri* and *B. erecta* exhibited ferric ion-reducing capacity of 29.36 ± 0.75 , 74.61 ± 0.73 , 37.02 ± 2.40 , and 126.65 ± 2.44 $\mu\text{g AAE/mg}$, respectively [37]. Nonetheless, the qualitative trend drawn from these comparisons robustly suggests that *B. subalternans* samples possess an antioxidant potential within the *Bidens* genus. Taken together, these findings highlight the unprecedented evaluation of the antioxidant capacity in vitro of specific solvent fractions of *B. subalternans*, reinforcing its potential as a promising natural source of bioactive compounds.

4.6. Lethality Test Against *Artemia salina*

The lack of toxicity observed for *B. subalternans* partially corroborates data reported in the literature for other species of the genus *Bidens*, although the lethality profile varies significantly depending on the species, the plant part, and the solvent used. Previous studies with the aerial parts of *B. pilosa* using ethanolic extract and chloroform and ethyl acetate fractions demonstrated that such samples do not exhibit toxicity toward *A. salina* [48], which was later confirmed by Cázares-Jaramillo et al. [49] in their evaluation of the methanolic extract and the chloroform and ethyl acetate fractions from the aerial parts of the same species.

In contrast, significant variations in lethality have been reported for other samples within the genus: the methanolic extract of the whole-plant extract of *B. chinensis* resulted in mortality rates of 63.3%, 43.33%, and 26.66% at concentrations of 1000, 100, and 10 $\mu\text{g/mL}$, respectively [50]. Zahara et al. [31], while evaluating three *Bidens* species (*B. pilosa*, *B. bipinnata*, and *B. biternata*), reported that root extracts were the most toxic to *A. salina*. Ethanolic root extracts caused mortality rates exceeding 60% for the first two species and 86.67% for the latter. The same study also demonstrated that a wide range of extracts (hexane, acetone, ethanol, and aqueous) obtained from leaves, stems, flowers, and achenes of these three species resulted in mortality rates above 60% in the crustacean [31].

This heterogeneous behavior highlights the importance of the present study, since a search of specialized scientific literature revealed no previous reports describing the toxicity of the ethanolic extract and fractions of *B. subalternans* towards *A. salina*. Therefore, these findings represent the first formal report of this biological property for the species.

4.7. Limitations of This Study

Some limitations of the present study should be acknowledged. System suitability parameters and carryover checks were not performed for the GC-MS analysis. Peaks detected during the first 10 min of the run were excluded because the ethyl acetate solvent contained certain contaminants. *Bis*(2-ethylhexyl) phthalate (DEHP) was confirmed as a trace plasticizer contaminant present in the ethyl acetate solvent used for sample preparation; therefore, the peak associated with DEHP in the EE and DCMF samples ($RT \approx 33.401$ min) was excluded from compound analysis (Supplementary Material Figure S1).

5. Conclusions

The results demonstrated that *B. subalternans* DC. leaves possess a diverse phytochemical composition, including steroids, saponins, alkaloids, coumarins, flavonoids, and tannins. GC-MS and HPLC-DAD analyses suggested the presence of lipophilic constituents, phenolic acid derivatives, and flavonoids. The dichloromethane and ethyl acetate fractions exhibited the highest levels of phenolic compounds, with the ethyl acetate fraction standing out for its superior flavonoid content. All samples displayed antioxidant activity in vitro; the ethyl acetate and dichloromethane fractions were the most promising. Assays using *A. salina* showed no acute toxicity at the tested concentrations, indicating a preliminary

safety profile. These findings expand current knowledge regarding *B. subalternans*, providing evidence of its chemical profile via GC-MS and HPLC-DAD, as well as its potential antioxidant in vitro as a source of natural antioxidants, while laying the groundwork for future research into its bioactive components and potential applications in biotechnology and the food, cosmetic, and pharmaceutical industries.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/nutraceuticals6040069/s1>, Figure S1. Total ion chromatogram (TIC) by GC-MS, (A) solvent (ethyl acetate); (B) ethanol extract (EE) of *Bidens subalternans* leaves; (C) dichloromethane fraction (DCMF) of *Bidens subalternans* leaves; Table S1: Chromatographic and Spectral Parameters of Authentic Reference Standards; Table S2. Phytochemical Profiling of the Ethanol Extract (EE) of *Bidens subalternans* Leaves; Table S3. Phytochemical Profiling of the Dichloromethane Fraction (DCMF) of *Bidens subalternans* Leaves; Table S4. Phytochemical Profiling of the Ethyl Acetate Fraction (EAF) of *Bidens subalternans* Leaves; Table S5. Phytochemical Profiling of the Hydroethanol Fraction (HEF) of *Bidens subalternans* Leaves; Figure S2. Fitted curves for ethanol extract and fractions of *B. subalternans*; Table S6. % DPPH inhibition ($n = 3$ technical replicate wells); Table S7. Non-linear regression fitting parameters and summary statistics for DPPH radical scavenging assay using the log(inhibitor) vs. response-variable slope (four parameters) model in GraphPad Prism 7.0; Figure S3. Ascorbic acid calibration curve—absorbance vs. concentration; Table S8. Absorbances for the FRAP blank; Table S9. Ascorbic acid concentration and absorbances used to construct the calibration curve, Table S10. Absorbances measured for the samples for the calculation of FRAP ($\mu\text{g AAE/mg}$).

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Abbreviations

AAE	Ascorbic acid equivalents
<i>B.</i>	<i>Bidens</i>
BHT	Butylated hydroxytoluene or 2,6-di- <i>tert</i> -butyl-4-methylphenol
DCMF	Dichloromethane fraction
DPPH	1,1-diphenyl-2-picrylhydrazyl or 2,2-diphenyl-1-picrylhydrazyl
EAF	Ethyl acetate fraction
EE	Ethanol extract
FRAP	Ferric reducing antioxidant power
GAE	Gallic acid equivalents
GC-MS	Gas chromatography coupled to mass spectrometry
HEF	Hydroethanol fraction
HPLC-DAD	High-performance liquid chromatography coupled to a diode-array detector
QE	Quercetin equivalents
UV	Ultraviolet

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