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Metabolomic Profiling, Antioxidant and Antimicrobial Activity of *Bidens pilosa*

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Abstract: *Bidens pilosa* L. (fam. Asteraceae) is an annual herb used globally in phytotherapy and each plant material or the whole plant have been declared to be effective. Therefore, the aim of the present study was to conduct metabolomic profiling of different plant materials, including the quali-quantitative composition of phenolic compounds. The intrinsic scavenging/reducing properties and antimicrobial effects of the extracts were assayed against numerous bacterial, Candida and dermatophytes species, whereas docking runs were conducted for tentatively unravelling the mechanism of action underlying antimicrobial effects. Oligosaccharide, disaccharide and fatty acids were present at higher concentrations in root rather than in the other plant parts. Monoglycerides were more abundant in stem than in the other plant parts, whereas peptide and diterpenoid were prominent in leaf and root, respectively. By contrast, amino acids showed very different distribution patterns in the four plant parts. Regarding the phenolic composition, appreciable levels of caftaric acid were found in most of the analyzed methanol extracts, that were also particularly efficacious as antiradical and anti-mycotic agents against *C. albicans* and dermatophytes. The docking experiments also showed a micromolar affinity of caftaric acid towards the lanosterol 14α-demethylase, deeply involved in fungal metabolism. In conclusion, the present study corroborates the *B. pilosa* as a phytotherapy remedy against infectious disease.

Keywords: *Bidens pilosa*; metabolomic profile; anti-mycotic effects; phenolic compounds; caftaric acid; bioinformatics

Citation: Angelini, P.; Matei, F.; Flores, G.A.; Pellegrino, R.M.; Vuguziga, L.; Venanzoni, R.; Tirillini, B.; Emiliani, C.; Orlando, G.; Menghini, L.; et al. Metabolomic Profiling, Antioxidant and Antimicrobial Activity of *Bidens pilosa*. *Processes* **2021**, *9*, 903. https://doi.org/10.3390/pr9060903

Academic Editors: Adriana Trifan, Luigi Menghini and Claudio Ferrante

Received: 27 April 2021
Accepted: 17 May 2021
Published: 21 May 2021

1. Introduction

Plants are an important source of pharmacologically active secondary compounds, that can be used in medicine to maintain and improve human health and also to treat specific conditions or illnesses [1,2]. Among these compounds, phenolics are very common and easy to find in plants, and they have demonstrated beneficial advantages in terms of antioxidant and antimicrobial activities. In particular, antioxidant metabolites can be used against different oxidative stress-induced diseases [3], while the antimicrobial properties of such compounds can rehabilitate the clinical application of older antibiotics by improving their efficacy and, therefore, by preventing the development of resistance [4]. *Bidens pilosa* L. (fam. Asteraceae) is an annual herb native to South America that is spread worldwide, especially in tropical and subtropical regions [5]. *B. pilosa* L. is used globally in phytotherapy

and each plant material or the whole plant have been declared to be effective in treating many illnesses such as malaria, flu, cancers, headache, inflammation, wounds, angina, metabolic syndrome, immunological disorders, and digestive and infectious diseases [6]. The plant has been widely used in Taiwan as a traditional medicine and as a major ingredient of herbal tea, which is believed to prevent inflammation and cancer [7]. Phytochemical and pharmacological analyses of *B. pilosa* employing roots [8], leaves [9], or the whole aerial parts [10,11] have also been published. In this regard, the studies indicated the presence of phenolic compounds that could explain, albeit partially, antioxidant and antimicrobial activities [12–20]. In a study by Abajo et al. (2004) [15], the antioxidant activity of an aqueous infusion of *B. pilosa* has been investigated by studying its protective effect on the hemolysis induced by an initiator of radicals such as 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH). The amount of *B. pilosa* infusion that halved the hemolysis induced by AAPH was 6 µL ($_{IC50}$: 1.19 mg mL^{-1} dry weight). Chiang and colleagues (2004) [21] evaluated the free radical scavenging activity of the crude extract, and fractions of *B. pilosa* using 1,1-diphenyl-2-picrylhydrazyl (DPPH) and hypoxanthine/xanthine oxidase assays. They found that the *B. pilosa* crude extract and the ethyl acetate, butanol, and water fractions had free radical scavenging activity and that the ethyl acetate and butanol fractions were more active than the water fraction and crude extract [21]. A complementary study by Muchuweti et al. (2007) [22] determined antioxidant activity of *B. pilosa* methanol extract. It also showed 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity [22]. Ashafa et al. (2009) [18] reported that the methanol and acetone extract of *B. pilosa* roots displayed antibacterial activities against *Bacillus cereus, Escherichia coli, Klebsilla pneumonia, Micrococcus kristinae, Pseudomonas aeruginosa, Staphylococcus aureus, S. epidermidis, Serratia marcescens, Shigelea flexneri,* and *Streptococcus faecalis.* Deba et al. (2007) [23] first evaluated the antifungal effect of the hot water extracts of the *B. pilosa* roots, stems, and leaves against *Corticium rolfsii*, *Fusarium solani*, and *Fusarium oxysporum. C. rolfsii* was particularly sensitive to the treatment with *B. pilosa* as its growth was reduced at almost all the tested concentrations, followed by *F. oxysporum* and *F. solani*. However, the fungicidal activities of the stems and roots were greater than the leaves [23]. The composition analysis of the extracts revealed the presence of different phenolic compounds that could be at the basis of the fungicidal effects. Essential oils appeared to have better fungicidal activity than water extracts [17]. Acetone, methanol, and water extracts of the *B. pilosa* roots also showed antifungal activities against *Aspergillus niger*, *A. flavus*, and *Penicillium notatum* using the agar dilution method [18]. The methanol extract of the *B. pilosa* roots at 10 mg/mL was also effective against *Candida albicans* [18]. Shandukani et al. (2018) [20] investigated the *B. pilosa* antibacterial activity against waterborne diarrhoeagenic bacteria. All the bacterial species tested were sensitive to the effect of different extracts of *B. pilosa*. Moreover, Nthulane et al. (2020) [24] determined the antimicrobial activities of plant extracts against the bacteria causing common sexually transmitted infections. The results showed that dichloromethane extract of *B. pilosa* exhibited good activities against *Neisseria gonorrhoeae* and *Gardnerella vaginalis*, whereas ethyl acetate, dichloromethane and methanol extracts of *B. pilosa* exhibited good activities against *C. albicans.* Some classes of compounds such as flavonoids, aliphatics, terpenoids, phenylpropanoids, aromatics and porphyrins were isolated from *B. pilosa* and related to the bio-pharmacological properties of this plant [5,19]. Also, saponins and steroids were identified in the phytocomplex of *B. pilosa.* These compounds were suggested to be involved in the antioxidant [21], antibacterial and antimicrobial activities [5,20]. In recent years, metabolomics, which is defined as the monitoring of metabolite concentration in a cell, tissue, organ or in the whole plant, has become prominent as a part of systems biology. Nonetheless, differentiation between different plant materials of *B. pilosa* based on their metabolomic profiling has not been carried out yet. For a suitable comparative antioxidant and antimicrobial activity of different plant materials of this precious plant, we proposed the in vitro antimicrobial activity study of the roots, leaves, stems and the whole plant extracts of *B. pilosa* by using various solvents. This study evaluates such activity towards some selected Gram-positive, Gram-negative bacteria and fungal species. Further-

more, a mass spectrometry ultra-performance liquid chromatography mass spectrometry (UHPLC)–QTOF method, coupled with different multivariate data analyses such as principle component analysis (PCA), was applied to *B. pilosa* metabolome aiming at investigating the metabolomic variation among the different plant materials of the same species and at evaluating this species as a potential antioxidant and antimicrobial. A liquid chromatography coupled to diode array and mass spectrometer (HPLC–DAD-MS) analysis was also conducted for measuring the levels of phenolic compounds in the extracts, whereas the intrinsic scavenging/reducing properties were determined via colorimetric methods. Finally, a docking approach was carried out for unravelling the putative mechanisms underlying the observed antimicrobial effects.

2. Materials and Methods

2.1. Chemical and Reagents

Mueller–Hinton broth (MHB), Rose Bengal Chloramphenicol Agar (RBCA), Malt Extract Agar (MEA), Sabouraud Dextrose Agar (SDA), RPMI (Roswell Park Memorial Institute) 1640 medium, and purity grade organic solvents (n-hexane, Ethyl acetate, Methanol, and Dimethyl Sulfoxide), were purchased from Sigma (Sigma-Aldrich, Milan, Italy).

2.2. Plants Material

The mature seeds of *B. pilosa* L. were collected in July 2018, at an altitude of 1500–1800 m in Yabaramba zone, Kicukiro district, Rwanda. The seeds were cleaned and sterilized with ethylic alcohol solution 70% for 1 min and washed thoroughly 3 times with sterile distilled water. The sterilised seeds were planted in a garden pot containing the sterilised garden soil with NPK 12:11:18:2. The plants full grown were separated into roots, leaves and stems. The plant materials were separated into 4 samples that are leaves, roots, stems and whole plants. Afterwards, they were dried in an autoclave at 40 °C. The dried plant materials separated in leaves, roots, stems and whole plants were finely grounded and macerated in methanol for 7 days at 20 °C (1:10 w/v). The resulting extracts were then filtered through Whatman GF/C filters (Sigma, Germany), and the solvent evaporated under reduced pressure (40 °C, 218 mbar) using a rotary vacuum evaporator (Rotavapor R-100, Büchi, Switzerland). The residue was kept at −20 °C until further use.

2.3. Untargeted LC-MS/MS-Based Metabolomics

Untargeted metabolomics was carried out by using ultra-performance liquid chromatography mass spectrometry (UHPLC)–QTOF employing a 1260 ultra-high-performance liquid chromatograph and a G6530A QTOF mass spectrometer (Agilent Technologies, Santa Clara, CA, USA). An Agilen JetStream ionization source in positive and negative polarity was also used. LC separation was performed using an Ascentis Express Peptide ES-C18 Supelco column (2.1 × 750 mm, 2.7 µm) with a gradient elution of mobile phase A (water + 0.1% Formic Acid) and mobile phase B (Acetonitrile with 0.1% Formic Acid). LC gradient consisted of holding solvent (A/B: 98/2) for 2 min, then linearly converting to solvent (A/B: 40/60) for 5 min, linearly converting to solvent (A/B: 20/80) for 1 min and holding for 2 min, then linearly converting to solvent (A/B: 98/2) for 0.5 min, and holding for 3 min for re-equilibration. The flow rate and column temperature were set to 0.45 mL/min and 45 °C, respectively. The mass spectrometer was operated in iterative Data Dependent Acquisition mode (50–1000 m/z), with a nominal resolution of 40,000 FWHM (full width at half maximum) and in the extended dynamic range mode using 5 precursors per cycle with collision energies of 30 eV. Peak picking and alignment were performed by MS-DIAL (ver. 4.38) with the following parameters: accurate mass tolerance (MS1) tolerance, 0.01 Da; MS2 tolerance, 0.025 Da; maximum charge number, two; smoothing method, linear weighted moving average; smoothing level, 3; minimum peak width, five scans; minimum peak height, 1000; mass slice width, 0.1 Da; sigma window value, 0.5; MS2Dec amplitude cut off, 0; exclude after precursor, true; keep isotope until, 0.5 Da; relative abundance cut off, 0; top candidate report, true; retention time tolerance for alignment,

0.1 min; MS1 tolerance for alignment, 0.015 Da; peak count filter, 0; adduct ion setting, [M + H]+, [M + NH4]+, [M + Na]+, in positive ion mode and [M-H]−, [M + CH3COO]− in negative ion mode. Compound annotation was made comparing the experimental MS/MS spectra to those available in the NIST2020 Tandem Mass Spectral Library. An m/z window of 0.005 Da and a relative intensity threshold of 0.5 were selected as input parameters. Only the compounds with an identification score cut off >80% were retained for further analysis. Principle component analysis (PCA) and Heatmap were performed with MetoboAnalyst 5.0 for either annotated metabolites or ontology grouped metabolites. For PCA and Heatmap, samples were normalized by median, followed by pareto scaling.

2.4. Determination of the Antioxidant Activity

The antiradical activity was determined by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging method. Each sample was mixed with 900 µL of 100 mM Tris-HCl buffer, pH 7.4, and then added to 1 mL of 0.5 mM DPPH in methanol (250 µM in the reaction mixture). The control sample was prepared using methanol. Trolox was employed as a reference antioxidant substance. Absorbances of the mixtures were measured at 517 nm. The activity was calculated as IC_{50} Trolox equivalent. All tests and analyses were run in triplicate and averaged. For ABTS (2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonate) radical scavenging) assay, the procedure followed the method of Arnao et al. with some modifications. The stock solutions included 7 mM ABTS solution and 2.45 mM potassium persulfate solution. The working solution was then prepared by mixing the two stock solutions in equal quantities and allowing them to react for 14 h at room temperature in the dark. The solution was then diluted by mixing 1 mL ABTS solution with 60 mL methanol to obtain an absorbance of 0.706 ± 0.01 units at 734 nm. Fresh ABTS solution was prepared for each assay. Plant extracts (1 mL) were allowed to react with 1 mL of the ABTS solution and the absorbance was taken at 734 nm after 7 min using a spectrophotometer. The ABTS scavenging capacity of the extract was compared with that of Trolox and the activity was calculated as IC_{50} Trolox equivalent. All determinations were performed in triplicate. The antioxidant capacity of methanolic solutions was estimated according to the procedure described by Benzie and Strain with some modifications. Briefly, 900 µL of FRAP (ferric reducing antioxidant power) reagent, prepared freshly and warmed at 37 °C, was mixed with 90 µL of distilled water and 30 µL of test sample. The final dilution of the test sample in the reaction mixture was 1/34. The FRAP reagent contained 2.5 mL of a 10 mmol/L TPTZ solution in 40 mmol/L HCl plus 2.5 mL of 20 mmol/L $FeCl_3$,$6H_2O$ and 25 mL of 0.3 mol/L acetate buffer, pH 3.6. The absorbance was measured at 593 nm against the blank after 4 min. Methanolic solutions of known Fe(II) concentrations in the range of 100–2000 µmol/L ($FeSO_4$,$7H_2O$) were used for calibration. FRAP value was calculated and expressed as mM Fe^{2+} equivalent (FE) per 100 g sample using the calibration curve of Fe^{2+}. All determinations were performed in triplicate. In this assay, antioxidant capacity was determined by measuring the inhibition of the volatile organic compounds and the conjugated diene hydroperoxides arising from linoleic acid oxidation. A stock solution of β-carotene/linoleic acid mixture was prepared as follows: 0.5 mg β-carotene (0.9 mM) was dissolved in 1 mL of chloroform, then 25 µL linoleic acid and 200 mg Tween 40 was added. Then, 100 mL distilled water saturated with oxygen (30 min, 100 mL/min) was added with vigorous shaking; 2.5 mL of this reaction mixture was dispensed into test tubes and 100 µL portions of the methanol extracts were added; the emulsion system was incubated for up to 24 h at room temperature under agitation. The same procedure was repeated with the synthetic antioxidant butylated hydroxytoluene (BHT) as positive control, and a blank. After this incubation period, absorbances of the mixtures were measured at 490 nm. The activity was calculated as % Antioxidant Activity (AA) using the following equation: $\%AA = 100 \times [1 - (As0 - Ast)/(Ac0 - Act)]$. As0 is the absorbance of sample at 0 min, Ast is the absorbance of sample at 4 h, Ac0 is the absorbance of control sample at 0 min, and Act is the absorbance of control sample at 4 h. All tests were run in triplicate and averaged.

2.5. HPLC–DAD-MS Determination of Phenolic Compounds

B. pilosa methanol extracts were analyzed for phenol quantitative determination using a reversed-phase high performance liquid chromatography coupled to diode array and mass spectrometer (HPLC–DAD-MS) in gradient elution mode. The separation was conducted within 30 min of the chromatographic run, starting from the following separation conditions: 0.23% formic acid, 93% water, 7% methanol, as previously described [25]. The separation was performed on InfinityLab Poroshell 120 reverse phase column (C_{18}, 150 × 4.6 mm i.d., 2.7 µm) (Agilent Santa Clara, CA, USA). Column temperature was set at 30 °C. Quantitative determination of phenolic compounds was performed via DAD detector. The extract was also qualitatively analyzed with an MS detector in negative ion mode, with the sole exception of rutin that was analyzed in positive ion mode. MS signal identification was realized through comparison with standard solutions and MS spectra present in the MassBank Europe database. Quantification was done through 7-point calibration curves, with linearity coefficients (R^2) > 0.999, in the concentration range 2–140 µg/mL. The limits of detection were lower than 1 µg/mL for all assayed analytes. The area under the curve from HPLC chromatograms was used to quantify the analyte concentrations in the extract.

2.6. Antimicrobial Tests

In vitro antimicrobial activity of n-hexan, ethyl acetate and methanol extracts from *B. pilosa* were assessed against: eight bacterial strains (CLSI M07-A9), namely *E. coli* (ATCC 10536), *E. coli* PeruMycA2, *E. coli* PeruMycA3, *B. cereus* (ATCC 12826), *P. aeruginosa* (ATCC 15442), *B. subtilis*, *S. typhi* (clinical isolate), and *S. aureus* (ATCC 6538); eight dermatophytes such as *T. interdigitale* CCF 4823, *T. tonsurans* CCF 4834, *T. rubrum CCF* 4879, *T. rubrum CCF* 4933, *T. rubrum CCF* 4879, *T. erinacei* CCF 5930, *A. crocatum* CCF 5300, *A. quadrifidum* CCF 5792, *Nannizzia gypsea* (*A. gypseum*) CCF 1229; and four yeasts, namely *C. tropicalis* (YEPGA 6184), *C. albicans* (YEPGA 6379), *C. parapsilosis* (YEPG 6551) and *C. albicans* (YEPG 6138). The MICs of the plant extracts were determined in sterile 48-well microplates using the broth microdilution method of the Clinical and Laboratory Standards Institute, M07-A10 (CLSI 2015) [26]. MICs have been determined using concentrations of the dry extracts in the range 1–0.031 mg mL^{-1}, derived from serial two-fold dilutions in Mueller–Hinton Broth (MHB). For the preparation of bacterial suspensions (inocula), three to five colonies of the bacterial strains used for the test were picked from 24 h cultures on tryptic soy agar plates (TSA) and pre-grown overnight in Mueller–Hinton broth (MHB) to reach a cell density of approximately 1–2 × 10^8 CFU mL^{-1} (analogous to the 0.5 McFarland standard). Hence, bacterial suspensions were diluted in fresh MHB and added to the MIC dilution series to reach 5 × 10^5 CFU mL^{-1} in each tube. This was confirmed by plating serial dilutions of the inoculum suspensions on Mueller–Hinton Agar (MHA). The set-up included bacterial growth controls in wells containing 10 µL of the test inoculum and negative controls without bacterial inoculum. MIC end-points were determined after 18–20 h incubation in ambient air at 35 °C [27]. MIC end-points were defined as the lowest concentration of either *B. pilosa* extracts or ciprofloxacin that totally inhibited bacterial growth [27]. Each test was done in triplicate. Geometric means and MIC ranges were calculated. Susceptibility testing against yeasts and filamentous fungi was performed according to the CLSI M38 (CLSI 2018) and M38-Ed3 (CLSI 2017) protocols [27–29]. RPMI (Roswell Park Memorial Institute) 1640 medium (Sigma) with L-glutamine and without sodium bicarbonate, supplemented with 2% glucose (*w*/*v*), buffered with 0.165 mol L^{-1} morpholinepropanesulphonic acid (MOPS), pH 7.0, was used throughout the study.

The inoculum suspensions were prepared from 7-day-old cultures grown on Sabouraud Dextrose Agar (SDA; Difco) at 25 °C and adjusted spectrophotometrically to optical densities that ranged from 0.09 to 0.11 (Mac Farland standard). Filamentous fungi (microconidia) and yeast inoculum suspensions were diluted to a ratio of 1:50 in RPMI 1640 to obtain twice an inoculum size ranging from 0.2 to 0.4 × $10^{4–5}$ CFU mL^{-1}. This was further confirmed by plating serial dilutions of the inoculum suspensions on SDA. MIC end-points (µg mL^{-1})

were determined after 24 h (for yeasts) and 72 h (for dermatophytes) of incubation in ambient air at 30 °C (CLSI 2017, CLSI 2018). For the plant extracts, the MIC end-points were defined as the lowest concentration that showed total growth inhibition [30]. The MIC end-points for fluconazole were defined as the lowest concentration that inhibited 50% of the growth when compared with the growth control [28]. Geometric means and MIC ranges were determined from the three biological replicates to allow comparisons between the activities of plant extracts.

2.7. Bioinformatics

Docking calculations were conducted through the Autodock Vina of PyRx 0.8 software, as recently described [31]. Crystal structures of target protein were derived from the Protein Data Bank (PDB) with PDB ID as follows: 5TZ1 (lanosterol 14α-demethylase). Discovery studio 2020 visualizer was employed to investigate the protein–ligand nonbonding interactions.

3. Results

3.1. Untargeted LC-MS/MS-Based Metabolomics

Using normalized peak intensity and principal component analysis (PCA) clustering of all annotated compounds, clear differences were observed in the metabolite profiles of the four plant parts (Figures 1 and 2; Tables 1 and 2). Although PCA analysis indicated that the different plant materials display similar metabolite profiles, oligosaccharides, disaccharides and fatty acids were found to be much more abundant in root than in the other plant parts. Monoglyceride fraction was particularly present in stem rather than in the other plant parts. By contrast, peptides and diterpenoids were found at higher levels in leaf and root, respectively, whereas amino acids showed very different distribution patterns in the four plant parts. In future experiments, to improve understanding of metabolic pathways and considering the complexity of compounds measured and their various physical and chemical properties, an internal standard for each ontology group could be used to address the differences between the extraction and ionization processes.

Table 1. *B. pilosa* metabolites identified by untargeted HPLC-MS analysis.

Sample	S-r1	S-r2	S-r3	R-r1	R-r2	R-r3	L-r1	L-r2	L-r3	SRL-r1	SRL-r2	SRL-r3
Label	Stem	Stem	Stem	Root	Root	Root	Leaf	Leaf	Leaf	Whole Plant	Whole Plant	Whole Plant
(10E,15Z)-9,12,13-Trihydroxyoctadeca-10,15-dienoic acid	102,173	96,783	102,660	107,873	71,433	99,821	196,627	188,188	189,856	321,988	331,819	318,475
(5.alpha.)-Androstane-3,11,17-trione	34,194	36,441	44,320	0	8046	21,702	213,508	389,303	663,509	242,485	211,420	272,362
(9Z,12Z)-15-Hydroxyoctadeca-9,12-dienoic acid	1065,623	105,5848	998,072	1,701,017	1,663,171	1,620,107	511,408	411,803	781,119	772,966	691,404	827,335
1-Amino-1-cyclobutanecarboxylic acid	2669,482	2,636,512	2,660,328	143,652	137,926	133,015	5,333,338	5,134,590	5,057,910	1,530,695	1,489,768	1,507,354
1-Kestose	38,408	36,278	38,266	405,333	395,093	385,401	2708	2676	3990	95,238	96,636	93,696
1-Monolinolenin	117,654	76,238	115,568	9897	37,743	42,994	359,371	313,651	367,023	181,659	181,033	124,774
1-Monolinoleoyl-rac-glycerol	272,465	212,759	229,670	20,577	76,455	187,741	91,642	76,617	145,129	62,188	69,493	58,052
1-Palmitoylglycerol	1181,775	1,196,756	1,197,374	793,127	958,065	1,029,851	938,824	1,007,960	980,759	882,266	887,517	894,507
1-Propanone,1-[4-(5′-chloro-3,5-dimethyl [2,4′-bipyridin]-2′-yl)-1-piperazinyl]-3-(methylsulfonyl)-	27,306	25,892	27,243	18,541	9878	18,272	113,474	115,616	117,522	131,924	121,542	121,718
1-Stearoyl-2-hydroxy-sn-glycero-3-phosphate	97,333	93,382	83,501	104,747	108,420	105,850	98,269	97,664	105,060	99,102	95,465	98,623
1,4-Dibutylbenzene-1,4-dicarboxylate	416,022	371,401	360,682	434,349	440,723	423,912	375,740	326,158	374,673	416,988	433,662	403,848
13-Keto-9Z,11E-octadecadienoic acid	849,783	806,623	815,040	1,095,797	1,059,482	1,052,804	290,096	265,444	273,350	423,990	445,814	390,688
13S-Hydroxy-9Z,11E,15Z-octadecatrienoic acid	552,453	537,008	496,079	325,373	308,474	310,760	1,425,663	1,557,364	1,508,194	933,262	825,946	908,150
15-Ketofluprostenol isopropyl ester	84,501	89,978	82,093	69,068	65,897	63,703	94,096	109,892	101,781	98,409	105,042	104,047

Table 1. *Cont.*

Sample	S-r1	S-r2	S-r3	R-r1	R-r2	R-r3	L-r1	L-r2	L-r3	SRL-r1	SRL-r2	SRL-r3
15-Oxo-11Z,13E-eicosadienoic acid	25,400	30,232	15,037	65,219	376,202	368,532	37,077	23,581	35,402	40,086	30,585	18,520
2-[5-[(3Z,6Z)-2,12-Dihydroxydodeca-3,6-dienyl]oxolan-2-yl]acetic acid	68,976	68,620	56,467	81,527	49,314	45,317	150,965	134,456	132,756	97,041	95,508	89,950
2-Amino-2-methylpentanoic acid	1,520,393	1,553,978	1,496,055	827,158	798,054	810,260	2,691,101	2,655,615	2,582,067	1,721,792	1,718,619	1,695,509
2-Chlorobenzhydrol	47,514	57,401	55,174	232,441	90,092	86,206	21,397	22,254	21,276	36,777	33,981	37,482
2-Palmitoyl-rac-glycerol	987,286	1,009,506	985,447	728,865	862,561	876,106	834,020	857,752	840,223	732,102	749,222	776,668
2,3-Dihydroxypropyl octadecanoate	1,133,703	1,196,710	1,244,961	1,030,349	1,164,072	835,097	1,154,587	868,611	1,207,623	843,702	579,951	779,486
3-[2-(1,3-Benzodioxol-5-yl)-2-oxoethyl]-5-bromo-3-hydroxy-1,3-dihydro-2H-indol-2-one	227,015	215,612	203,206	252,015	239,255	225,506	221,613	209,372	200,299	250,098	254,710	238,036
3-Cyclopentene-1-octanoic acid, 2-(3-hydroxy-1-penten-1-yl)-5-oxo-	100,681	107,678	93,923	62,210	85,529	82,842	240,640	231,413	230,760	209,629	175,091	174,184
3-Quinolinecarboxamide, 1,4-dihydro-6-(1-methylethyl)-4-oxo-1-pentyl-N-tricyclo[3.3.1.1(3,7)]dec-1-yl-	836,070	736,462	839,506	978,107	896,483	1,084,069	843,779	273,146	671,986	886,706	548,460	734,240
4,4′-Dimethoxy-2′-hydroxychalcone	643,936	619,740	577,619	751,830	751,240	727,338	198,064	189,351	197,834	706,887	682,076	696,318
4(15)-Selinene-11,12-diol	60,495	62,398	61,881	6961	7336	5863	168,662	207,043	204,807	105,752	118,421	81,926
5.alpha.-Androstane-3,17-dione	232,166	218,264	177,346	1,647,262	1,626,859	1,602,939	271,209	250,080	75,003	132,367	126,227	105,284
8-(3-Octyl-2-oxiranyl)octanoic acid	343,285	201,933	158,994	1,477,105	1,379,746	1,304,038	155,294	102,545	94,800	94,103	87,381	124,007
9-Hydroxy-9H-fluorene-9-carboxylic acid ethyl ester	121,171	126,353	124,089	272,125	268,880	279,196	817	299	740	82,251	82,078	82,875
Adenosine	124,413	120,181	125,404	56,006	59,374	61,803	171,186	172,298	175,864	93,050	87,825	92,119
Benzyldimethyltetradecylammonium cation	3,751,915	7,049,053	4,888,025	2×10^7	3.1×10^7	2×10^7	7,066,741	7,550,522	4,195,071	3,096,660	2,012,705	5,112,836
Choline cation	42,960	41,793	28,619	27,102	26,229	30,563	184,548	204,572	206,850	29,087	31,226	49,225
cis-Vaccenic acid	297,462	296,264	468,595	1,128,680	1,491,160	1,804,998	212,669	448,366	225,255	100,788	126,485	163,398
cis,cis-9,12-Octadecadien-1-ol	3,955,832	2,099,838	1,737,622	1.5×10^7	8,594,468	5,123,672	710,151	474,279	560,286	667,796	763,629	368,557
Cynarin	5467	4750	4372	124,950	118,904	129,559	31,993	31,242	31,062	39,844	36,948	40,683
Darendoside A	246,997	243,141	234,194	156,516	162,866	154,800	322,945	334,038	329,086	301,644	294,323	305,039
Diisooctyl phthalate	655,023	539,591	460,410	662,511	587,209	534,374	130,106	129,056	145,171	125,839	114,733	113,067
Gly-Gly-Val	590,655	579,052	581,744	340,701	347,372	323,871	2,281,278	2,223,467	2,070,998	917,095	867,045	896,749
L-Homoarginine	29,419	8015	33,727	35,426	56,567	50,880	321,122	273,969	265,960	115,573	142,021	120,612
L-Tryptophan	109,556	107,202	110,760	41,130	40,510	41,398	320,162	308,335	307,264	84,341	88,041	87,022
Labdanolic acid	47,859	59,733	37,730	258,351	293,240	278,953	12,472	13,866	16,874	26,643	21,161	42,621
Linoleoyl ethanolamide	151,265	152,477	146,410	116,457	116,544	123,219	32,107	46,621	33,168	77,323	94,694	68,613
Loliolide	141,912	139,791	146,221	60,562	57,027	35,675	435,314	448,573	448,272	274,405	254,800	263,034
Methyl arachidonyl fluorophosphonate	185,247	192,752	183,865	204,057	196,979	194,467	183,821	198,621	197,452	182,045	181,197	178,476
N-Benzyl-N,N-dimethyl-1-hexadecanaminium cation	3,372,469	3,200,520	1,997,818	7,575,669	1.3×10^7	8,243,737	2,263,083	1,815,563	2,871,648	375,748	582,177	882,638
N-Cyclohexyl-N′-(1-naphthyl)urea	84,932	71,905	69,777	40,831	60,361	32,076	214,537	368,038	224,414	168,117	127,194	170,491
N6-Carbamimidoyl-N2-((4E,6E,12E,14E)-3-hydroxy-2-(hydroxymethyl)-8,10,16-trimethyloctadeca-4,6,12,14-tetraenoyl)lysine	224,512	207,881	265,592	2,147,753	2,106,907	2,110,218	306,836	266,825	97,710	189,847	148,730	175,346
Oleamide	187,073	148,732	182,692	312,739	319,209	347,815	163,145	170,533	141,877	212,284	217,719	192,874
Oleoyl ethylamide	288,452	278,168	247,724	381,802	404,893	409,015	181,339	206,324	223,119	866,575	626,771	825,341
p-Coumaric acid	642	671	827	6247	7505	10,671	95,179	81,212	83,525	48,922	44,264	39,818
Pheophorbide a	882,828	636,397	802,593	315,815	317,935	237,413	2,603,775	4,186,462	3,212,948	1,264,546	1,153,771	1,085,994
Pipericine	140,872	139,382	135,285	305,766	368,281	383,514	291,684	296,772	169,931	1,184,817	1,198,274	1,130,158
Sucrose	171,039	175,897	181,013	664,452	656,404	655,640	30,340	28,754	32,234	254,888	246,343	248,963
Tetrahydrodicranenone B	62,007	30,813	63,402	14,760	12,860	18,913	224,458	221,431	222,159	79,141	109,472	75,175
Timosaponin B II	4125	33,780	20,202	4324	25,703	12,869	243,569	138,488	282,852	32,716	23,914	11,535
Tri(3-chloropropyl) phosphate	160,488	159,000	156,995	329,548	338,360	288,679	255,460	253,231	247,410	340,901	345,598	352,026

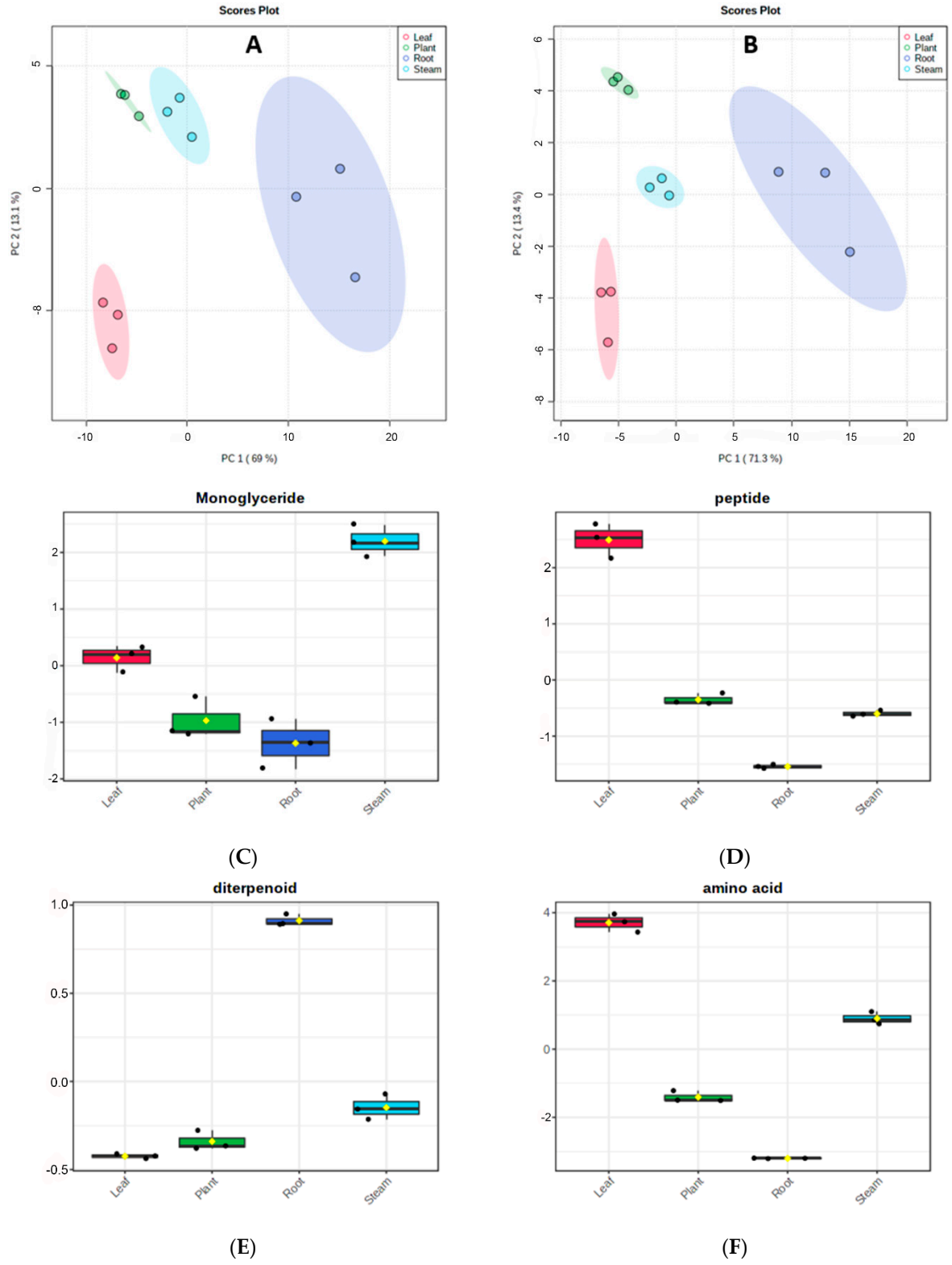

Figure 1. Principal Component Analysis (PCA) score plot of the four tested plant parts. (**A**) 67 annotated metabolites. (**B**) Ontology group of 34 annotated metabolites. Total quantitative variances of metabolites or ontology grouped metabolites were clustered to reveal the difference and relative similarities of different plant materials. 95% confidence regions (Hotelling's T2 eclipse) are displayed for each class. Monoglyceride fraction was particularly present in stem (**C**), rather than in the other plant parts. By contrast, peptides and diterpenoids were found at higher levels in leaf (**D**) and root (**E**), respectively, whereas amino acids were particularly present in the leaf (**F**).

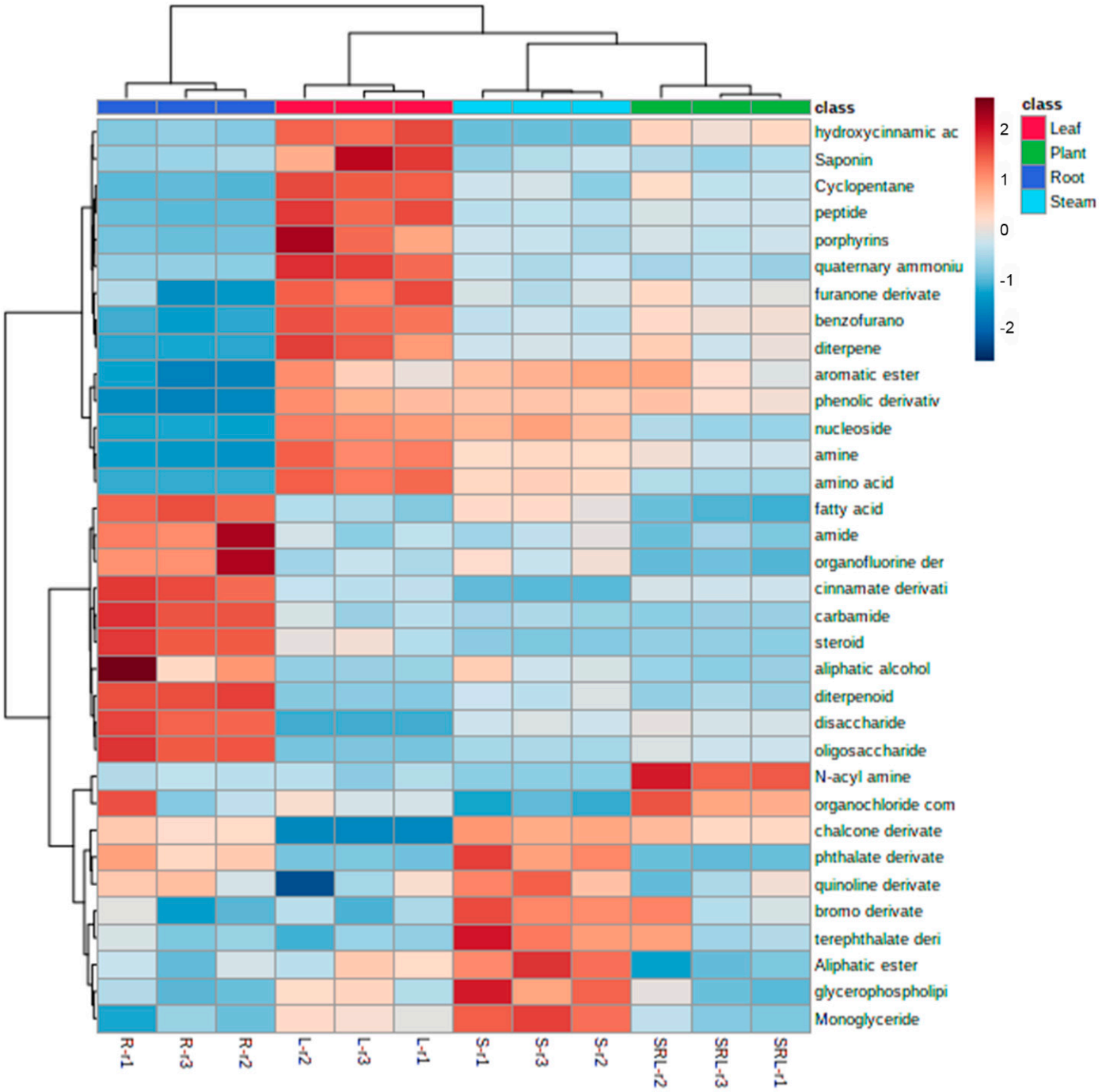

Figure 2. *Cont.*

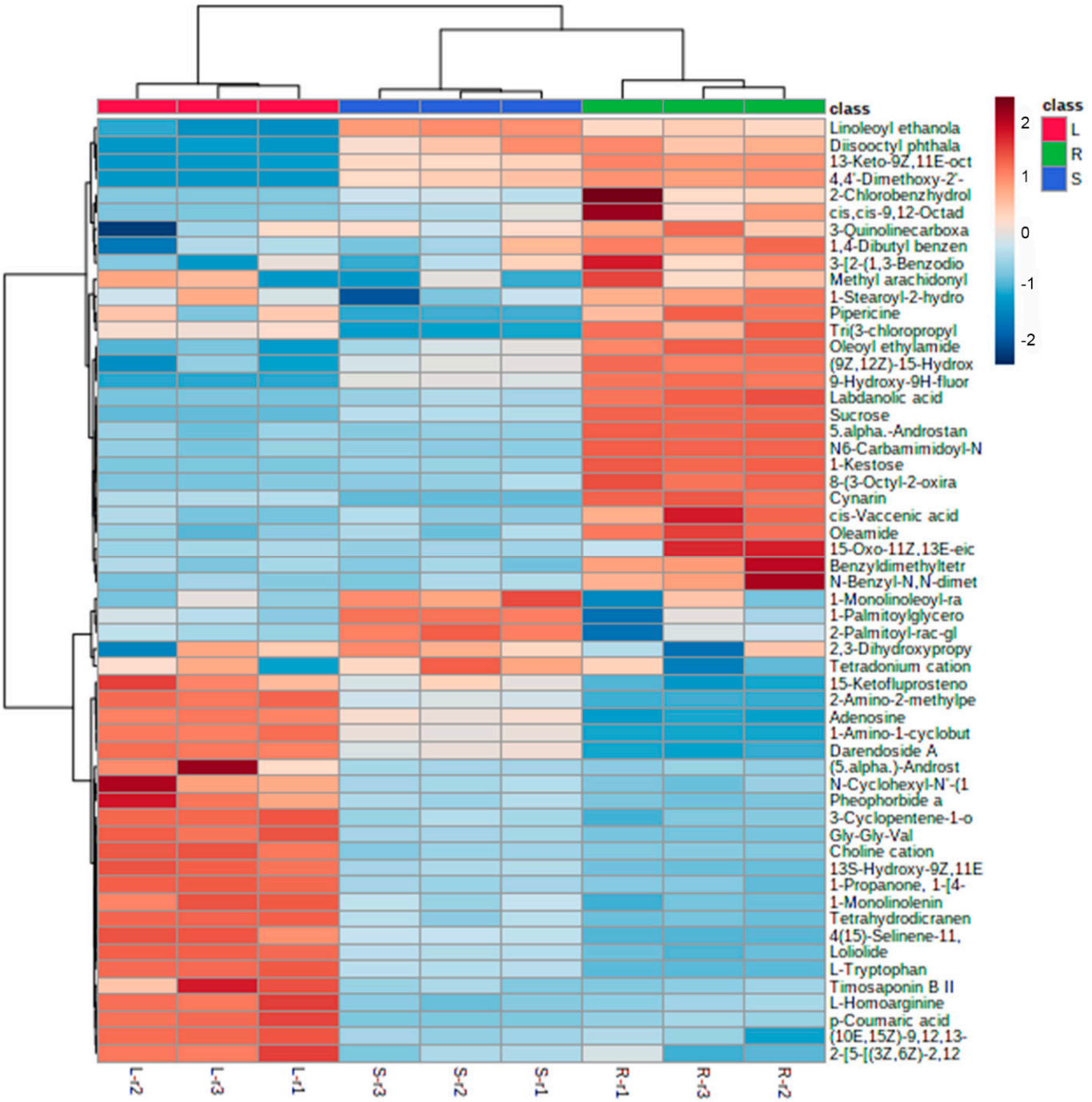

Figure 2. Heat maps showing the qualitative composition and the relative abundance of primary and secondary metabolites in root, stem, leaf and the whole plant. In the heat map columns (data reported in triplicate), red color indicates higher relative levels of metabolites, whereas the blue color suggests a minor content of them. Compound annotation was made comparing the experimental MS/MS spectra to those available in the NIST2020 Tandem Mass Spectral Library. An m/z window of 0.005 Da and a relative intensity threshold of 0.5 were selected as input parameters. Only the compounds with an identification score cut-off > 80% were retained for further analysis. Heatmap was performed with MetoboAnalyst 5.0 for either annotated metabolites or ontology grouped metabolites. Samples were normalized by median, followed by pareto scaling.

Table 2. Ontology based-metabolomics of *B. pilosa* plant materials.

	f.Value	*p*.Value	log10(p)	FDR	Fisher's LSD
amino acid	810.05	2.8488×10^{-10}	9.5453	9.6858×10^{-9}	Leaf—Plant; Leaf—Root; Leaf—Stem; Plant—Root; Stem—Plant; Stem—Root
diterpenoid	483.51	2.2226×10^{-9}	8.6531	3.3113×10^{-8}	Root—Leaf; Stem—Leaf; Root—Plant; Stem—Plant; Root—Stem
oligosaccharide	420.24	3.8807×10^{-9}	8.4111	3.3113×10^{-8}	Plant—Leaf; Root—Leaf; Stem—Leaf; Root—Plant; Plant—Stem; Root—Stem
disaccharide	419.83	3.8957×10^{-9}	8.4094	3.3113×10^{-8}	Plant—Leaf; Root—Leaf; Stem—Leaf; Root—Plant; Root—Stem
cinnamate derivative	387.96	5.3299×10^{-9}	8.2733	3.6244×10^{-8}	Root—Leaf; Leaf—Stem; Root—Plant; Plant—Stem; Root—Stem
peptide	339.51	9.0488×10^{-9}	8.0434	4.4266×10^{-8}	Leaf—Plant; Leaf—Root; Leaf—Stem; Plant—Root; Stem—Root
benzofurano	333.66	9.6956×10^{-9}	8.0134	4.4266×10^{-8}	Leaf—Plant; Leaf—Root; Leaf—Stem; Plant—Root; Plant—Stem; Stem—Root
hydroxycinnamic acid	327.68	1.0416×10^{-8}	7.9823	4.4266×10^{-8}	Leaf—Plant; Leaf—Root; Leaf—Stem; Plant—Root; Plant—Stem
nucleoside	283.3	1.8539×10^{-8}	7.7319	7.0037×10^{-8}	Leaf—Plant; Leaf—Root; Leaf—Stem; Plant—Root; Stem—Plant; Stem—Root
chalcone derivate	208.61	6.2137×10^{-8}	7.2067	2008×10^{-4}	Plant—Leaf; Root—Leaf; Stem—Leaf; Stem—Plant; Stem—Root
amine	206.27	6.4966×10^{-8}	7.1873	2.008×10^{-4}	Leaf—Plant; Leaf—Root; Leaf—Stem; Plant—Root; Stem—Plant; Stem—Root
fatty acid	176.56	1.199×10^{-4}	6.9212	3.3971×10^{-7}	Leaf—Plant; Root—Leaf; Stem—Leaf; Root—Plant; Stem—Plant; Root—Stem
quaternary ammonium compound	165.01	1.5641×10^{-7}	6.8057	4.0907×10^{-7}	Leaf—Plant; Leaf—Root; Leaf—Stem; Stem—Root
carbamide	149.31	2.3163×10^{-7}	6.6352	5.6252×10^{-7}	Root—Leaf; Root—Plant; Root—Stem
steroid	128.67	4.1484×10^{-7}	6.3821	9.4031×10^{-7}	Leaf—Plant; Root—Leaf; Leaf—Stem; Root—Plant; Root—Stem
phenolic derivative	122.17	5.0797×10^{-7}	6.2942	1.0794×10^{-6}	Leaf—Plant; Leaf—Root; Plant—Root; Stem—Root
N-acyl amine	117.35	5.9443×10^{-7}	6.2259	1.1889×10^{-6}	Plant—Leaf; Plant—Root; Plant—Stem
Cyclopentane	72.681	3.806×10^{-3}	5.4195	7.189×10^{-3}	Leaf—Plant; Leaf—Root; Leaf—Stem; Plant—Root; Stem—Root
Monoglyceride	65.417	5.7002×10^{-6}	5.2441	1.02×10^{-5}	Leaf—Plant; Leaf—Root; Stem—Leaf; Stem—Plant; Stem—Root
phthalate derivate	55.477	1.0687×10^{-5}	4.9711	1.8168×10^{-5}	Root—Leaf; Stem—Leaf; Root—Plant; Stem—Plant; Stem—Root
diterpene	53.245	1.2488×10^{-5}	4.9035	2.0219×10^{-5}	Leaf—Plant; Leaf—Root; Leaf—Stem; Plant—Root; Stem—Root
furanone derivate	27.794	0.00013939	3.8558	0.00021543	Leaf—Plant; Leaf—Root; Leaf—Stem; Plant—Root; Stem—Root
Saponin	23.322	0.00026134	3.5828	0.00036454	Leaf—Plant; Leaf—Root; Leaf—Stem
porphyrins	23.314	0.00026165	3.5823	0.00036454	Leaf—Plant; Leaf—Root; Leaf—Stem
Aliphatic ester	23.156	0.00026805	3.5718	0.00036454	Leaf—Plant; Stem—Leaf; Stem—Plant; Stem—Root
aromatic ester	21.937	0.00032451	3.4888	0.00042436	Leaf—Root; Plant—Root; Stem—Root
organofluorine derivate	21.655	0.00033966	3.469	0.00042771	Root—Leaf; Root—Plant; Stem—Plant; Root—Stem
amide	20.619	0.00040342	3.3942	0.00048987	Root—Leaf; Root—Plant; Root—Stem
glycerophospholipid	14.443	0.0013585	2.867	0.0015927	Stem—Leaf; Stem—Plant; Stem—Root
terephthalate derivate	9.818	0.0046632	2.3313	0.0052849	Stem—Leaf; Stem—Plant; Stem—Root
bromo derivate	8.1485	0.008147	2.089	0.0089355	Stem—Leaf; Stem—Root
aliphatic alcohol	6.3184	0.016665	1.7782	0.017707	Root—Leaf; Root—Plant; Root—Stem
organochloride compound	5.4374	0.024748	1.6065	0.025498	Plant—Stem; Root—Stem

3.2. Antimicrobial Effects

The antimicrobial effects of n-hexane, ethyl acetate and methanol extracts were compared with reference drugs and presented in Tables 3–5. Overall, clinical Gram-negative bacterial strains (PeruMyc 2, 3, 5 and 7) showed a somewhat lower susceptibility to plant extracts than that of Gram-positive ones. This was particularly true for the *B. cereus* strain PeruMycA 4, that showed the lowest MIC values (Table 3). Regardless of the bacterial strain used, n-hexane extracts showed the lowest antibacterial activity (Table 3). Table 4 shows the MIC ranges and geometric means of plant extract and fluconazole against the yeast species tested. *C. parapsilosis* (YEPGA 6551) were the most sensitive yeast strain to plant extracts, with MIC ranges of <0.031–0.198 mg mL^{-1}, while *C. albicans* (YEPGA 6379) showed the least sensitivity to the plant extract.

Table 3. Minimal inhibitory concentrations (MICs) of *B. pilosa* n-hexane, ethyl acetate and methanol extracts and ciprofloxacin against clinical Gram-positive and Gram-negative bacteria.

Extract Typology		Minimum Inhibitory Concentration (MIC)			
		n-hex (mg mL^{-1})	EtOAc (mg mL^{-1})	MeOH (mg mL^{-1})	Ciprofloxacin (μg mL^{-1})
Bacterial strain	Plant parts				
E. coli (ATCC 10536)	roots	<0.031	<0.031	<0.031	<0.12
	leaves	<0.031	<0.031	<0.031	
	stems	<0.031	<0.031	<0.031	
	whole	<0.031	<0.031	<0.031	
E. coli (PeruMycA 2)	roots	0.049 (0.031–0.062)	0.078 (0.062–0.125)	0.157 (0.125–0.25)	1.23 (0.98–1.95)
	leaves	0.049 (0.031–0.062)	0.039 (0.031–0.062)	0.157 (0.125–0.25)	
	stems	0.039 (0.031–0.62)	0.157 (0.125–0.25)	0.157 (0.125–0.25)	
	whole	<0.031	0.157 (0.125–0.25)	0.157 (0.125–0.25)	
E. coli (PeruMycA 3)	roots	0.099 (0.062–0.125)	0.157 (0.125–0.25)	0.157 (0.125–0.25)	0.62 (0.49–0.98)
	leaves	0.198 (0.125–0.25)	0.039 (0.031–0.062)	0.198 (0.125–0.25)	
	stems	0.078 (0.062–0.125)	0.315 (0.25–0.5)	0.157 (0.125–0.25)	
	whole	0.039 (0.031–0.62)	0.157 (0.125–0.25)	0.157 (0.125–0.25)	
B. cereus (PeruMycA 4)	roots	<0.031	<0.031	<0.031	<0.12
	leaves	<0.031	<0.031	<0.031	
	stems	<0.031	<0.031	<0.031	
	whole	<0.031	<0.031	<0.031	
P. aeruginosa (ATCC15442)	roots	0.039 (0.031–0.062)	0.078 (0.062–0.125)	<0.031	<0.12
	leaves	0.049 (0.031–0.062)	0.039 (0.031–0.062)	<0.031	
	stems	0.039 (0.031–0.062)	0.039 (0.031–0.062)	<0.031	
	whole	0.039 (0.031–0.62)	0.078 (0.062–0.125)	<0.031	
B. subtilis (PeruMyc 6)	roots	0.315 (0.5–0.25)	0.198 (0.125–0.25)	0.078 (0.062–0.125)	<0.12
	leaves	0.396 (0.25–0.5)	0.396 (0.25–05)	0.078 (0.062–0.125)	
	stems	0.315 (0.5–0.25)	0.198 (0.125–0.25)	<0.031	
	whole	0.315 (0.25–0.5)	0.098 (0.062–0.125)	<0.031	
S. typhy (PeruMyc 7)	roots	0.049 (0.031–0.062)	0.157 (0.125–0.25)	0.157 (0.125.0.25)	0.49
	leaves	0.198 (0.125–0.25)	0.198 (0.125–0.25)	0.078 (0.062–0.125)	
	stems	0.198 (0.125–0.25)	0.039 (0.031–0.062)	0.049 (0.031–0.062)	
	whole	0.157 (0.125–0.25)	0.198 (0.125–0.25)	0.049 (0.031–0.062)	
S. aureus (ATCC 6538)	roots	0.039 (0.031–0.062)	0.039 (0.031–0.062)	0.039 (0.031–0.062)	0.98
	leaves	0.049 (0.031–0.062)	0.039 (0.031–0.062)	0.039 (0.031–0.062)	
	stems	0.039 (0.031–0.062)	0.039 (0.031–0.062)	0.039 (0.031–0.062)	
	whole	0.039 (0.031–0.062)	0.039 (0.031–0.062)	0.039 (0.031–0.062)	

Table 4. Minimal inhibitory concentrations (MICs) of *B. pilosa* n-hexane, ethyl acetate and methanol extracts and fluconazole against clinical yeasts.

Extract Typology		Minimum Inhibitory Concentration (MIC)			
		n-hex (mg mL^{-1})	EtOAc (mg mL$^{-1)}$	MeOH (mg mL^{-1})	Fluconazole (μg mL^{-1})
Yeast strain	Plant parts				
C. albicans (YEPGA 6183)	roots	0.39 (0.25–0.5)	0.198 (0.125–0.250)	0.157 (0.125–0.250)	2
	leaves	0.314 (0.125–0.25)	0.315 (0.25–0.5)	0.198 (0.125–0.250)	
	stems	0.396 (0.25–0.5)	0.315 (0.25–0.5)	0.396 (0.25–0.5)	
	whole	0.198 (0.125–025)	0.315 (0.25–0.5)	0.315 (0.25–0.5)	
C. tropicalis (YEPGA 6184)	roots	0.051 (0.031–0.065)	0.099 (0.0625–0.125)	<0.031	2
	leaves	0.314 (0.25–0.5)	0.157 (0.125–0.250)	0.198 (0.125–0.250)	
	stems	0.198 (0.125–0.25)	0.198 (0.125–0.250)	0.099 (0.0625–0.125)	
	whole	0.198 (0.125–0.5)	0.099 (0.0625–0.125)	0.198 (0.125–0.250)	

Table 4. *Cont.*

Extract Typology		Minimum Inhibitory Concentration (MIC)			
		n-hex (mg mL^{-1})	EtOAc (mg mL^{-1})	MeOH (mg mL^{-1})	Fluconazole (μg mL^{-1})
C. albicans (YEPGA 6379)	roots	0.314 (0.25–0.5)	0.099 (0.0625–0.125)	0.157 (0.125–0.250)	1
	leaves	0.396 (0.25–0.5)	0.198 (0.125–0.250)	0.198 (0.125–0.250)	
	stems	0.314 (0.250–0.5)	0.315 (0.25–0.5)	0.396 (0.25–0.5)	
	whole	0.314 (0.125–0.5)	0.315 (0.25–0.5)	0.315 (0.25–0.5)	
C. parapsilosis (YEPGA 6551)	roots	<0.031	<0.031	<0.031	4
	leaves	0.157 (0.125–0.25)	<0.031	<0.031	
	stems	0.198 (0.125–025)	<0.031	<0.031	
	whole	<0.031	<0.031	<0.031	

Table 5. Minimal inhibitory concentrations (MICs) of *B. pilosa* n-hexane, ethyl acetate, methanol extracts and griseofulvin against clinical dermatophytes.

Extract Typology		Minimum Inhibitory Concentration (MIC)			
		n-hx (mg mL^{-1})	EtOAc (mg mL^{-1})	MeOH (μg mL^{-1})	Griseofulvin (μg mL^{-1})
Fungal strain	Plant parts				
T. rubrum (CCF4933)	roots	0.198 (0.125–0.25)	<0.031	<0.031	1.26 (1–2)
	leaves	0.396 (0.25–0.5)	<0.031	0.049 (0.031–0.062)	
	stems	0.315 (0.25–0.5)	0.049 (0.031–0.062)	0.157 (0.125–0.25)	
	whole	0.396 (0.25–0.5)	<0.031	<0.031	
T. mentagrofites (CCF 4823)	roots	0.198 (0.125–0.25)	<0.031	<0.031	1
	leaves	0.049 (0.031–0.062)	<0.031	<0.031	
	stems	0.315 (0.25–0.5)	0.049 (0.031–0.062)	0.157 (0.125–0.25)	
	whole	0.396 (0.25–0.5)	<0.031	<0.031	
T.rubrum (CCF4879)	roots	0.198 (0.125–0.25)	<0.031	<0.031	2
	leaves	0.198 (0.125–2.5	<0.031	0.049 (0.031–0.062)	
	stems	0.315 (0.25–0.5)	<0.031	0.157 (0.125–0.25)	
	whole	0.396 (0.25–0.5)	<0.031	<0.031	
T. tonsurans (CCF4834)	roots	0.198 (0.125–0.25)	<0.031	<0.031	0.125
	leaves	0.049 (0.031–0.062)	<0.031	198 (0.125–25))	
	stems	0.315 (0.25–0.5)	<0.031	0.157 (0.125–0.25)	
	whole	0.396 (0.25–0.5)	<0.031	<0.031	
A. crocatum (CCF5300)	roots	0.198 (0.125–0.25)	<0.031	<0.031	>8
	leaves	0.396 (0.25–0.5)	<0.031	0.078 (0.062–0.125)	
	stems	0.315 (0.25–0.5)	0.049 (0.031–0.062)	0.157 (0.125–0.25)	
	whole	0.396 (0.25–0.5)	<0.031	<0.031	
A. gypseum (CCF6261)	roots	0.315 (0.25–0.5)	<0.031	<0.031	1.587 (1–2)
	leaves	0.396 (0.25–0.5)	<0.031	0.157 (0.125–0.25)	
	stems	0.198 (0.125–0.25)	0.315 (0.25–0.5)	0.078 (0.062–0.125)	
	whole	0.198 (0.125–0.25)	<0.031	<0.031	
T. erinacei (CCF5930)	roots	0.315 (0.25–0.5)	<0.031	<0.031	0.25
	leaves	0.396 (0.25–0.5)	<0.031	0.157 (0.125–0.25)	
	stems	0.198 (0.125–0.25)	0.157 (0.125–0.250)	0.157 (0.125–0.25)	
	whole	0.198 (0.125–0.25)	<0.031	<0.031	
A. quadrifidum (CCF5792)	roots	0.315 (0.25–0.5)	<0.031	<0.031	>8
	leaves	0.396 (0.25–0.5)	<0.031	0.157 (0.125–0.25)	
	stems	0.198 (0.125–0.25)	0.157 (0.125–0.250)	0.157 (0.125–0.25)	
	whole	0.396 (0.25–0.5)	<0.031	<0.031	

3.3. Phenolic Profile

The HPLC analyses showed that gallic acid, caftaric acid, catechin, chlorogenic acid, epicatechin and caffeic acid were present in most of the analyzed plant materials (Figure 3A–D), with the only exception of leaf methanol extract. Among the identified compounds, caftaric acid (peak #3 in the Figure 3A–C; retention time: 9.15 min.) was revealed to be the prominent phenolic compound, especially in the extract prepared from the whole plant (3.03 µg/mL).

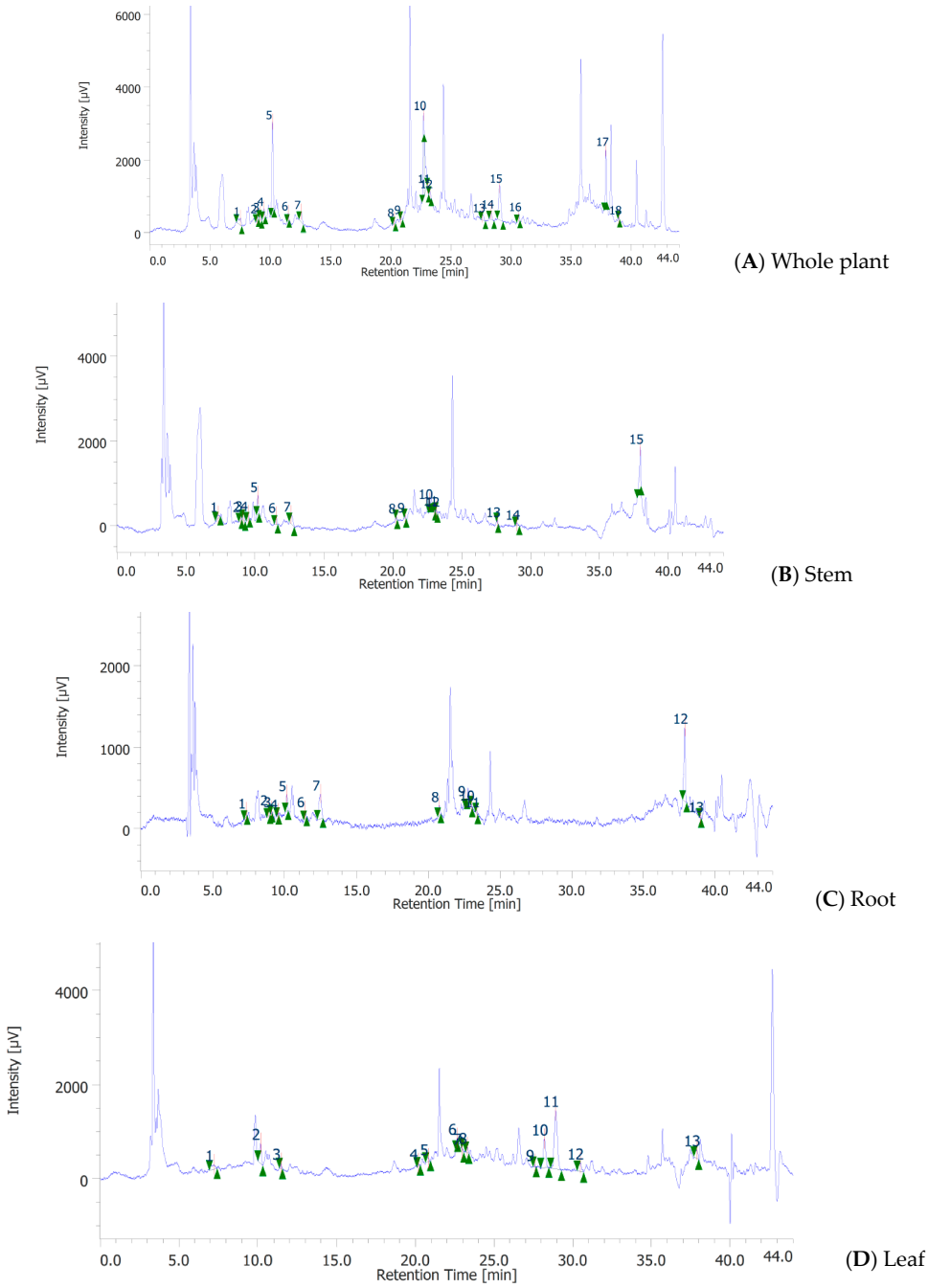

Figure 3. HPLC–DAD-MS analysis of methanolic extracts from *B. pilosa* plant materials. Among identified phenolic compounds in Table 1, caftaric acid (peak #3), catechin (peak #4), chlorogenic acid (peak #5) and epicatechin (peak #6) were identified in the whole plant (**A**), stem (**B**) and root (**C**). In the leaf (**D**), the principal phenolics identified in the extract were: gallic acid (peak #1), chlorogenic acid (peak #2) and epicatechin (peak #3).

3.4. In Silico Experiments

The results of the in silico experiment highlight the capability of caftaric acid to interact with the active site of the enzyme with a micromolar affinity, through the formation of both hydrogen bonds and alkyl interactions (Figure 4). The affinity of the caftaric acid was compared to that of the reference drug ketoconazole that, as expected, shows a much higher (sub-micromolar) affinity compared to that of caftaric acid.

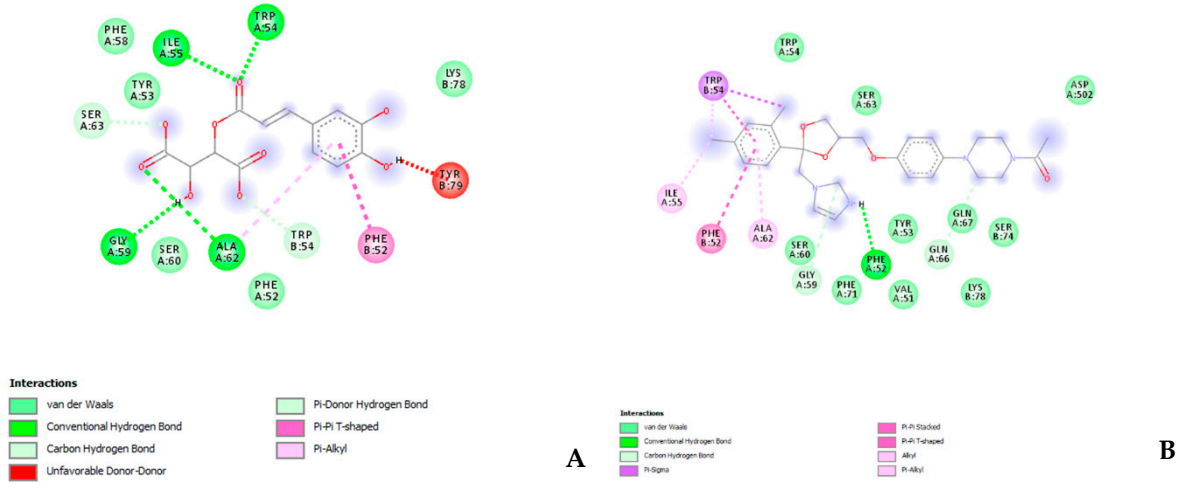

Figure 4. (**A**) Putative interactions between caftaric acid and lanosterol 14α-demethylase (PDB ID: 5TZ1). Free energy binding and affinity are −6.8 Kcal/mol and 10.5 μM, respectively. (**B**) Putative interactions between ketoconazole with lanosterol 14α-demethylase (PDB ID: 5TZ1). Free energy binding and affinity are −9.6 Kcal/mol and 0.1 μM, respectively.

3.5. Intrinsic Scavenging/Reducing Properties

Finally, the radical scavenging/reducing properties of the methanol extracts of *B. pilosa* plant materials were assayed (Table 6). The values of the DPPH assay are reported in comparison with the activity of the Trolox and the best activity is shown by the leaves with a rather good mean value of 9.9 IC_{50} referred to the Trolox. Decidedly low is the activity of the stems with a mean value of 101.4, whilst the roots have a medium–low mean value (15.2). The values of the ABTS assay are reported in comparison with the activity of the Trolox and analogously to the DPPH test the best activity is shown by the leaves, with a rather good mean value of 15.4 IC_{50} referred to the Trolox. Decidedly low is the activity of the stems with a mean value of 89.1, whilst the roots have a medium–low mean value (25.3). The FRAP assay shows the mM Fe(II)+ equivalent (FE) for an 100 g sample; interesting is the activity of the leaves, with a mean value of 73.2, while much lower are the values of the other extracts: plants > roots > stems with values of 17.7–15.3–10.1, respectively. In Beta Carotene/Linoleic acid assay values are expressed as % of antioxidant activity. The extracts do not show pro-oxidant activity but a good antioxidant value of the leaves (44.7) and of the plant (37.4). The antioxidant action of the stems is less than that of the leaves in all the tests carried out and the methanolic extract follows the order leaves > plants > roots > stems.

Table 6. Scavenging/reducing properties of methanol extracts from roots, stems leaves and whole plants of *B. pilosa*.

Plant Material	DPPH	ABTS	FRAP	β-Carotene/Linoleic Acid Assay
roots	15.2 ± 1.22	25.3 ± 2.16	15.3 ± 1.17	21.1 ± 1.74
stems	101.4 ± 8.23	89.1 ± 7.81	10.1 ± 0.87	44.7 ± 3.86
leaves	9.9 ± 0.84	15.4 ± 1.36	73.2 ± 6.5	27.4 ± 2.32
plants	3.7 ± 2.38	34.3 ± 3.05	17.7 ± 1.49	37.4 ± 3.78

4. Discussion

Considering the traditional ethnopharmacology and phytotherapy uses [6,7], in the present study, different materials from *B. pilosa* have been assayed in order to unravel plant material composition and extracts' antimicrobial effects. Oligosaccharide, disaccharide and fatty acids were found to be much more abundant in root than in the other plant parts. Monoglycerides were more abundant in stem than in the other plant parts, whereas peptide and diterpenoid were more abundant in leaf and root, respectively. By contrast, amino acids showed very different distribution patterns in the four plant parts. The microbiological study investigated the potential anti-bacterial and anti-fungal effects of the extracts against selected pathogen strains. All tested extracts showed fungal growth inhibition; particularly active were the ethyl acetate and methanolic extracts from root and leaves that showed the highest antifungal activity among all samples tested. Ethyl acetate and methanolic extract were also classified as potent for dermatophyte when compared to n-hexane. The results clearly demonstrated that the extracts were less effective when compared to the reference drugs, namely the anti-bacterial ciprofloxacin and the anti-mycotic fluconazole and griseofulvin. Nevertheless, the ethyl acetate and methanol extracts of *B. pilosa* displayed anti-mycotic activity on *C. albicans* (YEPGA 6379) and dermatophytes; this deserves further investigation. Considering the results of the antimicrobial tests pointing to promising activity of polar extracts from *B. pilosa* as anti-bacterial and anti-mycotic agents, a quali-quantitative HPLC–DAD-MS analysis was conducted on phenolic acids and flavonoids from *B. pilosa* methanol extracts, in order to unravel the putative mechanisms underlying the observed antimicrobial effects. In this regard, it is sensitive to highlight that phenolic compounds could explain, albeit partially, the anti-mycotic effects induced by polar extracts [32,33]. Specifically, the HPLC analyses showed that gallic acid, caftaric acid, catechin, chlorogenic acid, epicatechin and caffeic acid were present in most of the analyzed plant materials. Caftaric acid is known to be a phytocompound characterizing Echinacea species [34]. However, different studies suggest the presence of this phenolic compound in Bidens species, including *B. tripartita* and *B. pilosa* [28,29,35,36], thus further corroborating the results of the present phytochemical investigation. A docking approach was also conducted in order to predict putative interactions between the caftaric acid and the lanosterol 14α-demethylase, playing a master role in fungal metabolism. The results of the in silico experiment highlight the capability of caftaric acid to interact with the active site of the enzyme with a micromolar affinity, through the formation of both hydrogen bonds and alkyl interactions. The putative affinity of caftaric acid towards the selected target enzyme was lower compared to that of the reference drug ketoconazole. However, this putative affinity is consistent with the concentration of the phenolic compound in the extract, and also with the extract MIC values, above all against the Candida species. Therefore, the present docking experiments highlight the importance of phenolic compounds in mediating, albeit partially, the antimicrobial effects induced by *B. pilosa* methanolic extracts. Finally, the radical scavenging/reducing properties of the methanol extracts of *B. pilosa* plant materials were assayed. The intrinsic antioxidant effects of the extracts were evaluated through ABTS, DPPH and Beta-Carotene assays. The antioxidant action of the stems is less than that of the leaves in all of the tests carried out and the methanolic extract follows the order leaves > plants > roots > stems. Antioxidants attract a growing interest owing to their protective roles against oxidative deterioration in food and in the body, and against oxidative stress-mediated pathological processes. Screening of antioxidant properties of plants requires appropriate methods, which address the mechanism of antioxidant activity and focus on the kinetics of the reactions including the antioxidants. Many studies evaluating the antioxidant activity of various samples of research interest using different methods in food and human health have been conducted. Methods based on inhibited autoxidation are the most suited for termination-enhancing antioxidants and for chain-breaking antioxidants. In general, the methods for the determination of the antioxidant capacity of plant extract can deactivate radicals by two major mechanisms and were divided into two major groups: assays based on the single electron transfer (SET)

reaction, and assays based on hydrogen atom transfer (HAT). The end result is the same, regardless of mechanism, but kinetics and potential for side reactions are different. SET-based methods detect the ability of a potential antioxidant to transfer one electron to reduce any compound, including metals, carbonyls, and radicals [37]. HAT-based methods measure the ability of an antioxidant to quench free radicals by hydrogen donation [38]. For this purpose, the most common methods used in vitro determination of antioxidant capacity of plant raw extract were considered in this manuscript. The methanol extracts of roots, stems, leaves, and whole plants were tested with DPPH assay (1,1-diphenyl-2-picrylhydrazyl radical scavenging), ABTS assay (2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonate) radical scavenging), FRAP assay (ferric reducing antioxidant power), and beta carotene/linoleic acid assay (double bond antioxidant power). Methods based on the HAT reaction include the β-Carotene bleaching assays [37]. The SET-based methods include the following assays: 2,2-Diphenyl-1-picrylhydrazyl radical scavenging assay (DPPH·), ferric ion reducing antioxidant power assay (FRAP), and 2,2-Azinobis 3-ethylbenzthiazoline-6-sulfonic acid radical scavenging assay (ABTS). It was reported that ABTS methods used both HAT and SET mechanisms [38]. Phenolic compounds are secondary plant metabolites naturally present in almost all plant materials, including food products of plant origin. Many of the health-protective effects of phenolic compounds have been ascribed to their antioxidant, anticarcinogenic, antimutagenic, antimicrobial, anti-inflammatory and other biological properties [39,40]. The correlation matrix for Pearson coefficients provides a high correlation with the FRAP assay (0.90) and a very low inverse correlation with the linoleic assay (−0.53); intermediate values were for DPPH (−0.70) and ABTS (−0.74) assays. Flavonoids are cyclized diphenylpropanes that commonly occur in plants and particularly plant foods. They are polyphenolic compounds, which are very effective antioxidants that serve against chronic diseases. The intrinsic antioxidant properties have also been related to enzyme inhibition properties [41]. Flavonoids have been isolated from almost all parts of the plant such as leaves, stems, roots, fruits, or seeds. In general, the effective antioxidant ability of flavonoids depends on some factors: the metal-chelating potential that is strongly dependent on the arrangement of hydroxyls and carbonyl group around the molecule, the presence of hydrogen or electron-donating substituents able to reduce free radicals, and the ability of the flavonoid to delocalize the unpaired electron leading to formation of a stable phenoxy radical [39]. Similarly, the correlation matrix for Pearson coefficients provides a high correlation with the FRAP assay (0.88), a very low inverse correlation with the linoleic assay (−0.32), and intermediate values for DPPH (−0.64) and ABTS (−0.68) assays. The chemical complexity of the extracts, stemming from the fact that they are often mixtures of many compounds, with differences in functional groups, polarity, and chemical behavior, could lead to unpredictable results about their possible antioxidant activity.

5. Conclusions

The results of antimicrobial susceptibility testing were analyzed and thoroughly discussed, also with respect to results from similar studies. It should be born in mind, however, that comparisons between bioactivity results are always difficult, because working protocols may differ in terms of extraction methods, test organisms and test systems used [42]. In the present study, the microbiological assays pointed to the promising activity of polar extracts, namely methanol extracts, as anti-mycotic agents. The anti-mycotic effect could be partially mediated by phenolic compounds detected by colorimetric and HPLC–DAD-MS analyses. The pattern of phenolic compound composition could also explain the intrinsic scavenging/reducing properties of the methanol extracts. The present phytochemical determinations also validated previous studies suggesting the presence of caftaric acid, in the phytocomplex of *B. pilosa* [35]. Additionally, considering the intrinsic anti-inflammatory properties of caftaric acid [43], we cannot exclude its involvement in mediating, albeit partially, the anti-inflammatory effects of *B. pilosa* [44]. Therefore, future studies could be conducted in order to investigate anti-inflammatory effects induced by the present extracts from *B. pilosa.*

Author Contributions: Conceptualization, P.A., G.O., L.M. and C.F.; Methodology, P.A., L.M., G.O. and C.F.; Software, C.F.; Validation, P.A., G.O., L.M. and C.F.; Formal analysis, C.F.; Investigation, G.A.F., F.M., B.T., R.V., R.M.P., C.E. and L.V.; Resources, G.O. and C.F.; Data curation, P.A., G.O. and L.M.; Writing—original draft preparation, P.A.; Writing—review and editing, C.F., L.M. and G.O.; Visualization, R.V. and B.T.; Supervision, R.V.; Project administration, P.A., C.F., L.M. and G.O.; Funding acquisition, C.F., L.M. and G.O. All authors have read and agreed to the published version of the manuscript.

Funding: The study was supported by Italian Ministry funds (FAR 2020), granted to Giustino Orlando, Luigi Menghini and Claudio Ferrante.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: The present article is part of the third mission activities of the Botanic Garden "Giardino dei Semplici" planned for the 20th anniversary of the establishment.

Conflicts of Interest: The authors declare no conflict of interest.

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