Antioxidant and Free Radical Scavenging Activity of Phenolics from *Bidens humilis**

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Key words
- *Bidens humilis*
- Asteraceae
- flavonoids
- antioxidant activity

Abstract

A bioassay-oriented approach led to the isolation of 11 compounds, including three new natural flavonoids, (2*S*)-isookanin 7-*O*-*α*-L-arabinopyranoside (**1**), (2*S*)-isookanin 7-*O*-(2″-acetyl)-*α*-L-arabinopyranoside (**2**), and luteolin 7-*O*-*β*-D-glucopyranosyl-(1 → 6)-*β*-D-galactopyranoside (**6**), from *Bidens humilis* aerial parts. Their structures were determined via spectroscopic analyses including two-dimensional nuclear magnetic resonance. The antioxidant activity of all compounds was also tested by three different assays. The Relative Antioxidant Capacity Index (RACI) is applied herein, from the perspective of statistics, by integrating the antioxidant capacity data determined by these chemical methods.

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Introduction

The genus *Bidens* L. of the Asteraceae family comprises about 200 species of annual or perennial herbs, widely distributed in tropical and subtropical countries, especially in America. Plants of this genus are used in many indigenous systems of medicine as anti-inflammatory, antiallergic, antibacterial, antidiabetic, antimalarial, antiviral, antihypertensive, and antioxidant remedies [1]. *Bidens* species are mainly rich in flavonoids, polyacetylenes, and phenol glycosides [2, 3].

Bidens humilis Kunth (syn. *Bidens triplinervia* Kunth) is a plant used in Ecuadorian folk medicine for the treatment of headaches, fever, and liver diseases, while the flower infusion is used against gastric and abdominal pain. Moreover, the indigenous Salasacas used the yellow flowers to dye wool and other clothes. Nevertheless, no phytochemical study on this species has been reported in the literature. In the frame of our research on plants belonging to Ecuadorian flora, an antioxidant-oriented approach was carried out on *B. humilis* aerial part extracts, leading to the isolation and structural characterization of three new natural flavonoids, (2*S*)-isookanin 7-*O*-*α*-L-arabinopyranoside (**1**), (2*S*)-isookanin 7-*O*-(2″-acetyl)-*α*-L-arabinopyranoside (**2**), and luteolin 7-*O*-*β*-D-glucopyranosyl-(1 → 6)-*β*-D-galactopyranoside (**6**), together with eight known phenolic derivatives (**3**–**5** and **7**–**11**; **Fig. 1**). LC-HRESIMS and LC-HRESIMS/MS analyses of the *n*-BuOH extract were also performed. The antioxidant activity of extracts, fractions, and pure compounds was tested by three different assays. Multiple reaction mechanisms are usually involved in the antioxidant capacity evaluation of the natural compounds, therefore the Relative Antioxidant Capacity Index (RACI) was applied as an integrated approach to compare the results obtained by different methods.

Results and Discussion

The aerial parts of *B. humilis* were sequentially extracted with solvents of increasing polarity giving *n*-hexane, chloroform, and methanol residues. The methanol extract was partitioned between *n*-BuOH and water, yielding an *n*-BuOH fraction. Since a single assay cannot completely determine the antioxidant activity of plant extracts, three different complementary systems were carried out [4]. Thus, all residues were subjected to the 2,2-diphenyl-2-picrylhydrazyl (DPPH), *β*-carotene bleaching (BCB), and ferric reducing antioxi-

* Dedicated to Professor Dr. Dr. h.c. mult. Adolf Nahrstedt on the occasion of his 75th birthday.

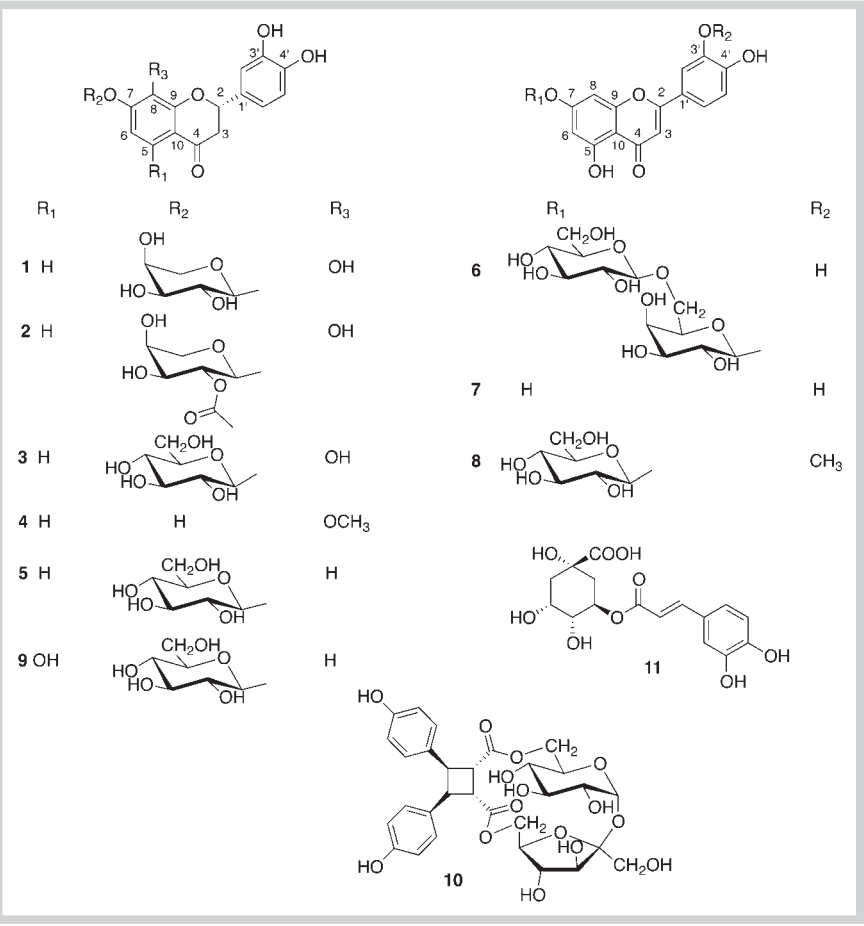


Fig. 1 Chemical structures of compounds **1**–**11**.

dant power (FRAP) assays to screen their antioxidant activity. Total phenolic content (TPC) was also measured. It was demonstrated that the *n*-BuOH fraction was the one containing the highest concentration of total phenolics (from 8 to 20 times higher than the other extracts) (**Table 1**). This extract was more active as a radical scavenger (DPPH) and antioxidant (FRAP) than as antilipoperoxidative (BCB). The high phenolic content of a plant extract is usually directly related to its antioxidant activity measured with DPPH and FRAP tests, while evidences demonstrated that with the BCB test, it is not the same [5]. This phenomenon can be explained by the affinity of the antioxidant complex for the lipids, and thus the lipophilic nature of the BCB test could be the determining factor [5,6]. RACI was calculated to examine the obtained results. TPC results were included in the RACI calculation since phenolics can act with other mechanisms (not measurable with our tests) and could contribute to the extract's health promoting value. In this way, RACI provided a more comprehensive assessment of the whole extract antioxidant potential, and confirmed the *n*-BuOH fraction as the most active (**Fig. 2a**). The *n*-BuOH extract was then subjected to Sephadex LH-20 column chromatography, collecting six major fractions. Also, in this case, all fractions were evaluated for their TPC and antioxidant activities (**Table 1**). All fractions showed to be rich in phenolics with a total content higher than 361.8 mg of gallic acid equivalents per g of the extract (mgGAE/g) (C) to 518.0 mgGAE/g for F, with the exception of A (90.1 mgGAE/g). Fraction E showed the highest DPPH and FRAP values [857.3 and 2208.8 mg of Trolox equivalent per g (mgTE/g) of fraction, respectively], while the highest BCB value was registred for fraction F. On the basis of the RACI results (**Fig. 2a**) and the evidence that the most promising fractions should have at least one value higher than their relative extract, fraction A was excluded from further analysis. All selected fractions were investigated for their phytochemical content. Fraction F showed the presence of one spot on TLC and was directly subjected to spectroscopic and spectrometric analyses. Fractions B–E were purified by RP-HPLC; a total of 11 compounds (**1**–**11**) were characterized (**Fig. 1**), of which three were new natural flavonoids (**1, 2**, and **6**).

A molecular formula of $C_{20}H_{20}O_{10}$ was assigned to compound **1** on the basis of its HR ESIMS (*m/z* 419.0984 $[M - H]^-$) and ^{13}C NMR data. The ESI MS spectrum of **1** showed a quasimolecular ion peak at *m/z* 419 $[M - H]^-$ and one peak at *m/z* 287 $[(M - 132)\text{-}H]^-$, due to the loss of one pentose moiety. Moreover, HR ESIMS/MS analysis revealed fragment ions at *m/z* 151.0058 $[C_7H_4O_4\text{-}H]^-$ and 135.0049 $[C_7H_4O_3\text{-}H]^-$, diagnostic for flavanoids carrying two hydroxyl groups on the A ring [7]. The UV absorption bands at 281 and 327 nm were suggestive of a flavanone skeleton. The ^1H and ^{13}C NMR spectra (**Table 2**) displayed signals for an oxygenated methine doublet of doublets at δ 5.46 (1H, dd, J = 12.5, 3.0 Hz, H-2), two methylene doublets of doublets at δ 3.14 (1H, dd, J = 17.0, 12.5 Hz, H-3_{ax}) and 2.82 (1H, dd, J = 17.0, 3.0 Hz, H-3_{eq}), and five aromatic protons, of which two were superimposable, that were assigned with the help of 1D TOCSY and DQF COSY, together with signals of a sugar residue. The chemical shifts of the two doublets at δ 7.40 (1H, d, J = 8.0 Hz, H-5) and 6.89 (1H, d, J = 8.0 Hz, H-6) suggested a 7,8-disubstituted A ring, while the three signals at δ 7.01 (1H, d, J = 2.0 Hz, H-2′), 6.89 (1H, dd, J = 8.5, 2.0 Hz, H-6′), and 6.81 (1H, d, J = 8.5 Hz, H-5′) were in accordance with a 1,2,4-trisubstituted B ring. The 20 ^{13}C NMR resonances (**Table 2**) were easily assigned

Table 1 Antioxidant activity and total phenolic content of *B. humilis* extracts, fractions and pure compounds using DPPH, BCB, FRAP, and Folin assays.

Sample	Test			
	DPPH mgTE/g*	BCB %AA**	FRAP mgTE/g*	Folin mgGAE/g***
Extract				
n-Hexane	34.9 ± 1.7	43.6 ± 2.9	6.5 ± 0.5	34.0 ± 2.2
$CHCl_3$	37.6 ± 2.4	37.9 ± 1.9	31.3 ± 2.1	13.3 ± 0.9
n-BuOH	173.8 ± 6.2	8.0 ± 0.7	790.1 ± 12.5	261.7 ± 11.0
Fraction				
A	27.4 ± 1.0	13.8 ± 0.4	115.0 ± 9.2	90.1 ± 5.2
B	147.1 ± 11.2	3.2 ± 0.4	675.1 ± 23.1	451.0 ± 21.3
C	163.8 ± 8.5	9.4 ± 0.2	620.7 ± 19.5	361.8 ± 22.2
D	296.3 ± 14.5	4.5 ± 0.5	954.3 ± 12.7	495.5 ± 24.2
E	857.3 ± 18.8	11.4 ± 0.7	2208.8 ± 45.2	511.1 ± 19.7
F	819.5 ± 21.2	40.6 ± 1.1	1442.3 ± 32.1	518.0 ± 16.5
Pure compound				
1	513.7 ± 28.7	16.8 ± 1.0	1404.2 ± 15.6	
2	484.7 ± 21.3	10.3 ± 0.8	777.6 ± 12.4	
3	356.5 ± 16.4	20.5 ± 1.5	707.4 ± 12.6	
4	255.3 ± 12.9	22.3 ± 1.1	1129.7 ± 24.0	
5	238.0 ± 7.7	23.2 ± 1.1	421.2 ± 9.2	
6	357.8 ± 17.5	17.1 ± 1.0	823.8 ± 11.4	
7	819.5 ± 22.5	40.6 ± 2.1	1442.3 ± 17.8	
8	151.5 ± 3.2	9.5 ± 0.7	170.5 ± 4.5	
9	477.1 ± 14.5	21.0 ± 0.9	770.8 ± 7.0	
10	317.5 ± 12.2	14.4 ± 0.9	297.5 ± 7.4	
11	470.7 ± 21.6	19.3 ± 1.7	953.1 ± 12.9	
BHT****		75 ± 4.8		

Values are the mean of three determinations ($p < 0.05$); * Milligrams of Trolox equivalents per g of extract/fraction/pure compound; ** antioxidant activity at 0.05 mg/mL; *** milligrams of gallic acid equivalents per g of extract/fraction/pure compound; **** BHT = butylhydroxytoluen

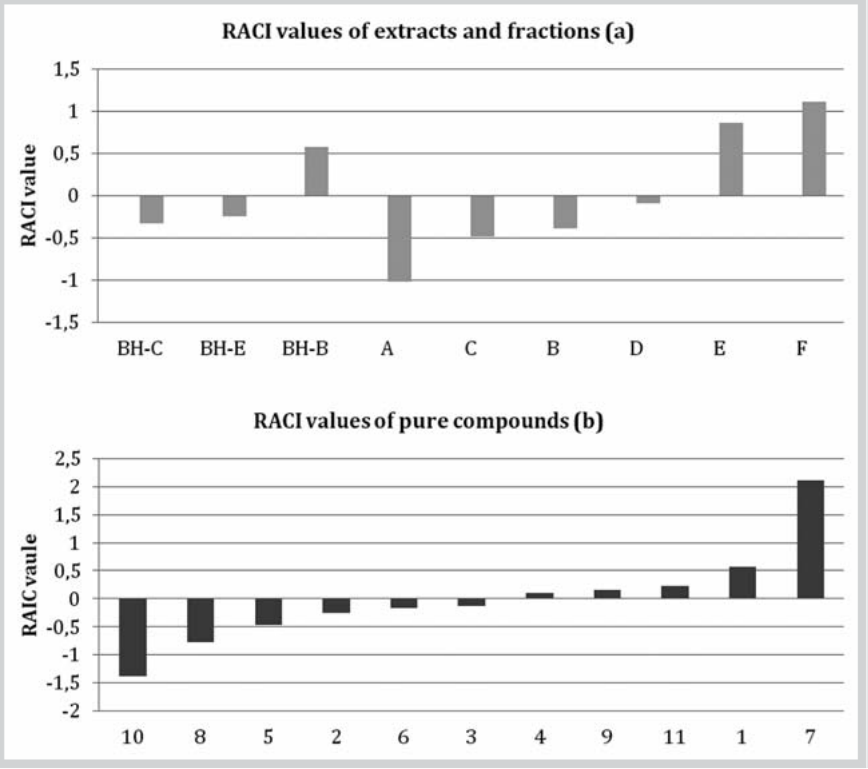


Fig. 2 RACI values of extracts (BH-C: chloroform; BH-E: *n*-hexane; BH-B: *n*-BuOH), Sephadex-LH20 fractions (A–F) (**a**), and pure compounds (**1**–**11**) (**b**) obtained from *B. humilis* aerial parts.

to a flavanone aglycone moiety and to an α-arabinose unit (δ_C 102.7, 73.1, 71.4, 68.9, and 66.6). These 1D NMR data, in combination with the observed 2D NMR correlations, suggested that compound **1** was an isookanin glycoside [8]. The position of the arabinose residue was deduced by the HMBC correlation between δ 4.97 ($H\text{-}1_{ara}$) and 151.8 ppm (C-7). Hydrolysis of **1** with 1 N HCl, followed by GC analysis through a chiral column of the trimethylsilylated sugar, led to the assignment of an arabinose configuration. The stereochemistry of C-2 was determined as *S* on the basis of a negative Cotton effect at 274 nm and a positive

Table 2 ^{1}H and ^{13}C-NMR data of compounds **1, 2**, and **6** (CD_3OD, 600 MHz, *J* in Hz).

Position	1		2		6	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
2	5.46 dd (12.5, 3.0)	81.0	5.43 dd (12.8, 3.0)	81.4		166.0
3ax	3.14 dd (17.0, 12.5)	45.0	3.14 dd (17.2, 12.8)	44.6	6.62 s	103.1
3eq	2.82 dd (17.0, 3.0)		2.77 dd (17.2, 3.0)			
4		193.0		193.3		182.9
5	7.40 d (8.0)	118.0	7.38 d (8.2)	118.0		161.4
6	6.89 d (8.0)	110.0	6.88 d (8.2)	111.3	6.62 d (2.0)	101.7
7		151.8		150.6		163.0
8		135.6		136.3	6.83 d (2.0)	95.2
9		155.0		155.4		157.8
10		113.9		113.0		105.9
1′		130.6		130.5		122.3
2′	7.01 d (2.0)	114.5	7.02 d (2.0)	114.9	7.45 d (2.0)	113.2
3′		145.0		145.0		145.9
4′		145.9		145.8		150.0
5′	6.81 d (8.5)	115.7	6.81 d (8.5)	115.9	6.94 d (8.0)	115.6
6′	6.89 dd (8.5, 2.0)	118.9	6.88 dd (8.5, 2.0)	119.2	7.45 dd (8.0, 2.0)	119.5
Ara 1	4.97 d (7.0)	102.7	5.17 d (6.8)	100.6		
2	3.92 dd (9.0, 7.0)	71.4	5.30 dd (9.0, 6.8)	73.0		
3	3.70 dd (9.0, 2.5)	73.1	3.88 dd (9.0, 2.5)	71.6		
4	3.94 m	68.9	3.94 m	68.6		
5a	3.95 dd (12.0, 2.0)	66.6	3.98 dd (12.0, 2.0)	66.7		
5b	3.74 dd (12.0, 3.5)		3.72 dd (12.0, 3.5)			
COCH_3				171.3		
CO**CH_3**			2.12 s	21.0		
Gal 1					5.09 d (7.5)	100.5
2					3.52 dd (9.0, 7.5)	73.9
3					3.53 dd (9.0, 4.0)	76.3
4					3.47 dd (4.0, 2.5)	70.3
5					3.83 m	76.6
6a					4.23 dd (12.0, 2.5)	69.3
6b					3.87 dd (12.0, 4.5)	
Glc1					4.39 d (7.8)	103.9
2					3.30 dd (9.0, 7.8)	74.0
3					3.29 t (9.0)	77.4
4					3.45 t (9.0)	70.6
5					3.37 m	77.0
6a					3.91 dd (12.0, 3.0)	62.0
6b					3.69 dd (12.0, 5.0)	

Data assignments were confirmed by DQF-COSY, 1D-TOCSY, HSQC, and HMBC experiments

Cotton effect at 306 nm in the CD spectrum of **1**. Consequently, **1** was characterized as (2*S*)-isookanin 7-*O*-*α*-L-arabinopyranoside.

The molecular formula of compound **2** ($C_{22}H_{22}O_{11}$) was established by ^{13}C NMR and HR ESIMS spectra (*m/z* 461.1090 for $[M - H]^-$). In the HR ESIMS/MS spectrum, fragments at *m/z* 401.1067 $[(M - 60)\text{-}H]^-$, 287.0865 $[(M - 174)\text{-}H]^-$, 151.0043 $[C_7H_4O_4\text{-}H]^-$, and 135.0041 $[C_7H_4O_3\text{-}H]^-$ were also observed. Its NMR spectral data (**Table 2**) suggested that the structure of **2** resembled that of **1**, but differed for the presence of an additional acetyl group. The acylation site was on C-2 of arabinose as evidenced by the strong deshielding of $H\text{-}2_{ara}$ at δ 5.30 and was confirmed by an HMBC experiment [9]. The stereochemistry at C-2 and the arabinose configuration were determined as reported for **1**. Thus, the structure of **2** was deduced to be (2*S*)-isookanin 7-*O*-(2″-acetyl)-*α*-L-arabinopyranoside.

Compound **6** ($C_{27}H_{30}O_{16}$) showed a quasimolecular ion peak at *m/z* 609.1461 $[M - H]^-$ in the negative HR ESIMS. The prominent fragment ions observed in the ESI MS spectrum at *m/z* 447 $[(M - 162)\text{-}H]^-$ and 285 $[(M - 162\text{–}162)\text{-}H]^-$, together with the UV absorption maxima at 270 and 335 nm, suggested the presence of a flavone glycoside. Analysis of the ^{1}H and ^{13}C NMR spectra (**Table 2**) of **6** clearly indicated that it was a luteolin derivative. The ^{13}C NMR spectrum allowed for assigning 15 signals to the aglycone moiety and 12 to the sugar residue, consisting of one inner *β*-galactose and one terminal *β*-glucose. The linkage between the sugar moieties was deduced by the HMBC correlation between the signals at δ 4.39 (1H, d, *J* = 7.8 Hz, $H\text{-}1_{glc}$) and 69.3 ($C\text{-}6_{gal}$). Hydrolysis of **6** with 1 N HCl, followed by GC analysis through a chiral column of the trimethylsilylated monosaccharides, permitted the assignment of the sugars configuration. Accordingly, compound **6** was assigned as luteolin 7-*O*-*β*-D-glucopyranosyl-(1 → 6)-*β*-D-galactopyranoside.

Compounds **3**–**4** and **7**–**11** were characterized as isookanin 7-*O*-*β*-D-glucopyranoside (flavanomarein) (**3**) [10], 8-methoxybutin (**4**) [11], butin 7-*O*-*β*-D-glucopyranoside (isocoreopsin) (**5**) [12], luteolin (**7**) [8], chrysoeriol 7-*O*-*β*-D-glucopyranoside (thermopsoside) (**8**) [13], eriodictyol 7-*O*-*β*-D-glucopyranoside (**9**) [8], 6′, 6″-sucrose ester of (1*α*,2*α*,3*β*,4*β*)-3,4-bis-(4-hydroxyphenyl)-1,2-cyclobutanedicarboxilic acid (**10**) [3], and chlorogenic acid

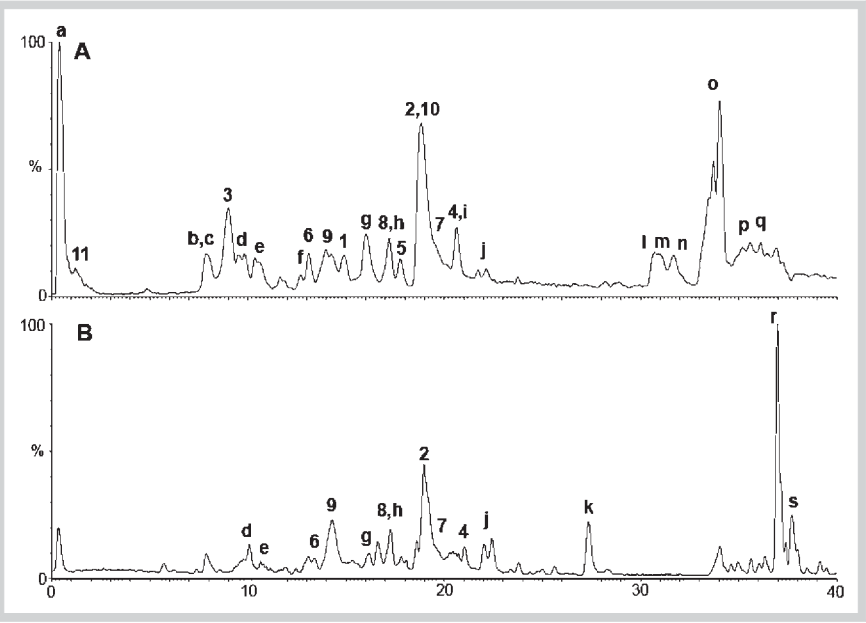


Fig. 3 Total ion current LC-HRESIMS chromatograms obtained for the *n*-BuOH fraction of the aerial parts of *B. humilis* in both the negative (**A**) and positive (**B**) ion modes. Peak numbers refer to compounds **1**–**11**; small case letters (**a**–**s**) indicate other compounds detected in the LC-MS analyses. MS data and tentative assignments for these compounds are reported in **Table 3**.

(**11**) [14] by spectrometric and spectroscopic data measurements and comparison with the literature data.

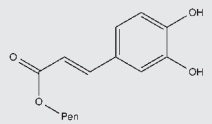
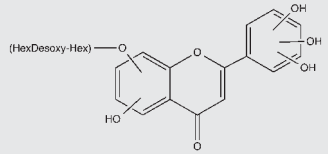
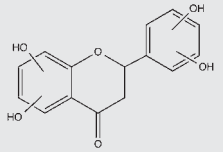
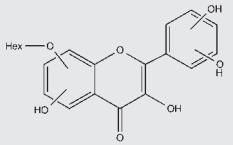
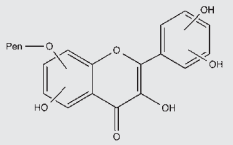
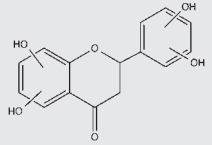
Consequently, the antioxidant activity of these new and known compounds using BCB, FRAP, and DPPH tests was measured (**Table 1**). Among the known compounds, luteolin (**7**) showed the highest values: 819.5 mgTE/g, 40.6% of antioxidant activity (AA), 1442.3 mgTE/g in DPPH, BCB, and FRAP tests, respectively. These findings are congruent with previous evidences [15, 16]. On the other hand, among new natural compounds, **1** showed to be the most active in DPPH and FRAP tests. On the basis of RACI (**Fig. 2b**), which combines the results obtained from all antioxidant tests, it is possible to assess that **7** and **1** are the most active compounds, followed by **11** and a group formed by compounds **9, 4, 3, 6**, and **2**. This group showed close but slightly descending RACI values. The lowest RACIs were observed for compounds **5, 8**, and **10**. All of the isolated compounds are flavonoid derivatives with the exclusion of **10** and **11**, and on the basis of their structures, it is possible to confirm the importance of the two hydroxyl groups in the *ortho*-diphenolic arrangement (compounds **7, 1, 11, 9, 4, 3, 6, 2**). The loss of activity of compound **8** could be ascribed to the presence of a methoxy group on the B ring. This result is congruent with previous findings where it was demonstrated that the presence of a methoxy group decreases, sensibly, the antioxidant capacity of flavonoids [17, 18]. Some of the pure compounds tested exerted interesting antioxidant activity, and their action could partially explain the activity shown by the fraction they come from. Thus, in order to deepen the polar fraction metabolome of *B. humilis*, the *n*-BuOH extract was subjected to LC-HRESIMS and LC-HRESIMS/MS analyses both in the positive and in negative ion modes (**Fig. 3**). Twenty-four major compounds were detected, including the 11 isolates. On the basis of accurate molecular weight determination, and taking into account the main fragments observed, the structure of a further 13 derivatives was proposed (**Table 3**). Compounds **1**–**11** were identified by comparing the HRMS and HRMS/MS data achieved in the LC/MS analyses with those obtained during the structural characterization on the isolated compounds. In the case of compounds **3** and **9**, the MS data were not informative enough to permit an unambiguous identification; therefore, separate injections of each pure compound were performed. Moreover, a comparison between our data and previously published results on the MS analyses of flavonoids [19, 20] and other phenols [21] allowed us to deduce the main structural features of five further flavones, one dimeric flavone, two flavanones, two flavonols, and two caffeoylquinic derivatives. Finally, one peptide was detected, whose amino acidic sequence was easily deduced from MS/MS data. The presence of these additional phenolic compounds could explain the high antioxidant activity showed by the *B. humilis n*-BuOH extract.

Materials and Methods

General experimental procedures

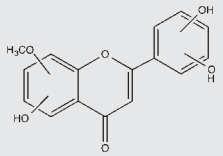
Optical rotations were measured on a Perkin-Elmer 241 polarimeter equipped with a sodium lamp (589 nm) and a 1 dm microcell. UV spectra were recorded on a Perkin-Elmer-Lambda spectrophotometer. CD spectra were measured on a JASCO J-810 spectropolarimeter with a 0.1-cm cell in MeOH at room temperature under the following conditions: speed 50 nm/min, time constant 1 s, and bandwidth 2.0 nm. NMR experiments were performed on a Bruker DRX-600 spectrometer at 300 K. The standard pulse sequence and phase cycling were used for DQF-COSY, TOCSY, HSQC, and HMBC experiments. ESIMS were obtained using a Finnigan LC-Q Advantage Termoquest spectrometer, equipped with Xcalibur software. HRESIMS were acquired in both the positive and negative ion modes on a Q-TOF premier spectrometer (Waters-Milford). Mass spectrometry analyses were performed on a Q-TOF premier spectrometer coupled with an Alliance HPLC module (Waters-Milford). TLC was performed on precoated Kieselgel 60 F_{254} plates (Merck); compounds were detected by spraying with $Ce(SO_4)_2/H_2SO_4$ (Sigma-Aldrich) and NTS (Naturstoffe reagent)-PEG (Poliethylene glycol 4000) solutions. Column chromatography was performed over Sephadex LH-20 (Pharmacia); reversed-phase (RP) HPLC separations were conducted on a Shimadzu LC-8A series pumping system equipped with a Shimadzu RID10A refractive index detector and a Shimadzu injector using a C_{18} μ-Bondapak column (30 cm × 7.8 mm, 10 µm, Waters-Milford) and a mobile phase consisting of MeOH-H_2O mixtures at a flow rate of 2 mL/min. GC analyses

Table 3 Identification of the major compounds observed in the HR-LCMS analysis of *B. humilis* extract in both the negative and positive ion modes. Peak numbering is consistent within the LC-MS profiles shown in **Fig. 3**.

Peak	Experimental MW	Positive ion mode (*m/z*)	Negative ion mode (*m/z*)	Main fragments (*m/z*)	Tentative assignment/compound[1]	Theoretical MW
a	312.1053		311.0874	179.07, 139.06, 135.06, 131.05, 89.01		312.0845
11	354.1002		353.0923	191.04, 179.04, 135.03	**11**	354.0951
b	516.1309		515.123	353.09, 191.07, 179.06, 135.03	Dicaffeoylquinic acid	516.1268
c	610.1576		609.1497	447.11, 301.09, 151.01		610.1534
3	450.1186		449.1107	287.09, 151.01, 135.01	**3**[2]	450.1162
d	288.0719	289.0696	287.0743	179.01, 151.01, 135.01		288.0634
e	464.1200	465.1005	463.1395	301.07, 179.01, 151.01, 135.01		464.0955
f	434.0940		433.0862	303.05, 151.01, 135.01		434.0849
6	610.1595	611.1603	609.1587	447.11, 285.10, 151.01, 135.00	**6**	610.1534
9	450.1168	451.1235	449.1089	287.08, 151.01, 135.01	**9**[2]	450.1162
1	420.1087		419.1008	287.09, 151.01, 135.01	**1**	420.1056
g	288.0718	289.0696	287.0741	179.01, 151.01, 135.01		288.0624
8	462.1183	463.1129	461.1237	446.13, 299.04, 285.04, 151.05	**8**	462.1162

cont.

Table 3 *Continued*

Peak	Experimental MW	Positive ion mode (*m*/*z*)	Negative ion mode (*m*/*z*)	Main fragments (*m*/*z*)	Tentative assignment/compound[1]	Theoretical MW
h	300.0695	301.0743	299.0648	284.06, 165.01, 151.09, 135.01		300.0634
5	434.1245		433.1166	271.05, 125.01	**5**	434.1213
2	462.1170	463.1167	461.1174	401.10, 287.09, 151.01, 135.01	**2**	462.1162
10	634.1905		633.1826	487.18, 353.17, 293.09, 163.01, 145.01	**10**	634.1898
7	286.0569	287.0403	285.0736	151.01, 135.01	**7**	286.0477
4	302.0852	303.0833	301.0871	286.08, 165.01	**4**	302.0790
i	530.1568		529.1489	367.13, 301.10, 287.09, 179.07, 161.05, 131.01, 135.01		
j	676.2078	677.2153	675.1999	463.16, 403.12, 211.11, 87.01		
k	824.3918	413.2037 $[M + 2H]^{2+}$		678.36, 621.28, 474.25, 276.18, 148.00	Peptide (FGAVVFGE)	824.4069
l	448.0892		447.0943	285.09, 151.01, 135.01	Glycosil-luteolin	448.1006
m	554.0938		553.0859	419.04, 413.03	Dimeric-luteolin	554.0849
n	326.2112		325.2033	255.10, 183.06, 97.00		
o	340.2192		339.2113	269.10, 183.07, 97.00		
p	286.0577		285.0498	151.01, 135.01	Luteolin	286.0477
q	254.0631		253.0552	151.01, 135.01	Chrysin	254.0579
r	370.0548	371.0627		259.06, 127.01		
s	426.1431	427.151		259.07, 127.03		

[1] Pen = pentose; hex = hexose; [2] This identification was confirmed by pure compound injection

were performed using a Dani GC 1000 instrument. All spectrophotometric measurements were done in 96-well microplates and cuvettes on a UV/VIS spectrophotometer SPECTROstar Nano (BMG Labtech).

Chemicals

Sodium acetate trihydrate, DPPH, 2,4,6-tripyridyl-s-triazine (TPTZ), iron (III) chloride ($FeCl_3$*$6H_2O$), β-carotene, linoleic acid, Tween 20, butylhydroxytoluen (BHT) (purity grade 99%), Folin-Ciocalteu reagent, sodium carbonate, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) (purity grade 97%), and gallic acid (purity grade 99%) were purchased from Sigma-Aldrich. *n*-Hexane, chloroform, methanol, hydrochloric acid, and glacial acetic acid were purchased from VWR.

Plant material

Aerial parts of *Bidens humilis* Kunth were collected in Tumbaco, Ecuador, in September 2011. The plant was identified at the Herbarium of Jardin Botanico de Quito, Quito, Ecuador. A voucher specimen (N. 9237 Bidens humilis/1) was deposited at Herbarium Horti Botanici Pisani, Nuove Acquisizioni, Pisa, Italy.

Extraction and isolation

The dried aerial parts of *B. humilis* (610 g) were extracted for 48 h with solvents of increasing polarity, *n*-hexane, $CHCl_3$, and MeOH by exhaustive maceration (2 L) to give 5.7, 16.0, and 26.8 g of the respective residues. The MeOH extract was partitioned between *n*-BuOH and H_2O to afford an *n*-BuOH residue (4.0 g). Part of the *n*-BuOH extract (2.5 g) was submitted to a Sephadex LH-20 column (5 × 70 cm, flow rate 1.0 mL/min) using MeOH as the eluent and collecting 56 fractions of 10 mL that were grouped by TLC into six major fractions (A–F). Fractions B (230 mg, 230–290 mL), C (122 mg, 300–330 mL), and E (93.6 mg, 390–480 mL) were subjected to RP-HPLC with MeOH–H_2O (3 : 7) as the eluent to give pure compounds **5** (2.4 mg, t_R = 33 min), **10** (3.6 mg, t_R = 46 min), and **6** (3.7 mg, t_R = 100 min) from fraction B; compounds **3** (5.1 mg, t_R = 23 min), **1** (5.3 mg, t_R = 32 min), and **9** (2.0 mg, t_R = 53 min) from fraction C; and compound **11** (2.2 mg, t_R = 16 min) from fraction E. Fraction D (185 mg, 340–380 mL) was purified by RP-HPLC with MeOH–H_2O (2 : 3) as the eluent to give pure compounds **2** (1.6 mg, t_R = 32 min), **4** (2.5 mg, t_R = 40 min), and **8** (4.0 mg, t_R = 90 min). Fraction F (490–560 mL) yielded pure compound **7** (14.5 mg). All of the compounds met the criteria of ≥ 95% purity, as inferred by HPLC and NMR analyses.

(2S)-Isookanin 7-O-α-L-arabinopyranoside (**1**): yellow amorphous powder; $[\alpha]_D^{25}$ − 22 (*c* 0.3, MeOH); UV (MeOH) λ_{max} (log ε): 281 (4.31), 327 (3.87); CD $[\theta]_{25}$ (*c* 0.05, MeOH, nm) − 5020 (274 nm), + 4380 (306 nm); ^1H and ^{13}C NMR data, see **Table 2**; ESI MS *m/z* 419 [M – H]$^-$, 287 [(M – 132)-H]$^-$; HR ESIMS [M – H]$^-$ 419.0984 (calcd. for $C_{20}H_{19}O_{10}$, 419.0978), 287.0602 [(M – 132)-H]$^-$, 151.0058 [$C_7H_4O_4$-H]$^-$, 135.0049 [$C_7H_4O_3$-H]$^-$.

(2S)-Isookanin 7-O-(2″-acetyl)-α-l-arabinopyranoside (**2**): yellow amorphous powder; $[\alpha]_D^{25}$ − 29 (*c* 0.06, MeOH); UV (MeOH) λ_{max} (log ε): 283 (4.12), 330 (3.98); CD $[\theta]_{25}$ (*c* 0.05, MeOH, nm) − 6400 (275 nm), + 4560 (307 nm); ^1H and ^{13}C NMR data, see **Table 2**; ESI MS *m/z* 461 [M – H]$^-$; HR ESIMS *m/z* 461.1090 [M – H]$^-$ (calcd. for $C_{22}H_{22}O_{11}$, 461.1084), 401.1067 [(M – 60)-H]$^-$, 287.0865 [(M – 174)-H]$^-$, 151.0043 [$C_7H_4O_4$-H]$^-$, and 135.0041 [$C_7H_4O_3$-H]$^-$.

Luteolin 7-O-β-D-glucopyranosyl-(1 → 6)-β-D-galactopyranoside (**6**): yellow amorphous powder; $[\alpha]_D^{25}$ − 45 (*c* 0.2, MeOH); UV (MeOH) λ_{max} (log ε): 270 (4.33), 335 (3.96); ^1H and ^{13}C NMR data, see **Table 2**; ESI MS *m/z* 609 [M – H]$^-$, 447 [(M – 162)-H]$^-$, 285 [(M – 162–162)-H]$^-$, 633 [M + Na]$^+$; HR ESIMS *m/z* 609.1461 [M – H]$^-$ (calcd. for $C_{27}H_{29}O_{16}$, 609.1458).

Acid hydrolysis of compounds 1, 2, and 6

A solution of each compound (2.0 mg) in HCl 1 N (1 mL) was stirred at 80 °C in a stoppered reaction vial for 4 h. After cooling, the solution was evaporated under a stream of N_2. The residue was dissolved in 1-(trimethylsilyl)imidazole and pyridine (0.2 mL), and the solution was stirred at 60 °C for 5 min. After drying the solution, the residue was partitioned between water and $CHCl_3$. The $CHCl_3$ layer was analyzed by GC using an L-Chirasil-Val column (0.32 mm × 25 m). Temperatures of the injector and detector were both 200 °C. A temperature gradient system was used for the oven, starting at 100 °C for 1 min and increasing up to 180 °C at a rate of 5 °C/min. Peaks of the hydrolysate were detected by comparison with the retention times of authentic samples of D-galactose, D-glucose, and L-arabinose (Sigma-Aldrich) after treatment with 1-(trimethylsilyl)imidazole in pyridine.

Liquid Chromatography-High-Resolution Electrospray Ionization Mass Spectrometry Analyses

Liquid chromatography separation was achieved using a Luna C_{18} column (150 × 2.1 mm, 2.5 µm, Phenomenex) and 0.1% formic acid in water (A) and acetonitrile (B). Compound elution was performed using a linear gradient from 1% to 50% of B in 45 min. Mass spectra were acquired both in the positive and negative ion modes, over the *m/z* 200–800 range. The source temperature was set at 120 °C, capillary voltage at 3300 eV, and cone voltage at 50 eV. HRESIMS/MS spectra were acquired for the three most abundant ions observed in each MS spectrum (dependent scan mode). When required, HRESIMS/MS spectra were acquired, setting a specific parent ion in the mass spectrometry method.

Antioxidant activity assays

2,2-Diphenyl-2-picrylhydrazyl radical scavenging activity: The ability to scavenge the DPPH free radical was monitored according to the method reported by Milella et al. (2014) with slight modifications [5]. All samples were tested individually at different concentrations by being added to a methanol solution of DPPH radical (100 µM). For each measure, 50 µL of appropriately diluted sample were added to 200 µL of DPPH reagent. The mixtures were stirred and allowed to stand in the dark at room temperature. In the control, 50 µL of methanol, instead of the diluted sample, were added to 200 µL of DPPH solution. The absorbance of the resulting solutions was measured at 515 nm after 30 min. In all experiments, Trolox radical scavenging activity was also determined and used as reference. Sample activity was expressed as mg of Trolox equivalents per gram of sample (mg TE/g) [22].

Ferric reducing antioxidant power (FRAP) assay: The FRAP assay was carried out as described by Russo et al. with slight modifications [23]. The FRAP reagent was made fresh before each experiment and it was prepared by mixing 300 mM acetate buffer in distilled water (pH 3.6), 20 mM $FeCl_3$*$6H_2O$ in distilled water, and 10 mM TPTZ in 40 mM HCl in a proportion of 10 : 1 : 1. For each sample, 25 µL of appropriately diluted sample (25 µL of methanol for the blank) and 225 µL of FRAP reagent were added and incubated at 37 °C for 40 min in the dark. Absorbance of the resulting solution was measured at 593 nm. Trolox was used as a reference antioxidant standard and FRAP values were expressed as mg Trolox equivalents per gram of sample (mg TE/g).

β-Carotene bleaching (BCB) assay: For this assay, the following reagents were mixed: β-carotene solution (0.2 mg of β-carotene dissolved in 0.2 mL of chloroform), linoleic acid (20 mg), and Tween 20 (200 mg). Chloroform was removed by using a rotary evaporator at room temperature [23]. Distilled water (50 mL) was added with oxygen and then 950 µL of the emulsion were transferred into several tubes containing 50 µL of sample (the final concentration for all tested samples was 0.05 mg/mL) or methanol as the blank. BHT was used as positive control. Next, 250 µL of emulsion-sample solution was transferred (250 µL/well) to the reaction plate. Since the reaction was temperature sensitive, close temperature control throughout the plate was essential in this assay; therefore, the outer wells were filled with 250 µL of water to provide a large thermal mass [24]. The microplate was immediately placed at 50 °C for 3 h and the absorbance was measured at 470 nm at 0′, 30′, 60′, 90′, 120′, 150′, and 180′. Results were expressed as the percentage of BCB inhibition and calculated as follows: $(A_{\beta\text{-carotene after 180 min}}/A_{\text{initial }\beta\text{-carotene}}) \times 100$ (AA%). BHT was used as a positive control.

Total polyphenolic content (TPC): TPC was determined according to the Folin-Ciocalteu method [5] by adding 75 µL of the diluted samples extract (in the blank, 75 µL of methanol) to 425 µL of distilled water, 500 µL of Folin-Ciocalteu reagent, and 500 µL of a sodium carbonate aqueous solution (10% w/v). The mixture was stirred and left in the dark at room temperature for 60 min and then the absorbance was measured at 723 nm. Gallic acid was used as reference standard and TPC was expressed as mg gallic acid equivalents (mgGAE)/g of sample.

Statistical analysis

For each spectrophotometric test, three independent experiments were carried out. Results are presented as mean ± standard deviation (mean ± SD) and a value of $p < 0.05$ was considered as significant. The RACI calculation, regression analysis, and statistical analysis were carried out using Microsoft Excel. Calibration curves of the standards were considered linear if R^2 resulted > 0.99.

Supporting information

NMR spectra of compounds **1, 2**, and **6** are available as Supporting Information.

Conflict of Interest

The authors declare no conflict of interest.

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