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Targeted Metabolite Analysis and Antioxidant Potential of *Rumex induratus*

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Targeted metabolite analysis of aqueous extract of *Rumex induratus* leaves, in terms of phenolic compounds and organic acids, and the study of its antioxidant activity against the DPPH$^{\bullet}$ radical, a reactive oxygen species, hypochlorous acid, and a reactive nitrogen species, nitric oxide ($^{\bullet}$NO), were performed. The samples were collected in several locations, spontaneously occurring or from greenhouse culture, at different stages of development and seasons. The phenolic composition was achieved by high-performance liquid chromatography (HPLC)−diode array detection, and four hydroxycinnamic acid derivatives and 10 flavonoid glycosides (*C*- and *O*-heterosides) were determined. Organic acids composition was established by HPLC-UV, revealing five compounds. The total amount of phenolic compounds and organic acids were affected by growing conditions and developmental phase. The aqueous extract exhibited a dose-related activity against all tested reactive species.

KEYWORDS: *Rumex induratus*; phenolic compounds; organic acids; antioxidant activity

INTRODUCTION

Metabolic change is a major feature of plant genetic modification and plant interactions with pathogens, pests, and their environment. The metabolome composition expresses the cellular processes controlling the biochemical phenotype of the cell, tissue, or entire organism. The determination of the metabolites of a plant reflects its biochemical status. However, in the case of plants, metabolomics renders difficult by the complexity of metabolites in any species (*1*). Phenolic compounds and organic acids are known for their influence in the organoleptic properties of plant foods (*2*) and for their utility in the authenticity and quality control of these matrices (*3*−*5*). On the other hand, antioxidants present in fruits and vegetables, such as phenolic compounds and organic acids, have been implicated in oxidative damage prevention (*6, 7*). A dietary food choice that includes plant species, good sources of these antioxidants, may have relevance in health preservation, decreasing the risk of degenerative diseases by reduction of oxidative stress and inhibition of macromolecular oxidation (*8*).

Several species of the *Rumex* (Polygonaceae) genus, namely, its leaves and roots, have been used in traditional medicine for inflammation, blood purification, and constipation (*9*−*11*).

Table 1. *R. induratus* Samples Characterization

sample	growing environmental conditions	harvesting	stage of development
GFe	greenhouse (fall)	23-11-2005	early stage
GWm	greenhouse (winter)	22-12-2005	midstage
GWo	greenhouse (winter)	02-02-2006	optimal stage
GWl	greenhouse (winter)	03-03-2006	late stage
GSe	greenhouse (spring)	11-04-2006	early stage
GSm	greenhouse (spring)	23-04-2006	midstage
GSo	greenhouse (spring)	09-05-2006	optimal stage
GSl	greenhouse (spring)	25-05-2006	late stage
Fm	Fervença	01-02-2006	midstage
Fo	Fervença	02-04-2006	optimal stage
Fl	Fervença	23-04-2006	late stage
Pm	Pombares	20-01-2006	midstage
Po	Pombares	02-04-2006	optimal stage
Pl	Pombares	24-04-2006	late stage
Mm	Macedo	04-02-2006	midstage
Mo	Macedo	02-04-2006	optimal stage
Ml	Macedo	25-04-2006	late stage
Rm	Romeu	04-02-2006	midstage
Ro	Romeu	02-04-2006	optimal stage
Rl	Romeu	26-04-2006	late stage
SNm	Sra das Neves	04-02-2006	midstage
SNo	Sra das Neves	02-04-2006	optimal stage
SNl	Sra das Neves	27-04-2006	late stage
Cm	Cerejais	04-02-2006	midstage
Co	Cerejais	02-04-2006	optimal stage
Cl	Cerejais	28-04-2006	late stage
CHm	Chãs	04-02-2006	midstage
CHo	Chãs	02-04-2006	optimal stage
CHl	Chãs	29-04-2006	late stage

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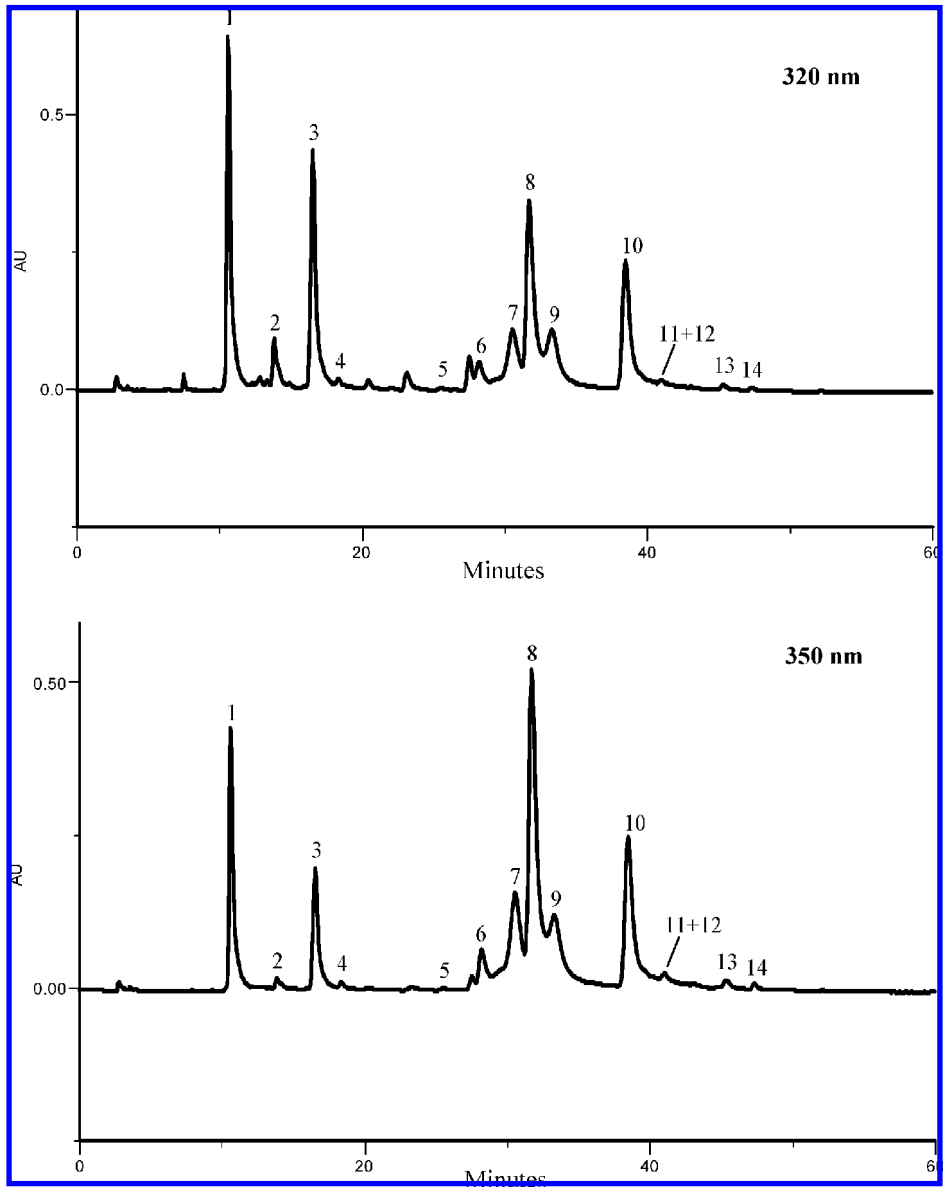

Figure 1. Typical HPLC-DAD chromatogram of the aqueous extract of *R. induratus* leaves. Peaks: 1, caffeoyl-hexoside; 2, *p*-coumaroyl-hexoside; 3, feruloyl-hexoside; 4, sinapoyl-hexoside; 5, 6-*C*-hexosyl-quercetin; 6, 8-*C*-hexosyl-luteolin; 7, 2″-*O*-pentosyl-8-*C*-hexosyl-luteolin; 8, 6-*C*-hexosyl-luteolin; 9, 2″-*O*-pentosyl-8-*C*-hexosyl-apigenin; 10, 6-*C*-hexosyl-apigenin; 11, 3-*O*-hexosyl-quercetin; 12, 3-*O*-rutinosyl-quercetin; 13, 7-*O*-hexosyl-diosmetin; and 14, 3-*O*-rutinosyl-isorhamnetin.

However, their high oxalic acid content has been implicated in oxalic intoxication, mainly in children (*10, 11*). Many *Rumex* species have been recently studied for their biological activities, which revealed a wide range of properties, such as effects in body weight and serum levels of amino acids and minerals (*12*), psychopharmacological (*13*), purgative (*14*), antioxidant and cytotoxic (*15, 16*), antifertility (*17*), antimicrobial and anti-inflammatory (*18*), antidiarrheal (*19*), and antiviral activities (*20*).

Rumex induratus Boiss. & Reuter is an endemic Iberian herb that prefers rocky habitats of the thermo Mediterranean region. It grows spontaneously in Northeast Portugal, where its leaves are highly consumed. This species is commonly served in salads and, to mitigate its acidity, is dressed with olive oil and sometimes mixed with boiled potatoes. Previous studies with *R. induratus* have concerned the determination of phenolic compounds in its leaves (*21, 22*), its oxalic acid and superoxide radical scavenging activities (*21*), its pollen allergenic action (*23*), its mercury bioaccumulation capacity (*24, 25*), and the characterization of DNA in its sex chromosomes (*26*). Regardless of its wide consumption, its biological potential remains little characterized.

The aim of the present work was to evaluate the influence of the growing environmental conditions and developmental stages in the phenolic compound and organic acid compositions of *R. induratus* leaves. For this purpose, targeted metabolite analysis was performed by high-performance liquid chromatography (HPLC)−diode array detection (DAD) and HPLC-UV for phenolic compounds and organic acids, respectively. In addition, the knowledge of the antioxidant capacity was extended, being tested against 2,2-diphenyl-1-picrylhydrazyl radical (DPPH$^{\bullet}$), a reactive oxygen species (hypochlorous acid), and a reactive nitrogen species (nitric oxide).

MATERIALS AND METHODS

Standards and Reagents. Caffeic, *p*-coumaric, ferulic, and sinapic acids, 7-*O*-glucosyl luteolin, 7-*O*-rutinosyl-diosmetin, 7-*O*-glucosyl apigenin, 3-*O*-glucosyl isorhamnetin, 3-*O*-galactosyl quercetin, and 3-*O*-

Table 2. Phenolic Compounds in *R. induratus* leaves (mg/kg of Lyophilized Extract)[a]

	phenolic compound[b]													
sample	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11 + 12**	**13**	**14**	total
GFe	2309.5 ± 0.9	159.2 ± 0.3	1747.6 ± 18.4	623.4 ± 1.3		198.5 ± 3.7	419.6 ± 4.7	787.9 ± 2.1	2784.4 ± 65.9	4885.2 ± 11.4				13914.1
GWm	2554.7 ± 65.9	156.9 ± 10.0	1677.1 ± 50.7	207.4 ± 7.3		290.0 ± 1.1	408.7 ± 10	1257.4 ± 35.0	2609.1 ± 82.5	5235.5 ± 52.8	119.3 ± 16.6			14516.3
GWo	1683.9 ± 5.8	359.2 ± 3.3	2538.7 ± 9.7	70.4 ± 0.3	NQ	976.5 ± 20.4	1475.9 ± 3.2	4820.3 ± 7.9	5608.0 ± 27.6	8821.3 ± 338.5	466.4 ± 10.3	89.4 ± 13	NQ	26910.1
GWl	1503.6 ± 43.8	427.3 ± 10.2	2654.1 ± 106.3	102.7 ± 4.1		1381.3 ± 15.4	3772.2 ± 9.9	9448.7 ± 54.5	7576.5 ± 54.2	10340.5 ± 366.6	618.6 ± 113.4	291.9 ± 76.9	57.2 ± 6.6	38174.6
GSe	2161.1 ± 1.2	188.0 ± 0.7	1485.0 ± 7.6	218.6 ± 2.1		374.9 ± 2.1	1000.2 ± 2.8	1275.9 ± 5.7	3116.8 ± 97.4	6715.2 ± 19.8				16535.6
GSm	3037.1 ± 129.4	196.3 ± 4.4	2034.5 ± 88.2	270.5 ± 2.3		457.1 ± 2.0	809.8 ± 12.8	1794.5 ± 5.0	4163.2 ± 45.4	8957.8 ± 171.4	NQ			21720.7
GSo	4256.0 ± 39.7	355.1 ± 1.7	2677.7 ± 3.4	390.1 ± 7.6		484.6 ± 0.9	665.2 ± 6.0	2491.2 ± 23.1	4583.0 ± 21.5	9159.9 ± 63.5		NQ		25062.9
GSl	3922.9 ± 10.1	242.9 ± 0.7	2999.6 ± 2.9	310.4 ± 0.7		286.3 ± 3.6	343.4 ± 1.8	1203.6 ± 3.3	3735.8 ± 5.7	6598.5 ± 32.0				19643.3
Fm	3017.6 ± 128.5	219.8 ± 0.6	2318.8 ± 41.9	270.9 ± 0.7	32.9 ± 3.2	1712.2 ± 27.2	1365.2 ± 9.8	6224.6 ± 51.0	5537.1 ± 21.4	9113.9 ± 250.2	394.9 ± 8.4	58.9 ± 1.0	46.2 ± 04	30313.0
Fo	2703.5 ± 26.1	559.3 ± 2.1	2645.5 ± 2.8	36.7 ± 1.7	52.8 ± 0.9	3018.1 ± 2.7	1913.4 ± 14.2	8769.2 ± 122.4	5926.8 ± 117.7	8974.9 ± 138.7	252.7 ± 11.9	117.5 ± 67.2	434.5 ± 3.9	35404.9
Fl	2905.6 ± 12.4	742.1 ± 7.3	3268.2 ± 4.7	51.2 ± 1.1	151.9 ± 5.1	3824.9 ± 98.6	5346.1 ± 36.9	16204.4 ± 187.2	8693.5 ± 72.5	10700.9 ± 65.3	712.7 ± 55.7	33.5 ± 4.7	456.0 ± 11.7	53091.1
Pm	1147.1 ± 20.8	278.7 ± 5.0	2003.5 ± 52.1	500.8 ± 6.1	34.5 ± 0.2	1741.7 ± 47.4	667.4 ± 17.1	5776.3 ± 137.4	4317.5 ± 171.2	8112.9 ± 30.9	403.0 ± 14.7		19.5 ± 0.6	25002.9
Po	3702.8 ± 5.6	292.2 ± 1.4	3765.8 ± 22.6	230.1 ± 3.4	111.6 ± 0.8	1819.2 ± 44.3	4506.1 ± 9.5	14087.6 ± 240.3	8633.7 ± 135.3	12648.9 ± 40.2	745.2 ± 36.6	348.5 ± 1.7	994.4 ± 35.6	47962.9
Pl	2932.0 ± 5.3	356.2 ± 12.7	3742.5 ± 95.2	132.0 ± 2.2	166.2 ± 11.0	2712.7 ± 3.5	4215.2 ± 160.7	17208.1 ± 130.0	8225.7 ± 26.5	17152.8 ± 234.7	1819.8 ± 161.1		1127.2 ± 77.8	59790.5
Mm	2971.9 ± 22.1	401.1 ± 0.1	3526.1 ± 5.4	331.0 ± 3.4	48.8 ± 0.9	1676.8 ± 16.5	1607.4 ± 62.9	6258.4 ± 48.3	6148.1 ± 61.2	10688.7 ± 225.2	446.0 ± 50.3	124.2 ± 4.1	287.0 ± 7.5	34515.7
Mo	3959.9 ± 10.6	322.0 ± 7.3	4287.1 ± 3.4	189.9 ± 0.8	86.1 ± 9.0	1026.4 ± 18.8	3731.5 ± 59.6	9249.4 ± 27.4	7438.8 ± 35.3	10723.2 ± 26.7	981.6 ± 2.0	671.2 ± 26.4	761.6 ± 10.7	43428.7
Ml	3278.8 ± 14.0	438.7 ± 3.6	2904.4 ± 14.0	35.3 ± 1.1	121.8 ± 35.2	1315.1 ± 72.2	5653.2 ± 234.1	15472.3 ± 297.4	8814.7 ± 130.8	11734.0 ± 226.0	1711.2 ± 7.1	562.1 ± 5.8	861.1 ± 70.1	52902.8
Rm	3970.7 ± 3.1	283.4 ± 2.0	3171.0 ± 92.3	207.2 ± 1.5		1107.8 ± 72.1	449.2 ± 1.3	3229.0 ± 43.4	2170.3 ± 30.0	4549.2 ± 105.6	574.3 ± 22.1	179.7 ± 0.1	143.2 ± 0.8	20035.0
Ro	4489.8 ± 43.6	439.8 ± 1.3	4177.4 ± 5.2	105.9 ± 20.8	133.0 ± 34.5	2862.7 ± 22.9	3002.0 ± 41.4	13388.9 ± 51.0	5910.5 ± 92.8	14202.3 ± 9.7	1016.2 ± 41.3	118.0 ± 20.0	741.0 ± 47.1	50587.6
Rl	2969.5 ± 52.4	293.4 ± 6.9	3064.7 ± 58.8	86.2 ± 4.9	41.0 ± 3.6	2475.0 ± 89.8	4325.7 ± 8.9	15080.6 ± 158.9	6402.2 ± 38.4	9972.2 ± 225.3	841.4 ± 20.5	375.4 ± 29.4	437.7 ± 48.2	46364.9
SNm	3257.0 ± 1.3	275.5 ± 0.8	2595.8 ± 41.5	344.4 ± 7.7	51.0 ± 0.4	1414.2 ± 25.3	1073.7 ± 11.4	7152.2 ± 30.3	5284.9 ± 43.0	9935.1 ± 65.5	670.3 ± 22.0	129.4 ± 6.2	49.2 ± 0.5	32232.8
SNo	6418.0 ± 69.6	862.3 ± 7.8	5163.9 ± 33.1	104.7 ± 3.0	107.6 ± 4.0	2029.1 ± 2.5	4590.9 + 45.3	17687.7 ± 14.6	7689.1 ± 231.3	14703.7 ± 52.9	2494.7 ± 85.5	747.1 ± 59.8	389.7 ± 0.1	62988.6
SNl	5991.3 ± 44.9	607.7 ± 11.8	5012.1 ± 90.6	217.8 ± 0.4	136.5 ± 15.2	1479.5 ± 25.0	4170.7 ± 99.1	18600.4 ± 21.5	5587.3 ± 239.1	13067.7 ± 461.9	2466.0 ± 150.3	689.6 ± 31.5	371.7 ± 15.7	58398.4
Cm	3627.8 ± 0.7	540.2 ± 1.4	4013.8 ± 25.7	726.7 ± 1.4		1391.6 ± 2.1	884.3 ± 34.3	5259.6 ± 20.8	3552.6 ± 63.1	7996.4 ± 45.1	352.8 ± 22.3	91.5 ± 10.7	NQ	28437.4
Co	3885.0 ± 0.9	991.3 ± 7.8	4339.3 ± 13.0	226.4 ± 0.3	19.9 ± 0.7	1926.2 ± 16.9	5109.5 ± 10.7	14664.3 ± 102.0	7079.2 ± 7.3	16374.2 ± 51.0	677.8 ± 0.3	398.8 ± 2.3	309.0 ± 4.3	56000.8
Cl	2999.8 ± 13.4	927.8 ± 1.7	3035.2 ± 15.2	157.6 ± 0.6	86.0 ± 1.6	1779.2 ± 5.5	5191.5 ± 111.4	14924.4 ± 40.4	6582.3 ± 7.3	14824.3 ± 128.5	858.7 ± 8.3	342.0 ± 0.2	295.9 ± 10.9	52004.7
CHm	1608.5 ± 19.3	447.0 ± 19.9	2888.0 ± 20.4	639.5 ± 6.1	43.5 ± 2.7	2152.5 ± 51.5	1226.8 ± 9.2	6526.4 ± 7.3	3486.4 ± 2.9	8119.3 ± 33.3	513.5 ± 32.3	102.8 ± 0.6	NQ	27754.1
CHo	5233.2 ± 63.4	913.4 ± 10.4	5793.9 ± 59.8	172.4 ± 1.4		1324.0 ± 14.0	3984.7 ± 231.2	12652.3 ± 460.7	5438.9 ± 11.9	12784.4 ± 243.7	634.6 ± 4.4	455.6 ± 2.7	300.1 ± 6.8	49687.4
CHl	2779.0 ± 2.8	934.4 ± 9.3	3346.1 ± 18.7	85.9 ± 2.0	45.7 ± 3.7	2512.3 ± 38.3	5572.8 ± 9.9	16013.4 ± 122.0	7286.1 ± 108.9	16105.4 ± 58.1	1211.7 ± 35.6	748.8 ± 3.0	331.0 ± 0.3	56972.6

[a] Values show means ± SD from triplicate. [b] Compounds: **1**, caffeoyl-hexoside; **2**, *p*-coumaroyl-hexoside; **3**, feruloyl-hexoside; **4**, sinapoyl-hexoside; **5**, 6-*C*-hexosyl-quercetin; **6**, 8-*C*-hexosyl-luteolin; **7**, 2″-*O*-pentosyl-8-*C*-hexosyl-luteolin; **8**, 6-*C*-hexosyl-luteolin; **9**, 2″-*O*-pentosyl-8-*C*-hexosyl-apigenin; **10**, 6-*C*-hexosyl-apigenin; **11**, 3-*O*-hexosyl-quercetin; **12**, 3-*O*-rutinosyl-quercetin; **13**, 7-*O*-hexosyl-diosmetin; **14**, 3-*O*-rutinosyl-isorhamnetin; and NQ, nonquantifiable.

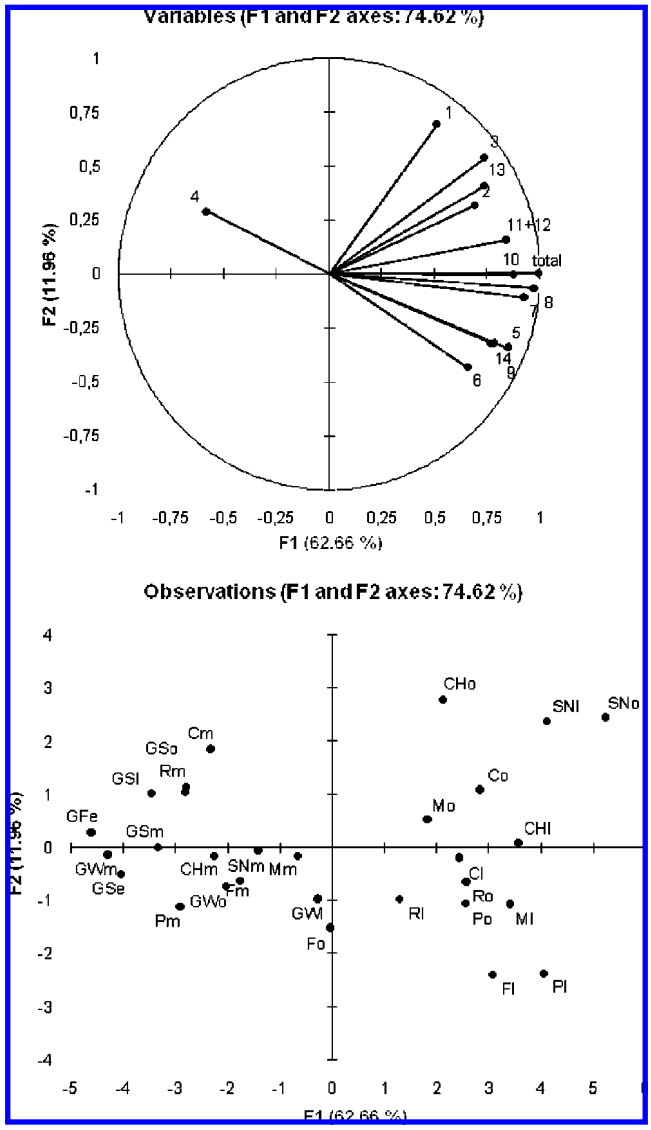

Figure 2. Principal components diagram of the phenolic compounds content from all analyzed samples: factor score plot 1-2. Components 1 and 2 account for 74.62% of the total variance. The identities of compounds are as in **Figure 1** and of samples are as in **Table 1**.

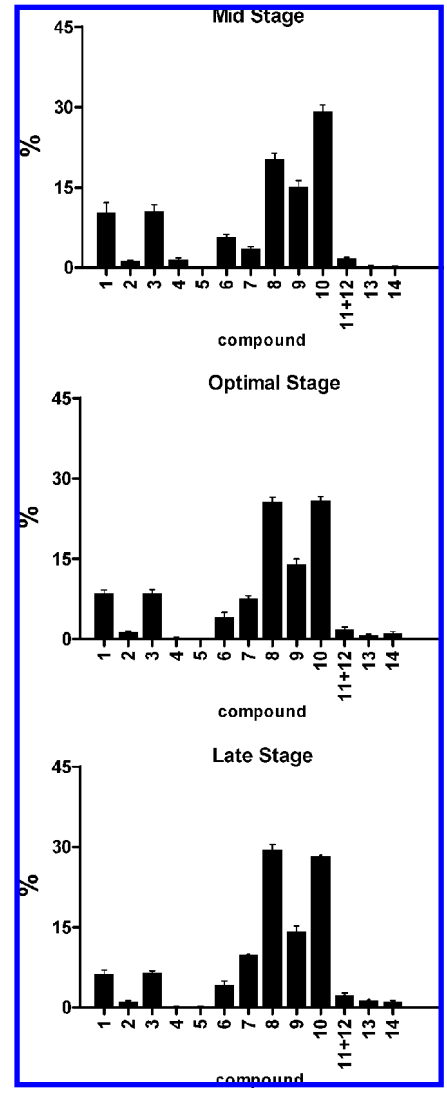

Figure 3. Phenolic compounds profile of field samples of *R. induratus* leaves. The identities of compounds are as in **Figure 1**.

rutinosyl quercetin were purchased from Extrasynthése (Genay, France). Oxalic, citric, malic, ascorbic, and shikimic acids and sulfanilamide were from Sigma (St. Louis, MO). *N*-(1-Naphthyl)-ethylen-diamine dihydrochlorid, methanol, formic, sulfuric, and *o*-phosphoric acid were purchased from Merck (Darmstadt, Germany). The water was treated in a Milli-Q water purification system (Millipore, Bedford, MA). Sodium nitroprussiate dehydrate (SNP) was obtained from Riedel-deHaën. DPPH$^{\bullet}$, sodium hypochlorite solution with 4% available chlorine (NaOCl), and 5,5-dithiobis(2-nitrobenzoic acid) (DTNB) were obtained from Sigma Chemical Co.

Plant Material. *R. induratus* samples spontaneously occurring in Bragança (Northeast Portugal) or growing in the greenhouse of Escola Superior Agrária (Instituto Politécnico de Bragança) were analyzed (**Table 1**). The greenhouse samples were developed in a double-wall polycarbonate greenhouse. The plants were grown under a mixture 3:1:1 of organic substrate, sand, and vermiculite. The substrate was a mixture of peat with other raw materials, such as coco fiber. The main properties of the substrate were 90% organic matter, 10% ash content, 50% moisture content, and pH 5.5−6.0. The nutrients in the substrate were as follows: total nitrogen, 2 mg kg^{-1}; phosphorus, 1 mg kg^{-1}; calcium, 5 mg kg^{-1}; magnesium, 1 mg kg^{-1}; iron, 2 mg kg^{-1}; and chloride, 1.5 mg kg^{-1}. The substrate included a compound N:P:K (15:10:20) fertilizer (plus micronutrients) and dolomitic limestone as additives. The plants were regularly irrigated from the automatic nebulization system of the greenhouse.

The early stage refers to plantules, the optimal stage corresponds to the growth phase at which they are usually consumed, and the late stage refers to samples near the end of the vegetative cycle, already exhibiting flower buds. After the plants were harvested, aerial parts were immediately transferred to the laboratory and kept in a freezer at −20 °C, prior to their lyophilization in a Labconco 4.5 Freezone apparatus (Kansas City, MO). The dried leaves were separated and powdered.

Sample Preparation. For the preparation of extracts, 3 g of powdered leaves was boiled in 500 mL of water for 30 min. The extract was then filtered over a Büchner funnel. The resulting aqueous extract was lyophilized in a Labconco 4.5 Freezone apparatus (Kansas City, MO). The lyophilized extracts were kept in an exsicator in the dark.

For the identification and quantification of phenolic compounds, the lyophilized extract (ca. 20 mg) was dissolved in 1 mL of water. For the determination organic acids, ca. 5 mg was thoroughly mixed with 1 mL of 0.01 N sulfuric acid.

HPLC-UV Analysis of Organic Acids. Twenty microliters of the redissolved extract was analyzed as previously reported (*21*), in a system consisting of an analytical HPLC unit (Gilson) in conjunction with a column heating device set at 30 °C, with an ion exclusion column, Nucleogel Ion 300 OA (300 mm × 7.7 mm). Elution was carried out isocratically at a solvent flow rate of 0.2 mL min^{-1}, with 0.01 N sulfuric acid. The detection was performed with an UV detector at 214 nm. Organic acids quantification was achieved

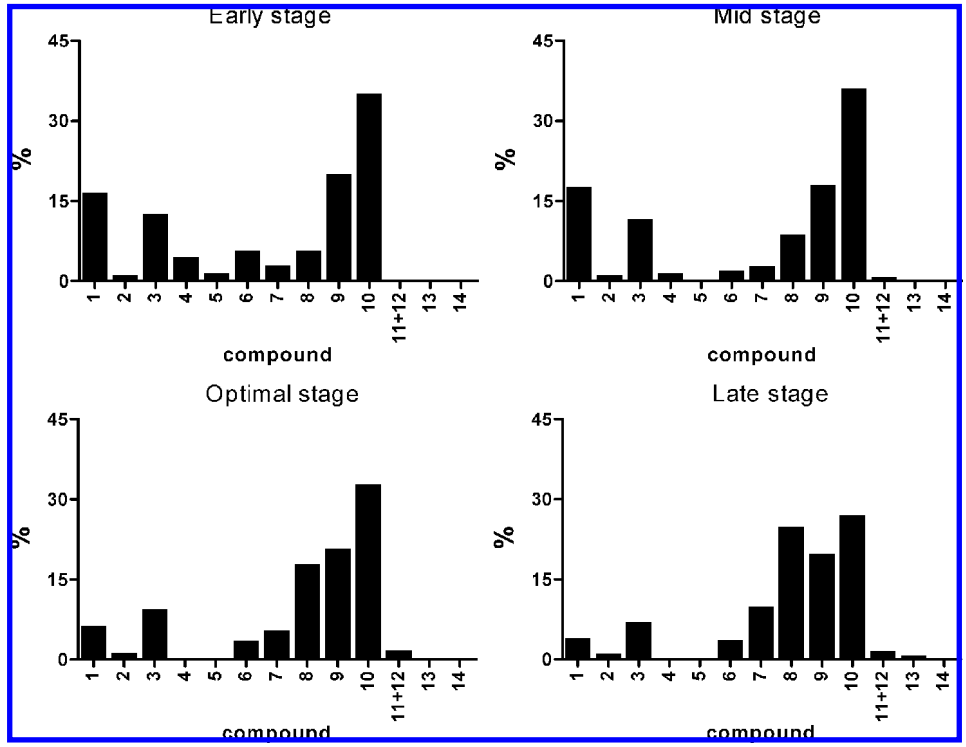

Figure 4. Phenolic compounds profile of greenhouse samples from fall/winter of *R. induratus* leaves. The identities of compounds are as in **Figure 1**.

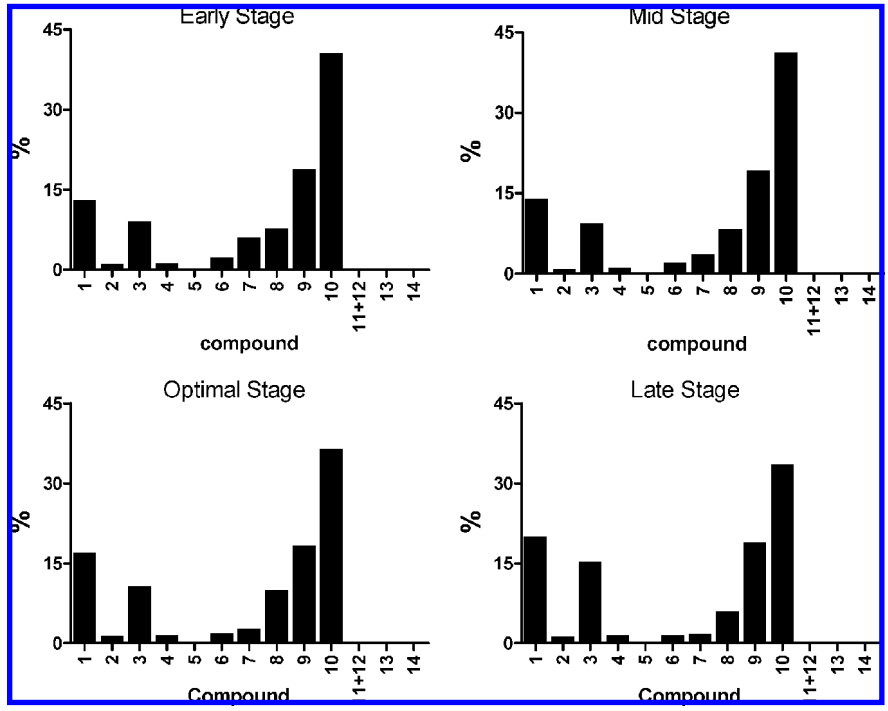

Figure 5. Phenolic compounds profile of greenhouse samples from spring of *R. induratus* leaves. The identities of compounds are as in **Figure 1**.

by the absorbance recorded in the chromatograms relative to external standards, and the peaks in the chromatograms were integrated using a default baseline construction technique.

HPLC-DAD Analysis of Phenolic Compounds. The redissolved aqueous lyophilized extracts (20 μL) were analyzed using an analytical HPLC unit (Gilson), with a Spherisorb ODS2 (25.0 cm $\times$ 0.46 cm; 5 μm, particle size) column and a solvent mixture of water−formic acid (19:1) (A) and methanol (B) (*21*). Elution was carried out at 0.9 mL min^{-1} starting with 5% B at 0 min and installing a gradient to obtain 15% B at 3 min, 25% B at 13 min, 30% B at 25 min, 35% B at 35 min, 45% B at 39 min, 45% B at 42 min, 50% B at 44 min, 55% B at 47 min, 70% B at 50 min, 75% B at 56 min, and 80% B at 60 min. Detection was achieved with a Gilson diode array detector. Spectral data from all peaks were accumulated in the 200−400 nm range, and chromatograms were recorded at 320 nm for phenolic acids derivatives and at 350 nm for flavonoidic compounds. The data were processed on Unipoint system software (Gilson Medical Electronics, Villiers le Bel, France).

Phenolic compounds quantification was achieved by the absorbance recorded in the chromatograms relative to external standards. Because standards of the compounds identified were not commercially available, caffeic, *p*-coumaric, ferulic, and sinapic acid derivatives were quantified as caffeic, *p*-coumaric, ferulic, and sinapic acids, respectively; luteolin derivatives were quantified as 7-*O*-glucosyl-luteolin, apigenin derivatives

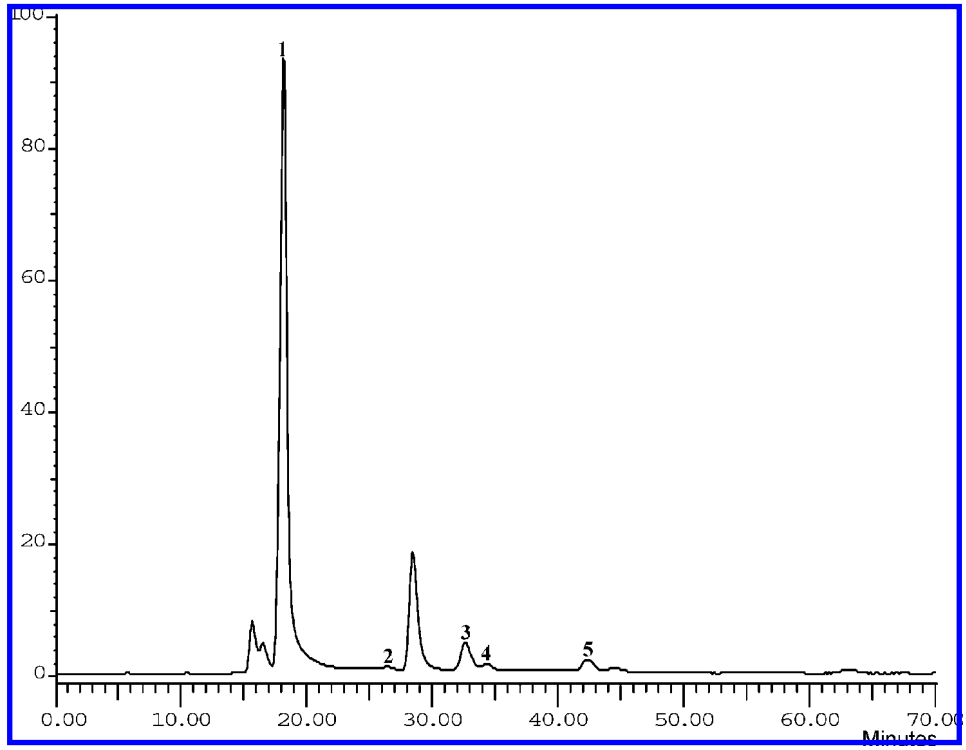

Figure 6. Typical organic acids profile of the aqueous extract of *R. induratus* leaves obtained by HPLC-UV. Detection at 214 nm. Peaks: 1, oxalic acid; 2, citric acid; 3, malic acid; 4, ascorbic acid; and 5, shikimic acid.

Table 3. Organic Acids in *R. induratus* Leaves (g/kg of Lyophilized Extract)[a]

	organic acid					
sample	oxalic	citric	malic	ascorbic	shikimic	total
GFe	362.8 ± 6.5	6.7 ± 3.1	79.6 ± 0.2	2.6 ± 0.4	0.16 ± 0.00	451.8
GWm	201.4 ± 0.1	9.6 ± 1.8	105.7 ± 1.3	2.4 ± 0.4	0.11 ± 0.01	319.3
GWo	112.8 ± 0.6		48.5 ± 1.8	1.8 ± 0.1	1.08 ± 0.00	164.3
GWl	114.8 ± 0.5	NQ	12.9 ± 0.4	1.5 ± 0.1	1.09 ± 0.03	130.3
GSe	354.1 ± 1.4	NQ	25.5 ± 0.3	0.9 ± 0.0	0.23 ± 0.00	380.7
GSm	388.0 ± 3.9	15.4 ± 1.4	33.2 ± 0.1	1.0 ± 0.0	0.14 ± 0.00	437.7
GSo	308.0 ± 1.3	5.9 ± 0.3	33.8 ± 0.7	0.6 ± 0.0	0.17 ± 0.00	348.4
GSl	293.7 ± 0.9	5.8 ± 1.9	46.1 ± 3.6	2.2 ± 0.3	0.18 ± 0.02	347.9
Fm	127.1 ± 0.3	5.3 ± 0.0	76.4 ± 0.9	2.1 ± 0.0	0.37 ± 0.00	211.3
Fo	196.8 ± 1.0	2.7 ± 0.2	24.0 ± 0.9	NQ	0.68 ± 0.00	224.2
Fl	159.5 ± 0.3	2.6 ± 0.1	28.3 ± 0.9	1.0 ± 0.6	0.95 ± 0.01	192.4
Pm	72.0 ± 0.3	7.2 ± 1.8	125.1 ± 0.4	NQ	0.16 ± 0.00	204.4
Po	209.3 ± 4.4	NQ	39.7 ± 3.4	2.4 ± 0.3	0.47 ± 0.00	251.9
Pl	156.2 ± 0.5	NQ	30.0 ± 4.0	NQ	3.19 ± 0.01	189.3
Mm	73.4 ± 0.5	12.9 ± 4.7	102.7 ± 1.3	NQ	0.29 ± 0.03	189.3
Mo	205.0 ± 1.6	0.0	77.6 ± 2.1	NQ	0.67 ± 0.02	283.2
Ml	161.2 ± 0.4	NQ	39.2 ± 1.9	1.9 ± 0.5	1.30 ± 0.02	203.6
Rm	96.8 ± 0.7	17.6 ± 4.0	189.8 ± 5.8	0.9 ± 0.2	0.51 ± 0.13	305.6
Ro	177.0 ± 0.7	NQ	47.9 ± 2.4	NQ	0.93 ± 0.01	225.8
Rl	153.7 ± 0.8	NQ	49.4 ± 1.0	NQ	1.19 ± 0.02	208.0
SNm	90.0 ± 0.8	3.5 ± 0.3	83.9 ± 2.0	1.9 ± 0.2	0.24 ± 0.00	179.5
SNo	163.0 ± 2.4		18.3 ± 0.2		0.87 ± 0.01	182.2
Snl	176.3 ± 2.2		25.4 ± 1.2		0.91 ± 0.00	202.6
Cm	160.9 ± 3.9	9.5 ± 0.1	109.7 ± 2.3	NQ	0.31 ± 0.02	280.4
Co	224.1 ± 2.0		29.3 ± 0.7	NQ	1.02 ± 0.11	254.4
Cl	182.5 ± 0.9		37.1 ± 2.7	NQ	0.68 ± 0.07	220.3
CHm	80.7 ± 2.0	8.2 ± 0.9	94.9 ± 0.2	NQ	0.14 ± 0.00	184.0
CHo	205.0 ± 0.9		23.2 ± 3.1	NQ	0.35 ± 0.01	228.5
CHl	108.9 ± 0.3		59.8 ± 7.7	NQ	0.42 ± 0.00	169.2

[a] Values show means ± SD from triplicate; NQ, nonquantifiable.

were quantified as 7-*O*-glucosyl-apigenin, 6-*C*-hexosyl-quercetin was quantified as 3-*O*-galactosyl-quercetin, diosmetin derivative was quantified as 7-*O*-rutinosyl-diosmetin, and isorhamnetin derivative was quantified as 3-*O*-glucosyl isorhamnetin. 3-*O*-Hexosyl-quercetin and 3-*O*-rutinosyl-quercetin were quantified together as 3-*O*-rutinosyl-quercetin.

DPPH• Radical Scavenging Activity. The antiradical activity was determined spectrophotometrically in a Multiskan Ascent microplate reader (Thermo Laboratory Systems) by monitoring the disappearance of DPPH• at 515 nm, according to a described procedure (*27*). The reaction mixtures in the sample wells consisted of 25 μL of aqueous

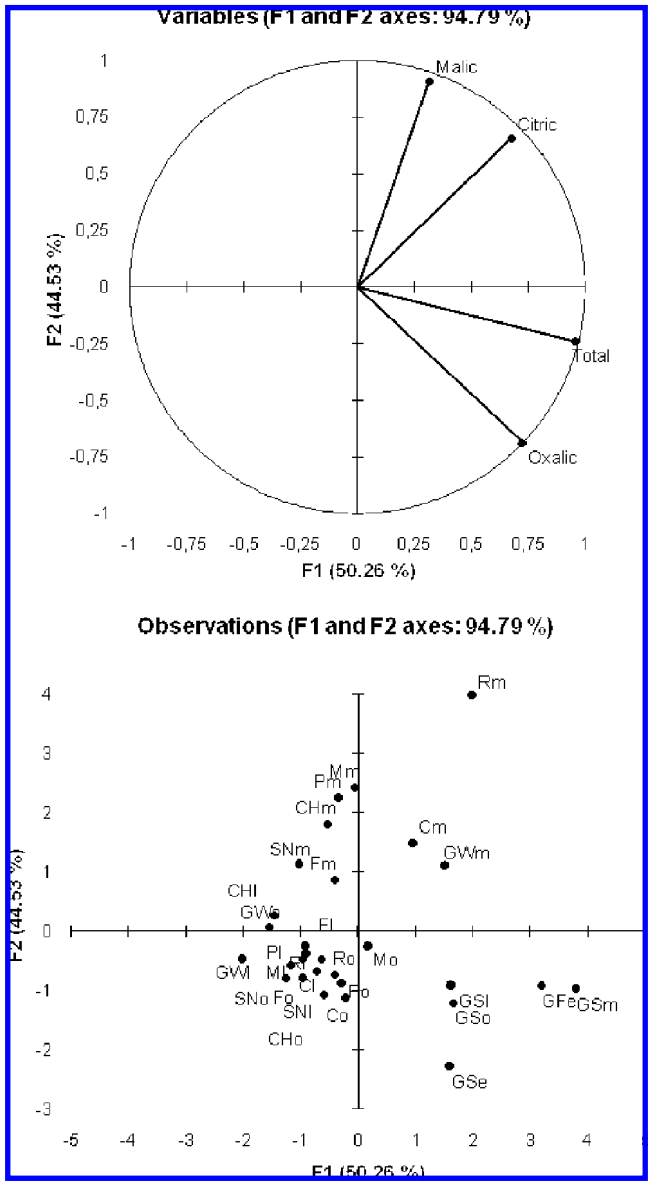

Figure 7. Principal components diagram of the organic acids content in all analyzed samples: factor score plot 1-2. Components 1 and 2 account for 94.79% of the total variance. The identities of samples are as in **Table 1**.

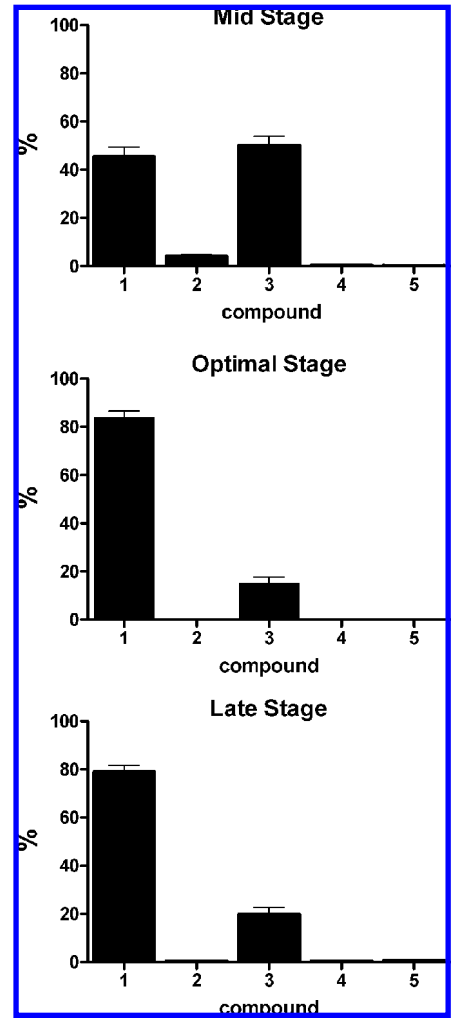

Figure 8. Organic acids profile of field samples of *R. induratus* leaves. The identities of compounds are as in **Figure 6**.

extract (five different concentrations) and 200 μL of DPPH$^{\bullet}$ dissolved in methanol. The reaction was conducted at room temperature (22−25 °C) for 20 min. Three experiments were performed in triplicate.

Nitric Oxide Radical Scavenging Activity. The antiradical activity was determined spectrophotometrically in a 96-well plate reader (Multiskan Ascent, Thermo Laboratory Systems) according to the described procedure (*27*). The reaction mixtures in the sample wells consisted of extract and SNP dissolved in saline phosphate buffer (pH 7.4), and plates were incubated at 25 °C for 60 min under light exposure. Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochoride in 2% H_3PO_4) was then added, and the absorbance was determined at 540 nm. Three experiments were performed in triplicate.

Hypochloride Radical Scavenging Activity. The inhibition of hypochlorous acid-induced 5-thio-2-nitrobenzoic acid (TNB) oxidation to DTNB, at room temperature (22−25 °C), was observed using a double beam spectrophotometer (Heλios α, Unicam), as previously described (*27*). Hypochlorous acid (75 mM) was prepared by adjusting a solution of NaOCl to pH 6.2 with diluted sulfuric acid and TNB by adding 20 mM sodium borohydride to a solution of DTNB [1 mM in potassium phosphate buffer (50 mM, pH 6.6) containing 5 mM EDTA] immediately before use. The amount of TNB remained unchanged after incubation and was calculated and expressed as a percentage of the initial value. Three experiments were performed in triplicate.

Statistical Analysis. Principal component analysis (PCA) was carried out using XLSTAT 2008.5 software. The PCA method shows similarities between samples projected on a plane and makes it possible to identify which variables determine these similarities and in what way.

RESULTS AND DISCUSSION

***R. induratus* Phenolic Compounds.** HPLC-DAD analysis of the aqueous extracts of *R. induratus* leaves revealed the presence of caffeoyl-hexoside, *p*-coumaroyl-hexoside, feruloyl-hexoside, sinapoyl-hexoside, 6-*C*-hexosyl-quercetin, 8-*C*-hexosyl-luteolin, 2″-*O*-pentosyl-8-*C*-hexosyl-luteolin, 6-*C*-hexosyl-luteolin, 2″-*O*-pentosyl-8-*C*-hexosyl-apigenin, 6-*C*-hexosyl-apigenin, 3-*O*-hexosyl-quercetin, 3-*O*-rutinosyl-quercetin, 7-*O*-hexosyl-diosmetin, and 3-*O*-rutinosyl-isorhamnetin (**Figure 1**). All of these compounds have been reported before in this species (*21, 22*).

The quantification of the detected phenolic compounds revealed some differences among the analyzed samples (**Table 2**). Samples produced in the field (samples Fm-CHl) clearly exhibited higher total phenolic contents than those from greenhouse samples (samples GFe-GSl) with the exception of those of the mid-development stage (**Figure 2**). It seems that the total amount of phenolic compounds in field samples increased throughout the plant cycle (**Table 2** and **Figure 2**).

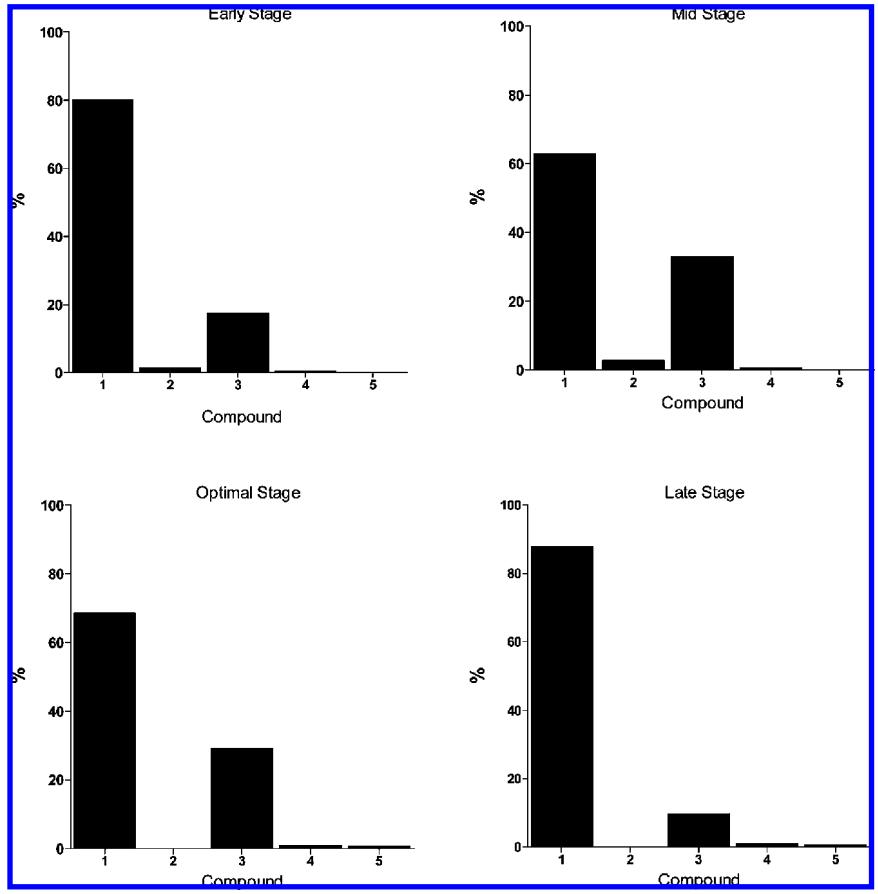

Figure 9. Organic acids profile of greenhouse samples from fall/winter of *R. induratus* leaves. The identities of compounds are as in **Figure 6**.

The phenolic profile of early stages of plant development of field samples showed 6-*C*-hexosyl apigenin as the most abundant compound, followed by 6-*C*-hexosyl luteolin. This relation is inverted during the plant development after the optimal stage for human consumption. Caffeoyl-hexoside, feruloyl-hexoside, and 2″-*O*-pentosyl-8-*C*-hexosyl-apigenin depict a slight decrease throughout plant growth, while 2″-*O*-pentosyl-8-*C*-hexosyl-luteolin increases during plant development. All other compounds have low and fairly stable amounts (**Table 2** and **Figure 3**). Synapoil hexoside appears to be a marker for later stages of maturation, decreasing during the plant development (**Table 2** and **Figure 2**).

As referred above, greenhouse samples (samples GFe-GSl) show lower amounts of phenolic compounds than those observed in field samples (**Table 2** and **Figure 2**), which can be due to more reduced environmental agression (*28*). Within these samples, those collected in the spring exhibited different behavior in what concerns the total amount of phenolic. In early stages of the plant cycle, spring samples showed higher contents than fall/winter samples, but both increased in the same manner. At the end of the plant development, the total amount of phenolic compounds in fall/winter samples continued to increase, but in spring samples, a slight decrease was noticed (**Table 2**).

The major compound is, in both sets of greenhouse samples and in all developmental stages, 6-*C*-hesoxyl apigenin. In fall/winter samples (GFe-GWl), 2″-*O*-pentosyl-8-*C*-hexosyl-apigenin, caffeoil-hexoside, and feruloyl-hexoside begin as second, third, and fourth most important compounds, respectively, but are, in later stages of the plant cycle, surpassed by 6-*C*-hexosyl luteolin. During the evolution, caffeoyl-hexoside swaps positions with feruloyl-hexoside, and both become far less important than 2″-pentosyl-8-*C*-hexosyl-apigenin. 2″-*O*-Pentosyl-8-*C*-hexosyl-luteolin exhibits an increase throughout the plant cycle (**Table 2** and **Figure 3**). In spring samples (GSe-GSl), 6-*C*-hexosyl luteolin does not evolve into becoming one major compound. 2″-*O*-Pentosyl-8-*C*-hexosyl-apigenin amounts are stable throughout the cycle, and it is the second most important compound just until the late stage of development, in which it is surpassed by caffeoyl-hexoside. Caffeoyl-hexoside and feruloyl-hexoside are the third and fourth most abundant compounds in early stages of development, increasing slightly in a parallel manner (**Table 2** and **Figure 4**). Samples collected during fall/winter in the greenhouse presented a greater resemblance to field samples of early stages (**Figure 2**), although subsequently 6-*C*-hexosyl luteolin with a high increase and 6-*C*-hesoxyl apigenin with a marked decrease, characteristic of the latter, are less marked (**Table 2** and **Figures 3** and **4**).

***R. induratus* Organic Acids.** The HPLC-UV analysis of the aqueous lyophilized extracts revealed the presence of oxalic, citric, malic, ascorbic, and shikimic acids (**Figure 6**). With the exception of oxalic acid, the remaining ones are reported for the first time in this species. Greenhouse samples exhibited higher organic acids contents than those from field growth, with the exceptions of those from the more developed stages of fall/winter harvest (samples GWo and GWl) (**Table 3** and **Figure 7**). In a general way, field samples (Fm-CHl) depicted a slight decrease in the total amount of organic acids during plant growth, with the exception of samples collected in Sra das Neves (samples SNm-SNl) (**Table 3**).

In these samples, malic and oxalic acid are the major compounds in the early stage of plant development (**Figure 8**). However, malic acid amounts decrease very rapidly, and oxalic acid becomes the most abundant compound for the rest of the plant's cycle (**Figure 7** and **8**). These findings are consistent with the literature, reporting several *Rumex* species as having high contents of oxalic acid (*9*−*11*).

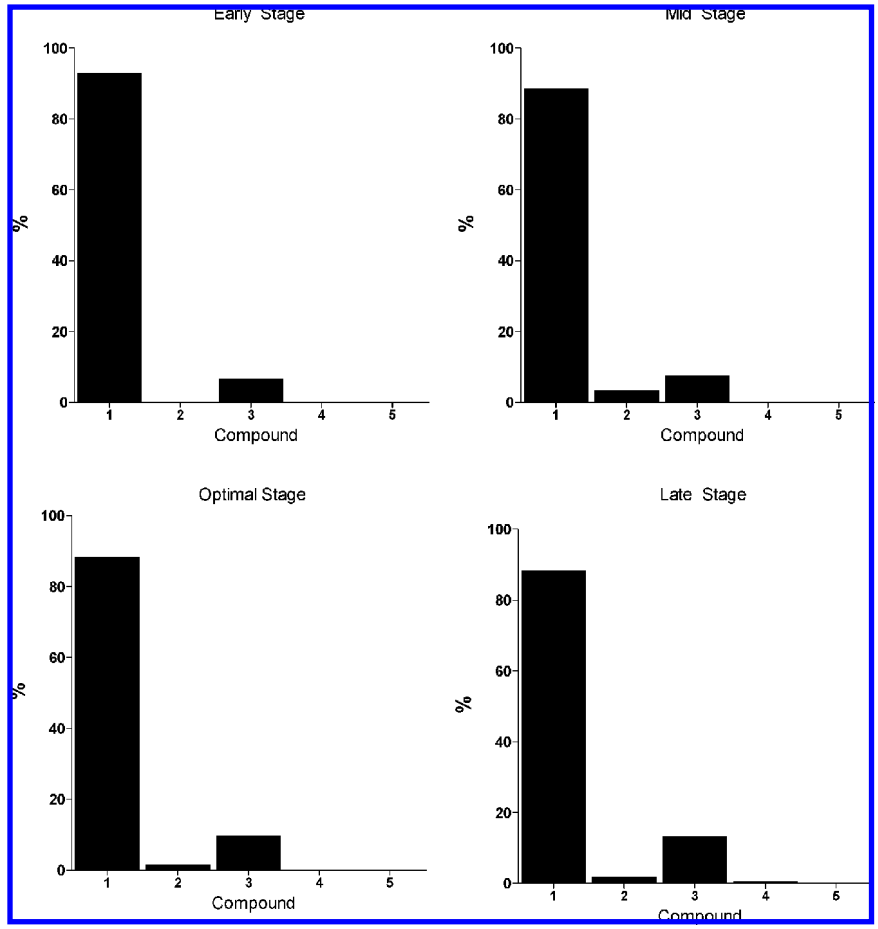

Figure 10. Organic acids profile of greenhouse samples from spring of *R. induratus* leaves. The identities of compounds are as in **Figure 6**.

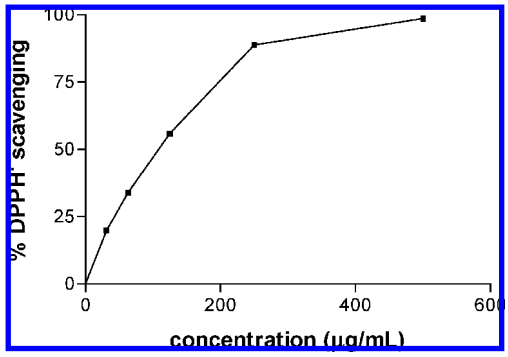

Figure 11. Effect of *R. induratus* on DPPH• reduction. Values show means ± SE from three experiments performed in triplicate.

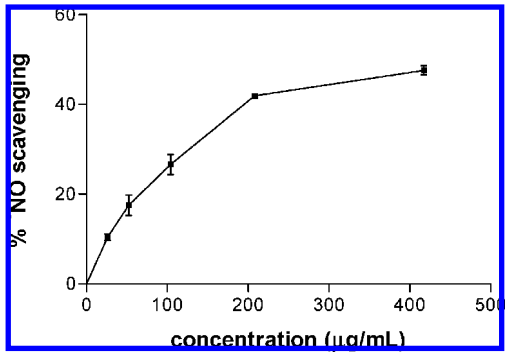

Figure 12. *R. induratus* scavenging of nitric oxide. Values show means ± SE from three experiments performed in triplicate.

In fact, this compound was found in very high amounts in the lyophilized extract (**Table 3**), especially in those stages that

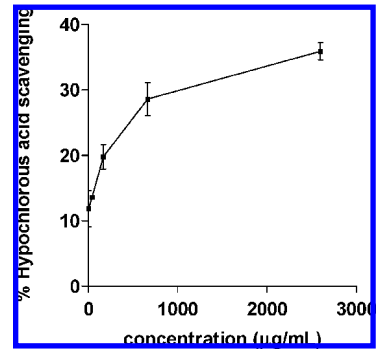

Figure 13. *R. induratus* scavenging of hypochlorous acid. Values show means ± SE from three experiments performed in triplicate.

correspond to optimal development for human consumption. The high oxalic acid level may be responsible for the acidity of the plant and must be a cause for concern due to its ability to form insoluble calcium salts, disturbing the calcium concentrations, and ultimately affecting the blood coagulation mechanism (*11, 29*). Besides, the ingestion of oxalates may trigger gastrointestinal symptoms, and the systemic absorption can lead to kidney damage (*10*). Generally, field samples at the middle stage exhibit the lowest amount of oxalic acid than in any other stage of development (**Figure 7**). Nevertheless, all samples show high amounts of oxalic acid, and according to the above-mentioned, that should be taken into account when consuming *R. induratus* leaves. On the other hand, the high oxalic acid content may play an important role as a pH regulator and osmoregulator in the plant, accounting also for its protection against microbial pathogens and foraging animals and insects, by affecting taste, texture, and calcium availability (*29*). Malic acid seems to be related with the maturity degree of the

plant spontaneously occurring: considering the evolution observed, younger plants exhibit higher amounts of this compound. In fact, previous works have already reported some relation between the malic acid and the ripeness stage of fruits, either increasing or decreasing with maturation (*38, 39*). Our results appear to be in agreement with the latter.

In fall/winter samples from greenhouse growth (samples GFe-GWl), the total amount of organic acids decreases throughout the plant's cycle, but those from the spring harvest (samples GSe-GSl) showed a slight increase in the middle stages of development (**Table 3** and **Figure 7**). The decrease observed in total organic acids contents can also be noticed in most fruit during ripening, as a consequence of its use as respiratory substrates and as carbon skeletons for the synthesis of new compounds (*40*).

The organic acids profiles of greenhouse samples evolved differently from that of field samples, although oxalic acid is the most important compound in both sets of samples and in all stages of development (**Figures 9** and **10**). In fall/winter samples, oxalic acid amounts exhibit a reduction for the middle stages of development, in opposition to malic acid amounts that depict a peak in the same stages. In spring samples, oxalic acid is also the most important compound, but its contents are stable during plant growth. All other organic acids are present in very little amounts and show very little variation (**Table 3**). The organic acids profile of fall/winter samples is closer to that observed for field samples during the optimal stage of development for human consumption (**Figure 7**).

The amount of oxalic acid in greenhouse samples from spring is higher than that observed in field samples (**Table 3** and **Figure 7**), and it represents an increased risk for oxalic acid intoxication. Fall/winter samples show lower oxalic acid contents and, therefore, might pose less risk for human health when consumed.

Antioxidant Activity. The DPPH$^{\bullet}$ assay is a basic assessment for antiradical activity of extracts: the stable free radical, once reduced by the antioxidant, no longer displays absorbance at 515 nm (*27*). The lyophilized aqueous extract of *R. induratus* leaves (sample SNo) exhibited a strong concentration-dependent antioxidant potential ($IC_{50} = 106.5\ \mu g\ mL^{-1}$) (**Figure 11**).

Nitric oxide and hypochlorous acid can be responsible for the formation of more reactive species, such as hydroxyl radical (*30*). The aqueous extract of *R. induratus* leaves showed a potent scavenging activity against nitric oxide in a concentration-dependent manner, with an IC_{50} of $92.7\ \mu g\ mL^{-1}$ (**Figure 12**). The extract exhibited a lower activity against hypochlorous acid. Nevertheless, a concentration-dependent antioxidant potential was observed ($IC_{20} = 171.3\ \mu g\ mL^{-1}$) (**Figure 13**). These findings, along with the fact that *R. induratus* leaves can act as superoxide radical scavengers and xanthine oxidase inhibitors (*21*), are extremely valuable: The simultaneous scavenging capacity of superoxide radical and nitric oxide can prevent the formation of peroxynitrite and, ultimately, hydroxyl radical (*30*).

The IC_{50} found for the DPPH$^{\bullet}$ assay is lower than that previously reported for this radical species under the same assay conditions (*21*), revealing a higher antioxidant potential, possibly due to the higher content of phenolic compounds in our sample, in spite of a lower content in organic acids. These results seem to indicate that phenolic compounds account for bigger contributions to the antioxidant capacity of the species than organic acids. Antioxidative properties have been observed for hydroxycinnamic acids derivatives (*31*−*33*), luteolin, apigenin, quercetin, and diosmetin glycosides (*32, 34*−*36*). Organic acids, such as oxalic, ascorbic, and citric acid, have also been shown to exhibit antioxidant capacity in several models (*37*). This suggests that all of these compounds may contribute in some extent to the antioxidant activity of the extract.

R. induratus has been shown to actively remove Hg from the soil and could be used as a phytoremediation in contaminated soils (*24*). Results presented here may explain the oxidative stress resistance in the presence of Hg compounds in the aerial parts of the plant.

The results obtained herein confirm that this is an interesting dietary source of bioactive compounds, namely, phenolics. In a general way, field samples show higher phenolics contents than *Brassica oleracea* var. *costata* leaves and than inflorescences of *B. oleracea* var. *acephala* and *Brassica rapa* var. *rapa*, other vegetal materials also consumed (*41, 42*). In addition, organic acids are present in higher quantities relative to other vegetables, mainly due to the presence of oxalic acid in high amounts, which is not observed in other plant foods (*42, 43*).

In conclusion, the qualitative profile of phenolic compounds and organic acids in *R. induratus* leaves is maintained for all samples, suffering quantitative changes according to the plant development. Samples for all stages of development exhibit a high content in oxalic acid, which should be taken into account regarding oxalic intoxication, either by intentional or by nonintentional consumption. As the LD_{50} of oxalic acid is pointed to be within 50 and 500 mg/kg body weight, children are at greater risk than adults. The protective activity displayed against nitric oxide and hypochlorous acid, together with the one described before for superoxide radical, suggests that the consumption of this vegetable may prevent the formation of other more reactive species, which increases the interest for their intake.

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