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Metabolic and Bioactivity Insights into *Brassica oleracea* var. *acephala*

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Seeds of *Brassica oleracea* var. *acephala* (kale) were analyzed by HPLC/UV-PAD/MS*n*-ESI. Several phenolic acids and flavonol derivatives were identified. The seeds of this *B. oleracea* variety exhibited more flavonol derivatives than those of tronchuda cabbage (*Brassica oleracea* var. *costata*), also characterized in this paper. Quercetin and isorhamnetin derivatives were found only in kale seeds. Oxalic, aconitic, citric, pyruvic, malic, quinic, shikimic, and fumaric acids were the organic acids present in these matrices, malic acid being predominant in kale and citric acid in tronchuda cabbage seeds. Acetylcholinesterase (AChE) inhibitory activity was determined in aqueous extracts from both seeds. Kale leaves and butterflies, larvae, and excrements of *Pieris brassicae* reared on kale were also evaluated. Kale seeds were the most effective AChE inhibitor, followed by tronchuda cabbage seeds and kale leaves. With regard to *P. brassicae* material, excrements exhibited stronger inhibitory capacity. These results may be explained by the presence of sinapine, an analogue of acetylcholine, only in seed materials. A strong concentration-dependent antioxidant capacity against DPPH, nitric oxide, and superoxide radicals was observed for kale seeds.

KEYWORDS: ***Brassica oleracea* L. var. *acephala*; *Brassica oleracea* L. var. *costata*; seeds; *Pieris brassicae*; phenolic compounds; organic acids; acetylcholinesterase inhibition; antioxidant activity**

INTRODUCTION

It is well-known that Brassicaceae, namely *Brassica oleracea*, species are an important source of bioactive compounds, including phenolics (flavonoids and hydroxycinnamic acid derivatives) and glucosinolates (*1*, *2*). Kale (*Brassica oleracea* var. *acephala*) leaves have been studied for their content of phenolic compounds and organic acids (*3*), but kale seeds are yet to be characterized. Previously, seeds of another variety, *Brassica oleracea* var. *costata*, were revealed to have more hydroxycinnamic acid derivatives and fewer flavonols than its aerial parts (*4*, *5*). Furthermore, seeds of Brassicaceae members are characterized by the presence of sinapoylcholine (or sinapine), which is thought to serve as a storage form of choline and sinapic acid for germinating seedlings (*6*).

Acetylcholine (ACh) is a neurotransmissor found in vertebrates and arthropods and one of the major compounds by which electrical impulses carried by nerve cells are transmitted to another nerve cell or to voluntary and involuntary muscles (*7*). Acetylcholinesterase (AChE) inhibitors have therapeutic applications in Alzheimer's disease (AD), senile dementia, ataxia, myasthenia gravis, and Parkinson's disease (*8*). The search for plant-derived inhibitors of AChE has been focused on alkaloids, such as physostigmine obtained from *Physostigma venenosum* and galantamine extracted from *Galanthus woronowii* (Amaryllidaceae) and related genera. Other major classes of phytochemicals reported to have such activity are terpenoids, glycosides, and coumarins (*9*). Plant extracts containing phenolic compounds have been previously evaluated for their AChE inhibitory activity (*10*, *11*).

The structural similarities between sinapoylcholine and ACh led us to investigate the effects of kale seed aqueous extract on AChE activity.

Because excess production of reactive oxygen species (ROS) in the brain has been implicated in a number of neurodegenerative diseases, the antioxidant properties of some extracts can also contribute to neuroprotection (*12*). For this reason, the antioxidant activities of kale seed aqueous extracts were also screened against DPPH and further evaluated against the radicals superoxide and nitric oxide, important in biological events, in a cell-free system.

The scavenging of these two radicals can be of major importance due to its role in the formation of other reactive species, which can be extremely deleterious to cells (*13*). Although kale seeds organic extracts have already been characterized in terms of phenolic acids, and antioxidant and antibacterial activities (*14*),

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the aqueous extract has never been characterized before. The aqueous extract analysis is important because it is representative of the way kale is consumed. In addition, by using the aqueous extract, organic solvents, which can interfere in bioactivity assays, are avoided.

Pieris brassicae, an insect whose larvae constitute a frequent pest of kale cultures, also has been reported for its phenolics and organic acids profile (*3*). *P. brassicae* larvae were revealed to be able to sequester, metabolize, and excrete phenolics from their feeding material. In addition, in previous works, *P. brassicae* fed with *B. oleracea* varieties (*3*, *15*) and *Brassica rapa* (*16*) showed stronger antioxidant potential than its feeding material. On the basis of these facts, materials obtained from *P. brassicae* fed with kale were included in this work.

This work aimed to contribute to the knowledge of the metabolic profile of *B. oleracea* var. *acephala* seeds and to evaluate some of its biological capacities. The metabolic profile and bioactivity of kale seed aqueous extracts were compared with those of seeds of *B. oleracea* var. *costata*. Because it was expected that kale seeds and leaves have different chemical compositions, their activities were also compared. Additionally, *P. brassicae* at different stages of its life cycle (butterfly and larvae) and its excrements were analyzed to compare its biological potential with the vegetal materials.

MATERIALS AND METHODS

Standards. Reference compounds were purchased from various suppliers: Aconitic, pyruvic, citric, and sinapic acids, kaempferol-3-*O*-rutinoside, and isorhamnetin-3-*O*-glucoside were from Extrasynthése (Genay, France). Oxalic, malic, quinic, shikimic, and fumaric acids, DPPH, β-nicotinamide adenine dinucleotide (NADH), phenazine methosulfate (PMS), bovine serum albumin (BSA), nitroblue tetrazolium chloride (NBT), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), sulfanilamine, AChE (CAS 9000-81-1; EC 232-559-3) from electric eel (type VI-s, lyophilized powder), acetylthiocholine iodide (ATCI), and Tris-HCl were purchased from Sigma (St. Louis, MO). *N*-(1-Naphthyl)ethylenediamine dihydrochloride, sodium nitroprussiate dehydrate (SNP), methanol, and sulfuric and acetic acids were obtained from Merck (Darmstadt, Germany). NaCl was purchased from José M. Vaz Pereira, S.A. (Sintra, Portugal) and $MgCl \cdot 6H_2O$ from Fluka (Buchs, Switzerland). Water was treated in a Milli-Q (Millipore, Bedford, MA) water purification system.

Samples. Wild *P. brassicae* larvae were collected in Bragança (northeastern Portugal) and taken to the laboratory to complete their life cycle, including oviposition in kale (*B. oleracea* var. *acephala*) leaves. Identification was performed by José A. Pereira, Ph.D. (CIMO). Larvae fed with kale *ad libitum* were allowed to develop. Larvae at the fifth instar were collected and kept without food for 12 h before freezing. The excrements were also collected and frozen. Other larvae were allowed to reach the butterfly stage, being collected < 24 h after eclosion. Tronchuda cabbage and kale seeds were obtained from local farmers in Bragança, northeastern Portugal, in July 2005 and August 2008, respectively.

P. brassicae (larvae, excrements, and butterflies), kale (leaves and seeds), and tronchuda cabbage seeds were freeze-dried. Then, the dried material was powdered, mixed, and kept in a desiccator in the dark until analysis.

Voucher specimens are deposited at Department of Pharmacognosy from Faculty of Pharmacy of Porto University.

Sample Preparation. Aqueous extracts of kale leaves and *P. brassicae* materials were prepared by boiling ca. 0.5 g for 30 min in 400 mL of water. For seed extracts ca. 4.0 g was extracted with 400 mL of boiling water for 30 min. Aqueous extracts were filtered using a Büchner funnel and lyophilized. Yields of ca. 94.0 mg (butterflies), 237.8 mg (larvae), 173.8 mg (excrements), 764.9 mg (tronchuda seeds), amd 666.4 and 229.5 mg for seeds and leaves of host kale, respectively, were obtained. The lyophilized extracts were kept in a desiccator in the dark until analysis. For phenolics or organic acids determination, the seed extracts were redissolved in water or sulfuric acid (0.01 N), respectively. The other assays were performed after the aqueous extract had been redissolved in water or buffer.

HPLC-PAD-MS*n*-ESI Phenolic Compounds Qualitative Analysis. For the identification of phenolic compounds, lyophilized extract (100 mg/mL) was ultrasonicated (1 h), centrifuged (12000 rpm, 5 min), and filtered through 0.45 μm size pore membrane. Chromatographic separations were carried out on a 250 × 4 mm, 5 μm, RP-18 LiChroCART column (Merck) protected with a 4 × 4 mm LiChroCART guard column, with 1% acetic acid (A) and methanol (B) as solvents, starting with 15% B and using a gradient to obtain 40% B at 30 min, 60% B at 35 min, and 80% B at 37 min. The flow rate was 1 mL min^{-1} and the injection volume 5 μL. The HPLC system was equipped with an Agilent 1100 series diode array and a mass detector in series (Agilent Technologies, Waldbronn, Germany). It consisted of a G1312A binary pump, a G1313A autosampler, a G1322A degasser, and a G1315B photodiode array detector, controlled by ChemStation software (Agilent, v. 08.03). Spectroscopic data from all peaks were accumulated in the range of 240−400 nm, and chromatograms were recorded at 330 nm. The mass detector was a G2445A ion-trap mass spectrometer equipped with an electrospray ionization (ESI) system and controlled by LCMSD software (Agilent, v. 4.1). Nitrogen was used as nebulizing gas at a pressure of 65 psi, and the flow was adjusted to 11 L min^{-1}. The heated capillary and voltage were maintained at 350 °C and 4 kV, respectively. The full scan mass covered the range from m/z 100 to 2000. Collision-induced fragmentation experiments were performed in the ion trap using helium as collision gas, with voltage ramping cycles from 0.3 to 2 V. MS data were acquired in the negative ionization mode and in the positive ionization mode for the study of compound **8** (sinapine). MS*n* was carried out in the automatic mode on the more abundant fragment ion in MS($n - 1$).

HPLC-PAD Phenolic Compound Quantitative Analysis. For quantification of phenolic compounds, 20 μL of redissolved seeds lyophilized extract (100 mg/mL) was analyzed using a HPLC/UV-PAD unit (Gilson) and a Spherisorb ODS2 (25.0 × 0.46 cm; 5 μm, particle size) column. Elution was performed under the conditions described by Sousa et al. (*4*). Detection was achieved with a Gilson diode array detector. Spectral data from all peaks were accumulated in the range of 200−400 nm, and chromatograms were recorded at 330 nm. The data were processed on Unipoint system software (Gilson Medical Electronics, Villiers le Bel, France). Peak purity was checked by the software contrast facilities.

Phenolic compounds' quantification was achieved by the absorbance recorded in the chromatograms relative to external standards. Because standards of the identified compounds were not commercially available, sinapic acid derivatives were quantified as sinapic acid, kaempferol derivatives as kaempferol-3-*O*-rutinoside, and isorhamnetin derivatives as isorhamnetin-3-*O*-glucoside. Quercetin-3-*O*-diglucoside-7-*O*-glucoside was quantified together with 1-sinapoylgentiobioside and kaempferol-3-*O*-triglucoside-7-*O*-glucoside together with sinapoylglucoside isomer, both as sinapic acid.

HPLC-UV Analysis of Organic Acids. The separation of the organic acids in both seed varieties lyophilized extracts (100 mg/mL) was carried out as previously reported (*3*), in a system consisting of an analytical HPLC unit (Gilson) with an ion exclusion column, Nucleogel Ion 300 OA (300 × 7.7 mm) in conjunction with a column heating device set at 30 °C. Elution was carried out isocratically, at a solvent flow rate of 0.2 mL min^{-1}, with 0.01 N sulfuric acid. Detection was performed with a UV detector set at 214 nm.

Identification was performed by comparison of the retention times with those of authentic standards. Organic acids' quantification was achieved by the absorbance recorded in the chromatograms relative to external authentic standards. The peaks in the chromatograms were integrated using a default baseline construction technique.

AChE Inhibitory Activity. AChE inhibitory activity was determined spectrophotometrically in a Multiskan Ascent plate reader (Thermo; Electron Corp.) based on Ellman's method, according to a described procedure (*10*). In each well the mixture consisted of ACh in water, DTNB in buffer A (50 mM Tris-HCl, pH 8, containing 0.1 M NaCl and 0.02 M $MgCl \cdot 6H_2O$), buffer B (50 mM Tris-HCl, pH 8, containing 0.1% BSA), and sample dissolved in a solution of 10% methanol in buffer C (50 mM Tris-HCl, pH 8). The absorbance was read at 405 nm. After this step,

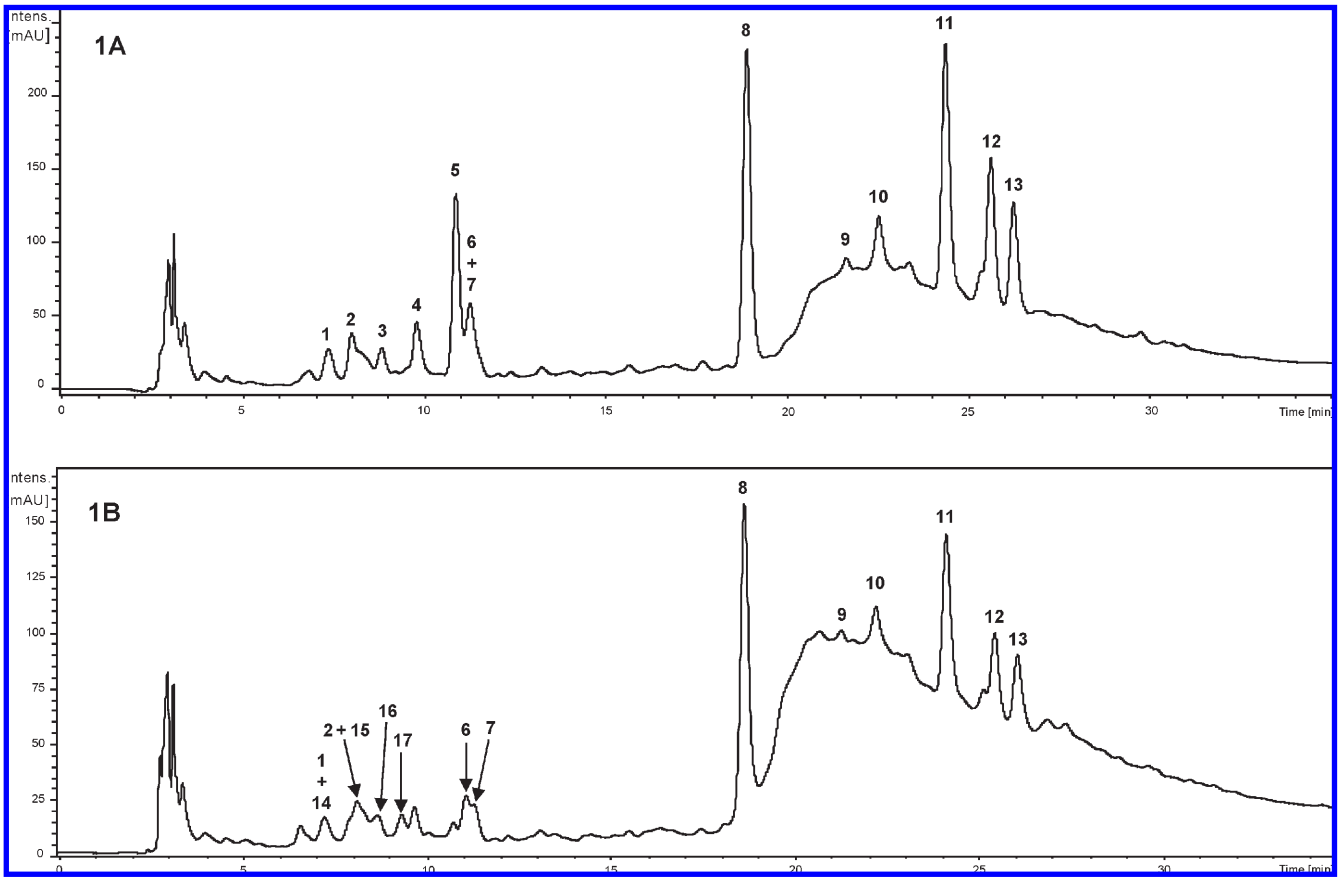

Figure 1. HPLC-UV phenolic profile of tronchuda cabbage seeds (**A**) and kale seeds (**B**). Detection was at 330 nm. Peaks: (1) sinapoylgentiobioside; (2) 1-sinapoylglucoside isomer; (3) sinapoylgentiobioside isomer; (4) 1-sinapoylglucoside isomer; (5) 1-sinapoylglucoside; (6) kaempferol-3-*O*-(sinapoyl)triglucoside-7-*O*-glucoside; (7) kaempferol-3-*O*-(sinapoyl)diglucoside-7-*O*-glucoside; (8) sinapoylcholine; (9) 1,2-disinapoylgentiobioside isomer; (10) 1,2-disinapoylgentiobioside isomer; (11) 1,2-disinapoylgentiobioside; (12) 1,2,2′-trisinapoylgentiobioside; (13) 1,2-disinapoylglucoside; (14) quercetin-3-*O*-diglucoside-7-*O*-glucoside; (15) kaempferol-3-*O*-triglucoside-7-*O*-glucoside; (16) kaempferol-3-*O*-diglucoside-7-*O*-glucoside; (17) isorhamnetin-3-*O*-diglucoside-7-*O*-glucoside.

AChE (0.44 U/mL) was added and the absorbance was read again. The rates of reactions were calculated by Ascent software version 2.6 (Thermo Labsystems Oy). The rate of the reaction before the addition of the enzyme was subtracted from that obtained after enzyme addition to correct eventual spontaneous hydrolysis of substrate. Percentage of inhibition was calculated by comparing the rates of the sample with the control (10% methanol in buffer C). Three experiments were performed in triplicate.

Antioxidant Activity. *DPPH Scavenging Assay*. The antiradical activity of the extracts was determined spectrophotometrically in a Multiskan Ascent plate reader (Thermo Electron Corp.), by monitoring the disappearance of DPPH at 515 nm, as before (*3*). The reaction mixture in the sample wells consisted of 25 μL of aqueous extract and 200 μL of methanolic solution of 150 mM DPPH. The plate was incubated for 30 min at room temperature after the addition of DPPH. Three experiments were performed in triplicate.

Superoxide Radical Scavenging Assay. Antiradical activity was determined spectrophotometrically at 562 nm, in a plate reader working in kinetic function, by monitoring the effect on reduction of NBT induced by superoxide radical.

Superoxide radicals were generated in a NADH/PMS system, according to a described procedure (*3*). All components were dissolved in phosphate buffer (19 mM, pH 7.4). Three experiments were performed in triplicate.

Nitric Oxide Scavenging Assay. Antiradical activity was determined spectrophotometrically in a 96-well plate reader according to the described procedure (*3*). The reaction mixtures in the sample wells consisted of extract and SNP, and plates were incubated at 25 °C for 60 min under light exposure. Griess reagent was then added, and the absorbance was determined at 540 nm. Three experiments were performed in triplicate.

RESULTS AND DISCUSSION

HPLC-PAD-MSn-ESI Phenolic Compounds Qualitative Analysis. The HPLC/UV-PAD/MSn-ESI analysis of both kale and tronchuda cabbage seeds revealed the presence of 17 phenolic compounds: (**1**) 1-sinapoylgentiobioside; (**2**) sinapoylglucoside isomer; (**3**) sinapoylgentiobioside isomer; (**4**) 1-sinapoylglucoside isomer; (**5**) 1-sinapoylglucoside; (**6**) kaempferol-3-*O*-(sinapoyl)triglucoside-7-*O*-glucoside; (**7**) kaempferol-3-*O*-(sinapoyl)-diglucoside-7-*O*-glucoside; (**8**) sinapoylcholine; (**9**) 1,2-disinapoylgentiobioside isomer; (**10**) 1,2-disinapoylgentiobioside isomer; (**11**) 1,2-disinapoylgentiobioside; (**12**) 1,2,2′-trisinapoylgentiobioside; (**13**) 1,2-disinapoylglucoside; (**14**) quercetin-3-*O*-diglucoside-7-*O*-glucoside; (**15**) kaempferol-3-*O*-triglucoside-7-*O*-glucoside; (**16**) kaempferol-3-*O*-diglucoside-7-*O*-glucoside and (**17**) isorhamnetin-3-*O*-diglucoside-7-*O*-glucoside (**Figure 1**).

The tronchuda cabbage seed phenolics profile was quite similar to that previously reported (**Figure 1A**; **Table 1**) (*4*). The flavonoid metabolites were slightly different: kaempferol-3,7-*O*-diglucoside-4′-*O*-(sinapoyl)glucoside, which was previously identified in trace amounts (*4*), was not detected in this sample. However, kaempferol-3-*O*-(sinapoyl)diglucoside-7-*O*-glucoside (**6**) usually found in *Brassica* species already studied by our group (*17*) was now found in tronchuda seeds. This compound coelutes with kaempferol-3-*O*-(sinapoyl)triglucoside-7-*O*-glucoside (**7**), with a

Table 1. *Rt* and MS*n* Data of Sinapoylglycosides, Flavonoids (−MS), and Sinapine (+MS) from Tronchuda Cabbage Seeds and Kale Seeds[a]

	Compounds[1]	*Rt* (min)	MSn, *m/z* (%)					
			monosinapoyl derivatives					
				MS2[M-H]$^-$				
			[M-H]$^-$	[M-H-18]$^-$	[$^{0,2}X_0$-18]$^-$	[Sinp-H]$^-$	[Sinp-H-18]$^-$	
1	SinpGentb	7.3	547	529(100)	247(65)	223(10)		
3	SinpGentb	8.8	547	529(55)	247(60)	223(100)		
2	1-SinpGlc	8.0	385	367(25)	247(100)	223(8)		
4	1-SinpGlc	9.8	385	367(20)	247(100)	223(5)		
5	1-SinpGlc	10.8	385	247(45)		223(100)	205(60)	
			disinapoyl derivatives					
				MS2[M-H]$^-$			MS3[(M-H)→(M-H-Sinp)]$^-$	
			[M-H]$^-$	[M-H-Sinp]$^-$	[Sinp-H]$^-$	[Sinp-H-18]$^-$	[Sinp-H]$^-$	[Sinp-H-18]$^-$
9	diSinpGentb	21.5	753	529(100)	223(3)		223(100)	205(20)
10	diSinpGentb	22.4	753	529(100)	223(4)		223(100)	205(55)
11	diSinpGentb	24.2	753	529(100)	223(5)		223(93)	205(100)
13	diSinpGlc	26.1	591	367(60)	223(100)	205(10)	223(60)	205(100)
			trisinapoyl derivatives					
				MS2[M-H]$^-$		MS3[(M-H)→(M-H-Sinp)]$^-$		
			[M-H]$^-$	[M-H-Sinp]$^-$		[M-H-Sinp-206]$^-$	[Sinp-H]$^-$	
12	triSinpGentb	25.5	959	735(100)		529(100)	223(80)	
			sinapoyl derivatives from flavonoid glycosides					
				MS2[M-H]$^-$	MS3[(M-H)→(M-H-162)]$^-$			
			[M-H]$^-$	[(M-H)-162]$^-$	[(M-H-162)-206]$^-$			
6	K-3(Sinp)triGlc-7Glc	11.0	1139	977(100)	771(100)			
7	K-3(Sinp)Soph-7Glc	11.3	977	815(100)	609(100)			
			flavonoid glycosides					
				MS2[M-H]$^-$	MS3[(M-H)→(M-H-162)]$^-$			
			[M-H]$^-$	[(M-H)-162]$^-$			[Aglic-H/2H]$^-$	
4	Q-3Soph-7Glc	7.4	787	625(100)	463(17)	445(90)	300(100)	
5	K-3triGlc-7Glc	8.0	933	771(100)		429(30)	285(100)	
6	K-3Soph-7Glc	8.6	771	609(100)	447(20)	429(70)	284(100)	
7	I-3Soph-7Glc	9.4	801	639(100)	477(50)		315(100)	
			Sinapoylcholine					
				MS2[M]$^+$	MS3[M→(M-(CH_3)$_3$N)]$^+$			
			[M]$^+$	[M-(CH_3)$_3$N]$^+$				
8	Sinapine	18.8	310	251(100)	207(55)	175(100)	147(25)	

[a] Glc, glucoside; Gentb, gentiobioside; Soph, sophoroside; Sinp, sinapic acid; K, kaempferol; Q, quercetin; I, isorhamnetin.

0.3 min delay in the new gradient, confirming that there are two distinct compounds. In our previous work (*4*), this compound was considered to be an artifact resulting from the loss of glucose from compound **7** during the ionization process, but the different retention times proved the existence of both compounds in the extracts. The reanalysis of the previous chromatogram of tronchuda cabbage seeds confirmed the existence of both compounds (**6** and **7**) in important amounts.

As in tronchuda, the phenolic profile of kale seeds (**Figure 1B**; **Table 1**) is characterized by the presence of sinapine (**8**), as the major compound, the two heterosides of kaempferol acylated with sinapic acid (**6** and **7**), and the disinapoylglycosides derivatives (**9**−**13**). In the previous work by our group with tronchuda cabbage seeds (*4*), monosinapoylglycoside derivatives were identified (compounds **1**−**5**). In kale seeds monosinapoylglycoside derivatives occur in trace amounts and coelute with other compounds, which makes the identification by their UV spectra difficult (**Figure 1**). Among these, quercetin-3-*O*-diglucoside-7-*O*-glucoside (**14**) coelutes with sinapoylgentiobioside (**1**) and kaempferol-3-*O*-triglucoside-7-*O*-glucoside (**15**) with sinapoylglucoside (**2**). Other flavonol derivatives such as kaempferol-3-*O*-diglucoside-7-*O*-glucoside (**16**) and isorhamnetin-3-*O*-diglucoside-7-*O*-glucoside (**17**) were identified (**Figure 1B**; **Table 1**).

Sinapoylglucoside (**5**), an abundant compound previously characterized in tronchuda cabbage seeds, was not detected in kale seeds (**Figure 1**; **Table 1**). Although Ayaz and collaborators (*14*) reported the presence of phenolic compounds, namely, phenolic acids, in hydromethanolic extract of kale seeds, all of the compounds described herein are presented for the first time in this matrix.

Phenolic Compound Quantification. To get a better characterization of the composition of the aqueous lyophilized extracts of tronchuda cabbage and kale seed, phenolic compounds were quantified by HPLC-PAD.

Kale seed total phenolics content, ca. 12.2 g/kg (**Table 2**), was similar to that previously reported for its leaves (ca. 11.1 g/kg) (*3*). However, kale seeds are richer in phenolic acids, whereas kale leaves were mainly characterized by the presence of flavonols (*3*). Both classes of compounds are formed in the phenylpropanoid pathway and fulfill important functions, being involved in the development and interaction of the plant with its environment. The higher amounts of hydroxycinnamic acids in seeds can be explained by the fact that these compounds are used as building blocks for lignin biosynthesis, important after seed germination for rigidifying cell walls and rendering them impermeable to water. Additionally, these compounds may be important for the resistance of both seed varieties to downy mildew (*18*) and insect pests (*19*), as they are known to exert a protective role against parasite attack (*20*). Leaves are richer in flavonoids because these metabolites protect plants against UV irradiation and act as signals in plant−symbiont interactions (*21*).

Kale seeds contain lower levels of phenolics (**Table 2**) than tronchuda cabbage seeds (ca. 24.0 g/kg). Sinapoylcholine (**8**) was the compound present in highest amounts in both seed varieties, representing ca. 28 and 42% of total compounds in tronchuda cabbage and kale, respectively (**Table 2**). 1,2-Disinapoylgentiobiose (**11**) was also a major compound, representing ca. 18 and 15% of total phenolics in tronchuda cabbage and kale seeds, respectively (**Table 2**).

Table 2. Quantification of Phenolic Compounds in Kale and Tronchuda Cabbage Seeds[a]

		mg/kg (dry basis)[a]	
	phenolic compounds	kale	tronchuda
1 + 14[b]	sinapoylgentiobioside + quercetin-3-diglucoside-7-glucoside	396.1 ± 24.4	672.8 ± 22.6
2 + 15[b]	1-sinapoylglucoside isomer + kaempferol-3-triglucoside-7-glucoside	243.0 ± 12.8	882.2 ± 23.1
3	sinapoylgentiobioside isomer		419.7 ± 29.3
4	1-sinapoylglucoside isomer		1058.6 ± 43.7
5	1-sinapoylglucoside		2716.8 ± 53.6
6 +	kaempferol-3-(sinapoyl)triglucoside-7-glucoside +	1526.9 ± 3.2	1892.9 ± 30.3
7	kaempferol-3-(sinapoyl)diglucoside-7-glucoside		
8	sinapoylcholine	5098.1 ± 7.1	6693.3 ± 67.0
9	1,2-disinapoylgentiobiose isomer	389.1 ± 8.8	752.9 ± 32.4
10	1,2-disinapoylgentiobiose isomer	482.3 ± 14.5	671.7 ± 25.8
11	1,2-disinapoylgentiobiose	1870.6 ± 8.3	4232.5 ± 43.7
12	1,2,2′-trisinapoylgentiobiose	690.6 ± 35.5	1943.8 ± 37.7
13	1,2-disinapoylglucose	1078.8 ± 3.0	2088.9 ± 28.9
16	kaempferol-3-diglucoside-7-glucoside	222.4 ± 18.3	
17	isorhamnetin-3-diglucoside-7-glucoside	229.1 ± 1.2	
	Σ	12227.0	24026.3

[a] Results are expressed as mean ± standard deviation of three determinations; Σ, sum of the determined phenolic compounds. [b] Found only in kale seeds.

Sinapoylgentiobioside isomer (**3**) was the minor compound in tronchuda cabbage seeds, accounting for 2% of total phenolics, whereas kaempferol-3-*O*-diglucoside-7-*O*-glucoside (**16**) and isorhamnetin-3-*O*-diglucoside-7-*O*-glucoside (**17**) were the compounds present in lower levels in kale seeds, representing each ca. 2% of total phenolics in this matrix (**Table 2**).

Furthermore, the most marked difference between the two seed varieties is in their flavonoid derivatives content. Quercetin and isorhamnetin derivatives were found only in kale seeds. Quercetin-3-*O*-diglucoside-7-*O*-glucoside (**14**), kaempferol-3-*O*-triglucoside-7-*O*-glucoside (**15**), kaempferol-3-*O*-diglucoside-7-*O*-glucoside (**16**), and isorhamnetin-3-*O*-diglucoside-7-*O*-glucoside (**17**) present in kale seeds were not detected in tronchuda cabbage seeds (**Figure 1**; **Table 2**). Kaempferol-3-*O*-(sinapoyl)-triglucoside-7-*O*-glucoside (**6**) and kaempferol-3-*O*-(sinapoyl)-diglucoside-7-*O*-glucoside (**7**) were the only flavonoids common to both seed varieties, representing ca. 8 and 12% of total compounds in tronchuda cabbage and kale, respectively (**Table 2**).

On the other hand, with regard to phenolic acids composition, tronchuda seeds were richer than kale ones (**Table 2**). In addition, tronchuda cabbage seeds contained sinapoylgentiobioside isomer (**3**), sinapoylglucoside isomer (**4**), and sinapoylglucoside (**5**), which represent 2, 4, and 11% of total compounds in this variety, respectively, not being found in kale seeds (**Table 2**).

The presence of isorhamnetin and quercetin derivatives in kale seeds is in accordance with the reported phenolic profile of kale leaves (*3*). In tronchuda cabbage, quercetin was found in only trace amounts in older leaves and isorhamnetin was absent (*4*, *5*). Thus, the differences found between both seeds may be used to distinguish these varieties.

Identification and Quantification of Organic Acids by HPLC-UV. The screening of kale seeds revealed a chemical profile composed by six identified organic acids: oxalic, aconitic, citric, pyruvic, malic, and fumaric acids (**Figure 2**). Comparison of this profile with that of the leaves showed that only two acids, oxalic (found in kale seeds) and shikimic (observed in kale leaves), were not present in both materials (**Figure 2**). The qualitative organic acids profile of tronchuda cabbage seeds revealed a similar composition, but in this variety quinic and shikimic acids were additionally detected (**Figure 2**). By comparison of the organic acids profile obtained in our previous work (*4*), we observed a similar composition, except for the compound with a retention time around 31 min. This compound was previously identified as ascorbic acid, but using other analysis conditions and according to the characteristic UV spectrum of ascorbic acid (maximum absorption at 245 nm), this identity was not confirmed. This compound was now identified as pyruvic acid, which was further confirmed by cochromatography with an external standard.

In quantitative terms, the total organic acids content of kale seeds (ca. 41.4 g/kg) was similar to that found in tronchuda cabbage ones (ca. 42.9 g/kg) (**Table 3**) and almost 3 times less than that previously found in kale leaves (ca. 112.3 g/kg) (*3*). This low quantity of organic acids found in kale seeds when compared with leaves can be justified by plant primary metabolism, much more active in leaves than in seeds due to their quiescent state (*22*).

As observed with kale leaves (*3*), malic and citric were the acids present in highest amounts in both seed varieties (**Table 3**): malic acid represented ca. 50.4 and 27.1% in kale and tronchuda cabbage seeds, respectively, and citric acid corresponded to 42.0 and 59.2%, respectively (**Table 3**). Oxalic, pyruvic, and fumaric acids were minor compounds, accounting for ca. 1.3, 0.8, and 0.4% of total acids, respectively, in kale seeds (**Table 3**). These acids represented ca. 1.2, 1.8, and 0.5%, respectively, in tronchuda cabbage seeds (**Table 3**). In tronchuda cabbage seeds, shikimic acid was present in the lowest amount (0.2%) (**Table 3**).

AChE Inhibitory Activity. AChE is the principal enzyme involved in the hydrolysis of ACh. As referred to above, given the structural similarities between sinapoylcholine and ACh, the effects of kale and tronchuda cabbage seed aqueous extracts on enzyme activity were assessed for the first time. Kale and tronchuda cabbage seeds exhibited a concentration-dependent AChE inhibitory capacity (**Figure 3**). Under the assay conditions the IC_{50} found for kale seed extract was 3438 μg/mL of dried lyophilized extract, containing 17.5 μg/mL of sinapine. For tronchuda cabbage seeds extract the IC_{50} obtained corresponded to 3399 μg/mL (**Figure 3**), containing 22.8 μg/mL sinapine. Sinapine had already been described for its potent AChE inhibitory activity (*23*). He and collaborators (*23*) demonstrated that sinapine significantly inhibited AChE present on rat cerebral homogenate and on rat blood serum. Thus, due to the closely related structure of sinapine with ACh, it may act as a competitive inhibitor for the enzyme (*24*). Sinapine has a quaternary nitrogen that probably binds reversibly to the site on the enzyme where the quaternary ammonium of AChE binds (*25*).

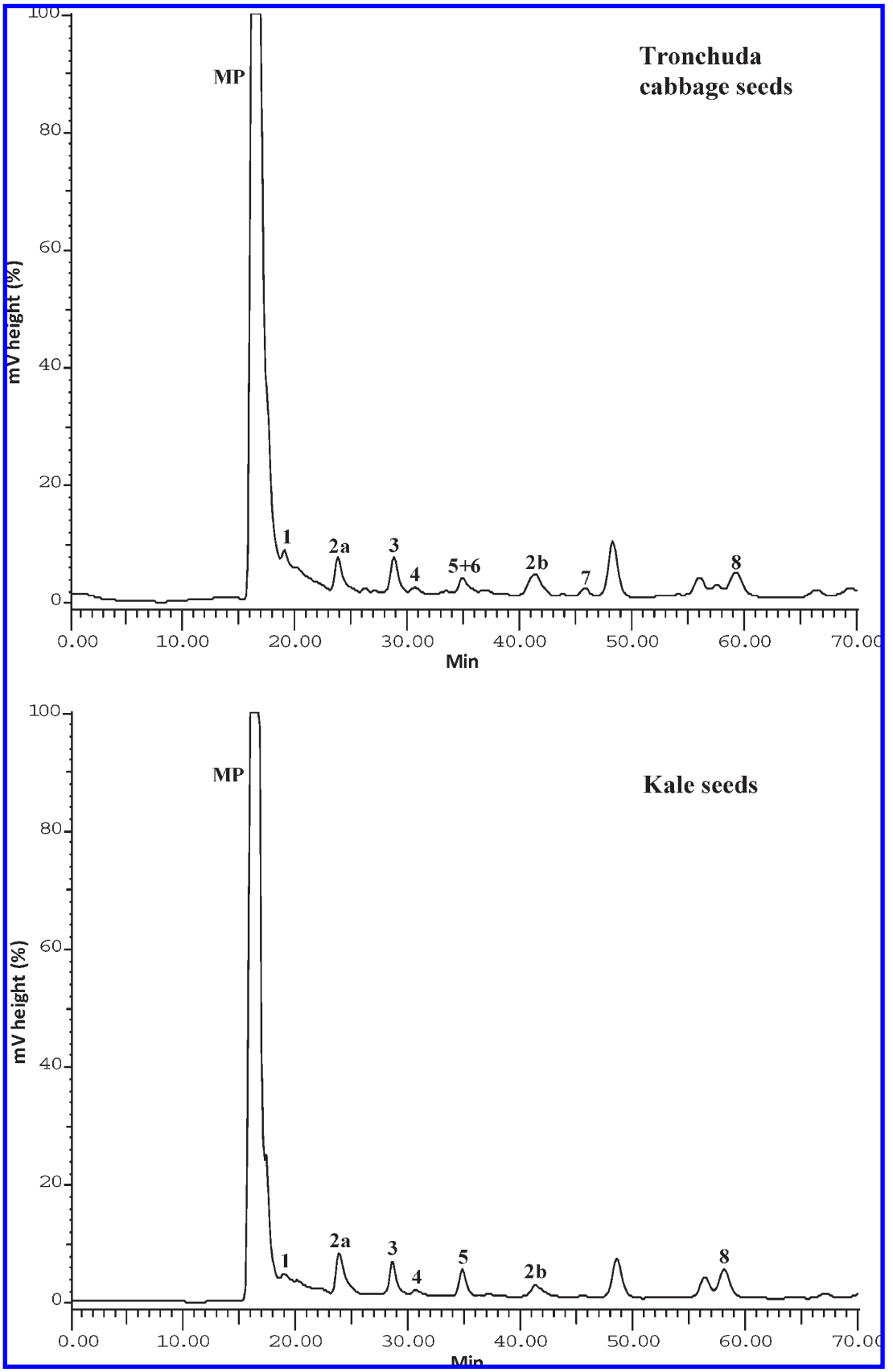

Figure 2. HPLC-UV organic acid profile of tronchuda cabbage and kale seeds. Detection was at 214 nm. Peaks: (MP) mobile phase; (1) oxalic acid; (2a and 2b) aconitic acid; (3) citric acid; (4) pyruvic acid; (5) malic acid; (6) quinic acid; (7) shikimic acid; (8) fumaric acid.

As in previous works involving *P. brassicae* reared on *B. oleracea* (*3*, *15*) and *B. rapa* varieties (*16*), the insect was revealed to be able to selectively sequester, metabolize, and excrete phenolic compounds from its feeding material and exhibited stronger antioxidant potential than its host plant; the AChE inhibitory capacity of the insect material, as well as that of

Table 3. Quantification of Organic Acids in Kale and Tronchuda Seeds[a]

		mg/kg (dry basis)[a]	
	organic acid	kale	tronchuda
1	oxalic	542.2 ± 2.3	506.3 ± 5.7
2a + **2b**	aconitic	2081.4 ± 307.3	4327.6 ± 20.1
3	citric	17418.3 ± 32.4	25372.8 ± 106.0
4	pyruvic	320.3 ± 28.4	754.4 ± 12.0
5 + **6**	malic + quinic	20886.2 ± 391.5[b]	11615.9 ± 78.3
7	shikimic		97.9 ± 0.3
8	fumaric	120.9 ± 2.3	195.0 ± 0.3
	Σ	41369.3	42870.0

[a] Results are expressed as mean ± standard deviation of three determinations; Σ, sum of the determined organic acids. [b] Only malic acid.

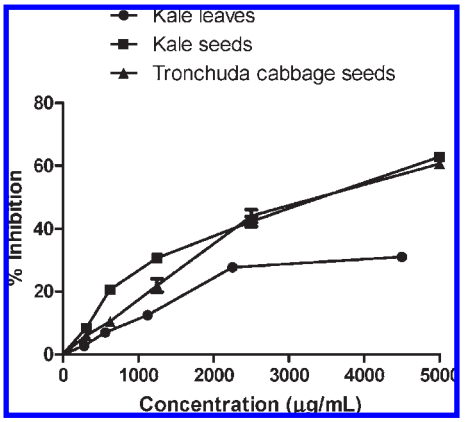

Figure 3. AChE inhibitory effect of kale leaves and seeds and tronchuda cabbage seed aqueous extract.

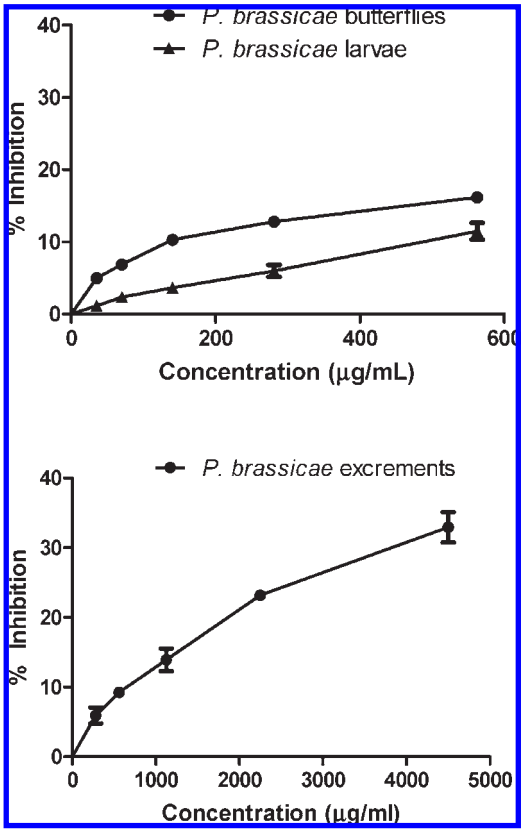

Figure 4. AChE inhibitory effect of *P. brassicae* materials (butterflies, larvae, and their excrements) aqueous extract.

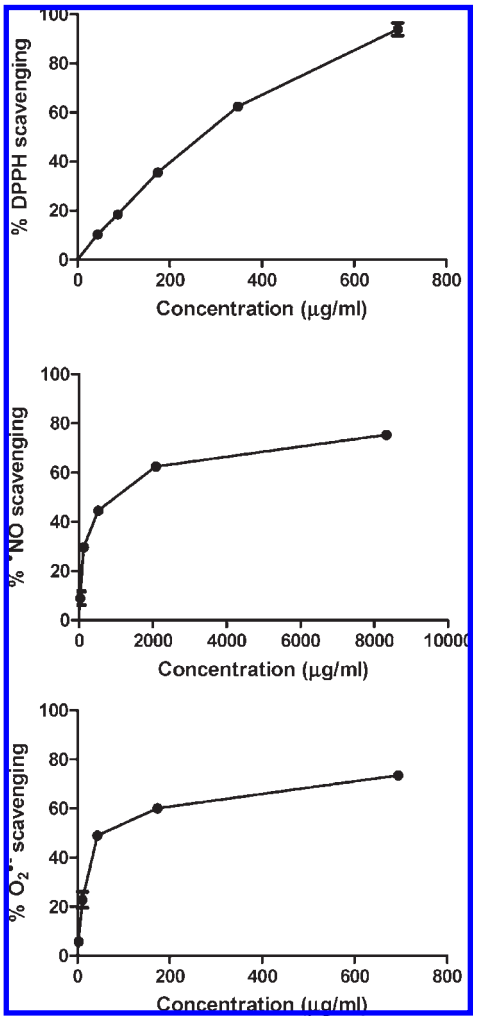

Figure 5. Effect of kale seed aqueous lyophilized extracts against DPPH, nitric oxide, and superoxide radical. Values show mean ± SE from three experiments performed in triplicate.

host kale leaves, was also evaluated for the first time, to be compared with that of *B. oleracea* seeds.

These extracts displayed some concentration-dependent AChE inhibitory potential: excrements were the *P. brassicae* material that was revealed to have stronger capacity to inhibit this enzyme (IC_{25} =2666 µg/mL) (**Figure 4**). For *P. brassicae* butterfly and larvae a very low activity was found (**Figure 4**). Kale leaves displayed a slightly better AChE inhibitory activity, with an IC_{25} of 2051 µg/mL (**Figure 3**).

Thus, the marked difference in the activity shown by the different analyzed extracts can be explained by the absence of sinapine in *P. brassicae* materials, as well as in host kale leaves, and its presence in high quantities in kale and tronchuda cabbage seeds. Therefore, this compound should make an important contribution to AChE inhibition.

Some flavonoids, such as quercetrin, quercetin, or 3-methoxy-quercetin, have also been described in the literature as AChE inhibitors (*26*). However, despite the presence of flavonoids in these matrices, namely, quercetin derivatives, none of the above-described compounds was found. Although the phenolic profile of the distinct matrices reveals the presence of several quercetin derivatives, they exhibit a more complex substitution pattern, which can impair their activity as AChE inhibitors.

Despite the AChE inhibitory activity shown by some of the tested aqueous extracts, especially the seeds, physostigmine used

as reference compound was more potent (IC_{50} =1.8 μg/mL under the same conditions).

Antioxidant Capacity. The antioxidant ability of the aqueous lyophilized extract of kale seeds was screened by the DPPH assay. In this assay, kale seeds exhibited a strong concentration-dependent antioxidant potential (IC_{25} =120 μg/mL) (**Figure 5**). The sequestration effect against DPPH had already been observed (*14*), but for different extracts and using different assay conditions.

Against nitric oxide, kale seeds also provided protection in a concentration-dependent way (**Figure 5**), with an IC_{20} = 151 μg/mL.

With regard to superoxide anion, kale seeds displayed a potent protective effect, as shown in **Figure 5**,with an IC_{25} at 19 μg mL^{-1}.

Kale seeds were revealed to have higher antioxidant potential than kale leaves (*3*) despite leaves being richer in phenolics than seeds. Although phenolic compounds and organic acids have already been reported to have antioxidant properties (*4*), other compounds present in the extracts may contribute to the overall antioxidant activity exhibited by seeds. The high antioxidant potential of the seed can be explained by the need to protect its storage lipids from oxidation and to ensure its viability, especially important during its germination when oxygen demand is high (*27*).

In a general way, comparison of the two seed varieties revealed that tronchuda cabbage seeds exhibited a higher protective effect than kale seeds (*4*, *28*). The observed differences can be, at least partially, explained by higher amounts of phenolic compounds in tronchuda cabbage seeds than in kale seeds.

In summary, this study provides further knowledge on kale and tronchuda cabbage seeds. The potential of these matrices as inhibitors of AChE activity was demonstrated for the first time. Other materials, such as *P. brassicae* (butterflies, larvae, and their excrements) and kale leaves, were less active. Phenolic compounds (namely, phenolic acids, flavonols, and sinapine) and organic acids can, at least partly, explain these activities. This opens another perspective for the medicinal use of these natural matrices as a source of bioactive compounds to treat chronic diseases, such as Alzheimer's. Additionally, they can be used as a source of bioactive compounds.

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