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Inflorescences of Brassicacea species as source of bioactive compounds: A comparative study

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ABSTRACT

Two *Brassica oleracea* varieties (*B. oleracea* L. var. *costata* DC and *B. oleracea* L. var. *acephala*) and *Brassica rapa* L. var. *rapa* inflorescences were studied for their chemical composition and antioxidant capacity. Phenolic compounds and organic acids profiles were determined by HPLC–DAD and HPLC–UV, respectively. *B. oleracea* var. costata and *B. oleracea* L. var. *acephala* inflorescences presented a similar qualitative phenolic composition, exhibiting several complex kaempferol derivatives and 3-*p*-coumaroylquinic acid, while *B. rapa* var. *rapa* was characterized by kaempferol and isorhamnetin glycosides and several phenolic acids derivatives. *B. oleracea* L. var. *costata* and *B. rapa* var. *rapa* showed the highest phenolics content. The three *Brassica* exhibited the same six organic acids (aconitic, citric, pyruvic, malic, shikimic and fumaric acids), but *B. oleracea* L. var. *acephala* presented a considerably higher amount. Each inflorescence was investigated for its capacity to act as a scavenger of DPPH radical and reactive oxygen species (superoxide radical, hydroxyl radical and hypochlorous acid), exhibiting antioxidant capacity in a concentration dependent manner against all radicals. These samples were also studied for its antimicrobial potential against Gram-positive and Gram-negative bacteria and fungi, displaying antimicrobial capacity only against Gram-positive bacteria.

1. Introduction

Brassica vegetables belong to the Cruciferous family, which includes a variety of economically significant horticultural crops. They are consumed all over the year as ingredients of different salads or after cooking of raw and frozen vegetables (Podsędek, 2007). Tronchuda cabbage (*Brassica oleracea* L. var. *costata* DC), kale (*B. oleracea* L. var. *acephala* DC) and turnip (*Brassica rapa* var. *rapa* L.) appear within the most consumed species.

Increasing attention has been paid to the role of diet in human health. In fact, food provides not only essential nutrients needed for life, but also other bioactive compounds for health promotion and disease prevention. It is generally assumed that the beneficial effects of vegetables are partly attributed to the complex mixture of phytochemicals possessing antioxidant activity (Liu, 2003; Podsędek, 2007). These comprise both phenolic compounds and organic acids (Liu, 2003; Podsędek, 2007; Pulido, Bravo, & Saura-Calixto, 2000; Silva et al., 2004), which contribute to their organoleptic features (Vaughan & Geissler, 1997), despite being applied in the quality control of several matrices (Fernandes et al., 2007; Ferreres et al., 2005; Sousa et al., 2005). Furthermore, plant compounds are known for their antimicrobial capacity (Cowan, 1999; Tim Cushnie & Lamb, 2005), which may be relevant considering the existing problem of resistance to antimicrobial agents.

The polyphenol composition of several materials from members of *Brassica* genus, or their byproducts, has been described (Llorach, Gil-Izquierdo, Ferreres, & Tomás-Barberán, 2003; Romani, Vignolini, Isolani, Ieri, & Heimler, 2006; Vallejo, Tomás-Barberán, & Ferreres, 2004), including that of *B. oleracea* var. *costata* (Ferreres et al., 2007, 2006, 2005; Sousa et al., 2005), *B. oleracea* var. *acephala* (Heimler, Vignolini, Dini, Vincieri, & Romani, 2006; Romani et al., 2003) and *B. rapa* var. *rapa* (Fernandes et al., 2007; Liang et al., 2006), referring distinct profiles between them. Recent publications also report the organic acids (Ayaz et al., 2006; Fernandes et al., 2007; Ferreres et al., 2007, 2006; Sousa et al., 2005) and the antioxidant potential (Fernandes et al., 2007; Ferreres et al., 2007, 2006; Heimler et al., 2006; Vrchovská et al., 2006) of these three species. However, information regarding their inflorescences is almost non-existent. As far as we know, only one study about the phenolic compounds and organic acids composition of *B. rapa* var.

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rapa was performed by our group, suggesting that this constitutes an interesting dietary source of protective compounds, displaying a DPPH scavenging activity stronger than that of the roots and leaves (Fernandes et al., 2007).

The objectives of this study were to define and compare the phenolics and organic acids composition and the biological potentials of the inflorescence of three *Brassica* varieties: *B. oleracea* var. *costata*, *B. oleracea* var. *acephala* and *B. rapa* var. *rapa*. For these purposes, the phenolic profile was established by reversed-phase HPLC–DAD analysis, while organic acids were determined by HPLC–UV. The antioxidant capacity was assessed by scavenging assays against DPPH radical and reactive oxygen species (superoxide radical, hydroxyl radical and hypochlorous acid). The antimicrobial potential was checked for three Gram-positive (*Bacillus cereus*, *B. subtilis* and *Staphylococus aureus*) and three Gram-negative bacteria (*Escherichia coli, Pseudomonas aeruginosa* and *Klebsiella pneumoniae*) and two fungi species (*Candida albicans* and *Cryptococcus neoformans*).

2. Materials and methods

2.1. Standards and reagents

Malic, shikimic, fumaric, caffeic, *p*-coumaric acids were purchased from Sigma (St. Louis, MO, USA). Aconitic, citric, pyruvic, ferulic and sinapic acids, kaempferol 3-*O*-rutinoside and isorhamnetin 3-*O*-glucoside were from Extrasynthése (Genay, France). Methanol, formic and acetic acids were obtained from Merck (Darmstadt, Germany) and sulphuric acid from Pronalab (Lisboa, Portugal). The water was treated in a Milli-Q water purification system (Millipore, Bedford, MA, USA). DPPH, xanthine, xanthine oxidase (XO) grade I from buttermilk (EC 1.1.3.22), β-nicotinamide adenine dinucleotide (NADH), phenazine methosulfate (PMS), nitroblue tetrazolium chloride (NBT), ferric chloride anhydrous ($FeCl_3$), ethylenediaminetetraacetic acid disodium salt (EDTA), ascorbic acid, trichloroacetic acid, thiobarbituric acid, deoxyribose, sodium hypochlorite solution with 4% available chlorine (NaOCl), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) were obtained from Sigma Chemical Co. (St. Louis, USA).

2.2. Samples

Inflorescences of *B. oleracea* L. var. *costata* DC, *B. oleracea* L. var. *acephala* and *B. rapa* L. var. *rapa* were collected in Carrazeda de Ansiães, Northeast Portugal, in February 2006. After harvesting, the material of three distinct individuals of each variety was immediately transferred to the laboratory and frozen at −20 °C, prior to their lyophilisation in a Labconco 4.5 Freezone apparatus (Kansas City, MO, USA). Then the dried material was powdered, mixed and stored in a desiccator, in the dark.

2.3. Sample preparation

An aqueous extract was used for the phytochemical characterization and in the biological activities assays: ca. 3.0 g of powdered inflorescences were boiled for 15 min in 600 ml water and then filtered over a Büchner funnel. The resulting extract was lyophilized in a Labconco 4.5 Freezone apparatus (Kansas City, MO, USA) and yields of ca. 1.2 g (*B. oleracea* L. var. *costata* DC), 1.2 g (*B. rapa* L. var. *rapa*), and 0.8 g (*B. oleracea* L. var. *acephala*) were obtained. The lyophilized extracts were kept in a desiccator, in the dark.

For the characterization and quantification of the phenolic compounds by HPLC–DAD, each lyophilized extract was redissolved in water. For organic acids determination they were redissolved in sulphuric acid 0.01 N prior to analysis by HPLC–UV.

2.4. HPLC–DAD analysis of phenolic compounds

Twenty microliters of inflorescences lyophilized extracts were analyzed using a HPLC unit (Gilson) and a Spherisorb ODS2 (25.0 × 0.46 cm; 5 μm, particle size) column. The *B. oleracea* varieties (*costata* and *acephala*) were analyzed as previously described (Ferreres et al., 2005), using a mixture of formic acid 5% (A) and methanol (B), with a flow rate of 1 ml/min, as follows: 0 min – 10% B, 25 min – 20% B, 40 min – 50% B, 45 min – 50% B, 46 min – 90% B, 50 min – 90% B, 55 min – 100% B, 58 min – 100% B, 60 min – 10% B.

The separation of *B. rapa* var. *rapa* phenolic compounds was achieved as before (Fernandes et al., 2007), with a solvent mixture of water (adjusted to pH 3.2 with formic acid at 10%, v/v) (A) and methanol (B). Elution was carried out at 1 ml/min and followed the gradient system 20% B at 0 min, 50% B at 35 min, 80% B at 45 min and 100% B at 50 min. 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. Since standards of several compounds identified in the lyophilized extracts were not commercially available, 3-*p*-coumaroylquinic acid was quantified as *p*-coumaric acid, and sinapic acid, kaempferol and isorhamnetin derivatives as sinapic acid, kaempferol 3-*O*-rutinoside and isorhamnetin 3-*O*-glucoside, respectively. The other compounds were quantified as themselves.

2.5. HPLC–UV analysis of organic acids

The separation of the organic acids present in the inflorescences lyophilized extracts was carried out as previously reported (Sousa et al., 2005), 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. Briefly, elution was carried out isocratically, at a solvent flow rate of 0.2 ml/min, with sulphuric acid 0.01 N. The detection was performed with an UV detector set at 214 nm.

Organic acids quantification was achieved by the absorbance recorded in the chromatograms relative to external standards.

2.6. DPPH· scavenging activity

The antiradical activity of the extracts was determined spectrophotometrically in a Multiscan Ascent plate reader (Thermo Electron Corporation), by monitoring the disappearance of DPPH· at 515 nm, according to a described procedure (Ferreres et al., 2006; Vrchovská et al., 2006). For each extract, a dilution series composed of five different concentrations was prepared in a 96 well plate. The reaction mixtures in the sample wells consisted of 25 μl aqueous extract and 200 μl of 150 μM DPPH· dissolved in methanol. The plate was incubated for 30 min at room temperature. Three experiments were performed in triplicate.

2.7. Superoxide radical-scavenging activity

Antiradical activity of the aqueous extracts was determined spectrophotometrically in a Multiscan Ascent plate reader (Thermo Electron Corporation), by monitoring at 562 nm the formation of formazan as a result of the superoxide radical-induced reduction of NBT.

2.7.1. Non-enzymatic assay

Superoxide radicals were generated by the NADH/PMS system according to a described procedure (Valentão et al., 2001). All components were dissolved in phosphate buffer (19 mM, pH 7.4). Experiments were performed in triplicate.

2.7.2. Enzymatic assay

Superoxide radicals were generated by the xanthine/xanthine oxidase (X/XO) system following as reported before (Valentão et al., 2001). Briefly, xanthine was dissolved in NaOH (1 μM) and subsequently in phosphate buffer (50 mM) with EDTA (0.1 mM, pH 7.8), xanthine oxidase in EDTA (0.1 mM) and the remaining components in phosphate buffer (50 mM) with EDTA (0.1 mM, pH 7.8). Experiments were performed in triplicate.

2.7.3. Effect on xanthine oxidase activity

The effect of the lyophilized extracts on xanthine oxidase activity was evaluated by measuring the formation of uric acid from xanthine in a double beam spectrophotometer (Heλios α, Unicam), at room temperature, according to a described procedure (Valentão et al., 2001). The reaction mixtures contained the same proportion of components as in the enzymatic assay for superoxide radical-scavenging activity, except NBT, in a final volume of 750 μl. The absorbance was measured at 295 nm for 2 min. Experiments were performed in triplicate.

2.8. Hydroxyl radical assay

The deoxyribose method for determining the scavenging effect of the aqueous extracts on hydroxyl radicals was performed as previously described (Valentão et al., 2002) in a double beam spectrophotometer (Heλios α, Unicam). Reaction mixtures contained 50 μM ascorbic acid, 40 μM $FeCl_3$, 2 mM EDTA, 2.8 mM H_2O_2, 2.8 mM deoxyribose and lyophilized extracts. All components were dissolved in KH_2PO_4–KOH buffer 10 mM, pH 7.4. This assay was also performed either without ascorbic acid or EDTA, in order to evaluate the extracts pro-oxidant and metal chelation potential, respectively. Experiments were performed in triplicate.

2.9. Hypochlorous acid scavenging activity

The inhibition of hypochlorous acid-induced 5-thio-2-nitrobenzoic acid (TNB) oxidation to 5,5′-dithiobis(2-nitrobenzoic acid) was performed according to a described procedure (Valentão et al., 2002), in a double beam spectrophotometer (Heλios α, Unicam). Hypochlorous acid and TNB were prepared immediately before use. Experiments were performed in triplicate.

2.10. Antimicrobial activity

2.10.1. Microorganisms and culture conditions

Microorganisms CECT were obtained from the Spanish type culture collection (CECT) of Valencia University, while microorganisms ESA were clinically isolated strains identified in the Microbiology Laboratory of Escola Superior Agrária de Bragança. Gram-positive (*B. cereus* CECT 148, *B. subtilis* CECT 498 and *S. aureus* ESA 7 isolated from pus) and Gram-negative (*E. coli* CECT 101, *P. aeruginosa* CECT 108 and *K. pneumoniae* ESA 8 isolated from urine) bacteria, and fungi (*C. albicans* CECT 1394 and *C. neoformans* ESA 3 isolated from vaginal fluid) were used to screen the antimicrobial potential of the three *Brassica* varieties. Microorganisms were cultured aerobically at 37 °C (Scientific 222 oven) in nutrient agar medium for bacteria, and at 30 °C (Scientific 222 oven) in Sabouraud dextrose agar medium for fungi.

2.10.2. Assay

The screening of antibacterial activities against Gram-positive and Gram-negative bacteria and fungi and the determination of the minimal inhibitory concentration (MIC) were achieved by an adaptation of the agar streak dilution method based on radial diffusion, as previously reported (Hawkey & Lewis, 1994; Pereira et al., 2006; Sousa et al., 2006). Suspensions of the microorganism were prepared to contain approximately 108 cfu/ml, and the plates containing agar medium were inoculated (100 μl; spread on the surface). Each sample (50 μl) was placed in a hole (3 mm depth, 4 mm diameter) made in the centre of the agar. The MIC was considered to be the lowest concentration of the tested sample able to inhibit the growth of bacteria or fungi, after 24 and 48 h, respectively. The diameters of the inhibition zones corresponding to the MICs were measured using a ruler, with an accuracy of 0.5 mm. Each inhibition zone diameter was measured three times (three different plates) and the average was considered. A control using only inoculation was also carried out.

3. Results and discussion

3.1. Phenolic composition of the inflorescences

The HPLC–DAD analysis allowed the identification of fourteen phenolic compounds in the inflorescences of *B. oleracea* var. *costata*: 3-*p*-coumaroylquinic acid, kaempferol 3-*O*-sophorotrioside-7-*O*-glucoside, kaempferol 3-*O*-(methoxycaffeoyl/caffeoyl)-sophoroside-7-*O*-glucoside, kaempferol 3-*O*-sophoroside-7-*O*-glucoside, kaempferol 3-*O*-sophorotrioside-7-*O*-sophoroside, kaempferol 3-*O*-sophoroside-7-*O*-sophoroside, kaempferol 3-*O*-tetraglucoside-7-*O*-sophoroside, kaempferol 3-*O*-(sinapoyl/caffeoyl)-sophoroside-7-*O*-glucoside, kaempferol 3-*O*-(feruloyl/caffeoyl)-sophoroside-7-*O*-glucoside, kaempferol 3-*O*-sophorotrioside, kaempferol 3-*O*-(sinapoyl)-sophoroside, kaempferol 3-*O*-(feruloyl)-sophorotrioside, kaempferol 3-*O*-(feruloyl)-sophoroside and kaempferol 3-*O*-sophoroside (Fig. 1A). All these compounds have been previously described in *B. oleracea* var. *costata* leaves (Ferreres et al., 2006, 2005; Sousa et al., 2005).

The same compounds were found in *B. oleracea* var. *acephala* inflorescences aqueous lyophilized extract (Fig. 1B), with the exception of kaempferol 3-*O*-tetraglucoside-7-*O*-sophoroside (compound 7). Among the detected phenolics, only kaempferol 3-*O*-sophoroside-7-*O*-glucoside has been reported in the leaves of this *B. oleracea* variety (Romani et al., 2003).

B. rapa var. *rapa* inflorescences exhibited several phenolic acids and flavonoids distinct from those found in the *B. oleracea* varieties, namely isorhamnetin derivatives. Besides 3-*p*-coumaroylquinic acid, kaempferol 3-*O*-sophoroside-7-*O*-glucoside, kaempferol 3-*O*-sophoroside-7-*O*-sophoroside, kaempferol 3-*O*-(feruloyl/caffeoyl)-sophoroside-7-*O*-glucoside and kaempferol 3-*O*-sophoroside detected in the above mentioned varieties, also identified were caffeic, ferulic and sinapic acids, kaempferol 3,7-*O*-diglucoside, isorhamnetin 3,7-*O*-diglucoside, 1,2-disinapoylgentiobiose, 1,2′-disinapoyl-2-feruloylgentiobiose, kaempferol 3-*O*-glucoside and isorhamnetin 3-*O*-glucoside (Fig. 2). These compounds have been already described in *B. rapa* var. *rapa* leaves and inflorescences (Fernandes et al., 2007). In addition, as observed before with other materials, isorhamnetin derivatives are present in *B. rapa* group and absent in *B. oleracea* (Romani et al., 2006).

The quantification of the identified phenolics in the three analyzed *Brassica* varieties inflorescences revealed that *B. oleracea* var. *costata* and *B. rapa* var. *rapa* present the highest contents (ca. 20 and 18 g/kg, respectively), corresponding to twice more the amount exhibited by *B. oleracea* var. *acephala* (ca. 9 g/kg) (Tables 1 and 2).

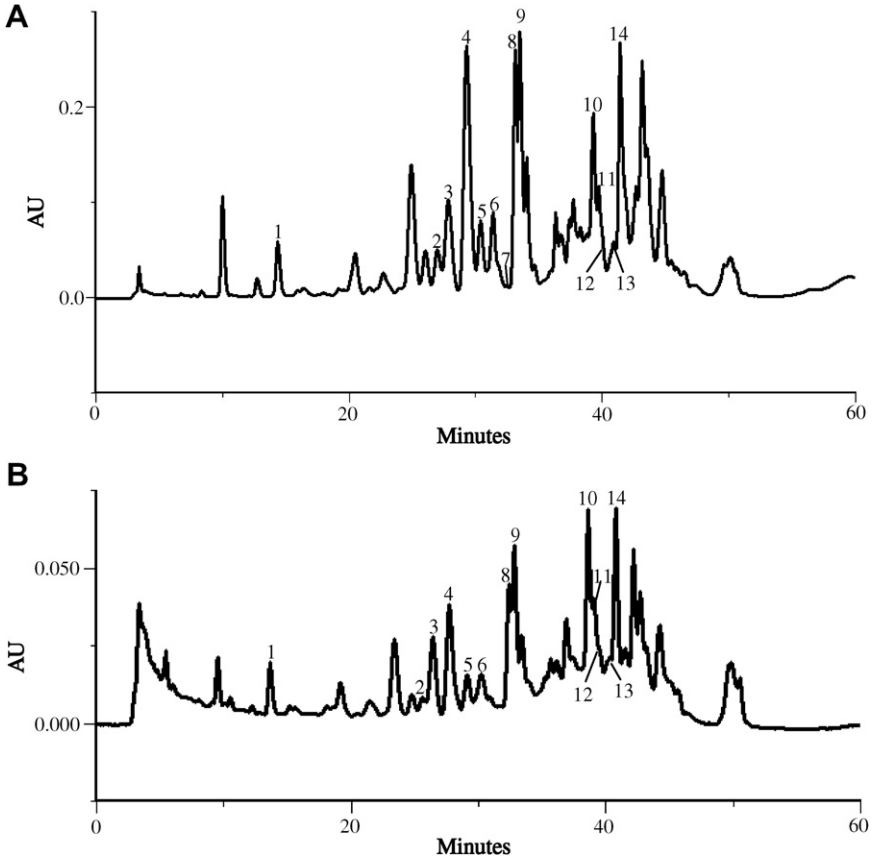

Fig. 1. HPLC–DAD phenolics profile of (A) *Brassica oleracea* var. *costata* and (B) *Brassica oleracea* var. *acephala* inflorescences aqueous lyophilized extracts. Detection at 330 nm. Peaks: (1) 3-*p*-coumaroylquinic acid; (2) kaempferol 3-*O*-sophorotrioside-7-*O*-glucoside; (3) kaempferol 3-*O*-(methoxycaffeoyl/caffeoyl)-sophoroside-7-*O*-glucoside; (4) kaempferol 3-*O*-sophoroside-7-*O*-glucoside; (5) kaempferol 3-*O*-sophorotrioside-7-*O*-sophoroside; (6) kaempferol 3-*O*-sophoroside-7-*O*-sophoroside; (7) kaempferol 3-*O*-tetraglucoside-7-*O*-sophoroside; (8) kaempferol 3-*O*-(sinapoyl/caffeoyl)-sophoroside-7-*O*-glucoside; (9) kaempferol 3-*O*-(feruloyl/caffeoyl)-sophoroside-7-*O*-glucoside; (10) kaempferol 3-*O*-sophorotrioside; (11) kaempferol 3-*O*-(sinapoyl)-sophoroside; (12) kaempferol 3-*O*-(feruloyl)-sophorotrioside; (13) kaempferol 3-*O*-(feruloyl)-sophoroside and (14) kaempferol 3-*O*-sophoroside.

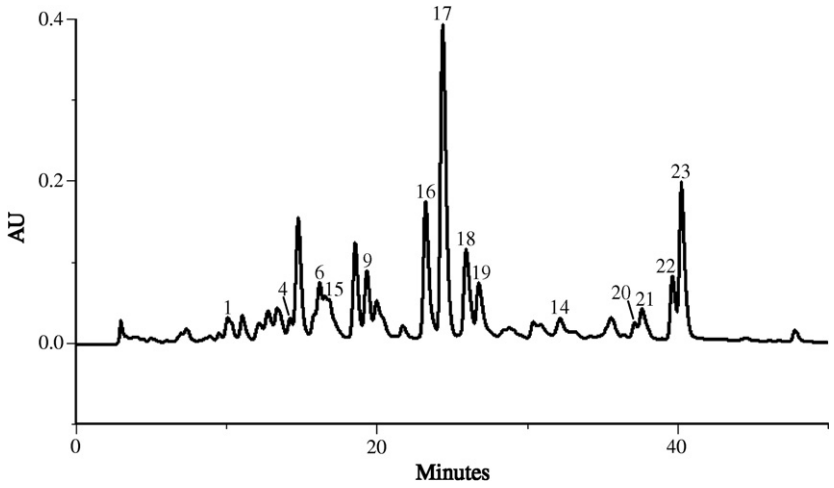

Fig. 2. HPLC–DAD phenolics profile of *B. rapa* var. *rapa* inflorescences aqueous lyophilized extract. Detection at 330 nm. Peaks: (1) 3-*p*-coumaroylquinic acid; (4) kaempferol 3-*O*-sophoroside-7-*O*-glucoside; (6) kaempferol 3-*O*-sophoroside-7-*O*-sophoroside (15) caffeic acid; (9) kaempferol 3-*O*-(feruloyl/caffeoyl)-sophoroside-7-*O*-glucoside; (16) kaempferol 3,7-*O*-diglucoside; (17) isorhamnetin 3,7-*O*-diglucoside; (18) ferulic acid; (19) sinapic acid; (14) kaempferol 3-*O*-sophoroside; (20) 1,2-disinapoylgentiobiose; (21) 1,2′-disinapoyl-2-feruloylgentiobiose; (22) kaempferol 3-*O*-glucoside and (23) isorhamnetin 3-*O*-glucoside.

Despite their similar qualitative composition, the two *B. oleracea* varieties showed distinct profiles. In *B. oleracea* var. *costata* inflorescences kaempferol 3-*O*-sophoroside-7-*O*-glucoside is the main compound (corresponding to ca. 19% of total phenolics) and kaempferol 3-*O*-tetraglucoside-7-*O*-sophoroside the minor one (less than 0.5%), while in *B. oleracea* var. *acephala* kaempferol 3-*O*-sophoroside is the compound present in highest amounts (representing ca. 21% of total phenolics) and 3-*p*-coumaroylquinic acid

Table 1
Quantification of phenolic compounds in *Brassica oleracea* varieties' inflorescences

	Compound	mg/kg (dry basis)[a]	
		var. *costata*	var. *acephala*
1	3- *p*-Coumaroyl quinic acid	305.1 ± 2.9	165.6 ± 2.5
2	Kaempferol 3-*O*-sophtr-7-*O*-gluc	630.8 ± 4.4	172.8 ± 9.5
3	Kaempferol 3-*O*- (methoxycaffeoyl/caffeoyl)-soph-7-*O*-gluc	1508.7 ± 4.9	783.2 ± 32.0
4	Kaempferol 3-*O*-soph-7-*O*-gluc	3678.1 ± 10.0	839.5 ± 37.4
5	Kaempferol 3-*O*-sophtr-7-*O*-soph	989.5 ± 5.3	308.7 ± 3.7
6	Kaempferol 3-*O*-soph-7-*O*-soph	1263.2 ± 3.5	426.8 ± 12.5
7	Kaempferol 3-*O*-tetragluc-7-*O*-soph	78.4 ± 1.2	nd
8	Kaempferol 3-*O*-(sinapoyl/caffeoyl)-soph-7-*O*-gluc	1809.0 ± 40.3	663.9 ± 67.0
9	Kaempferol 3-*O*- (feruloyl/caffeoyl)-soph-7-*O*-gluc	2322.1 ± 19.1	1055.5 ± 66.7
10	Kaempferol 3-*O*-sophtr	1783.8 ± 9.9	1353.5 ± 18.0
11	Kaempferol 3-*O*-(sinapoyl)-soph	1345.3 ± 3.8	1148.9 ± 58.4
12	Kaempferol 3-*O*-(feruloyl)-sophtr		
13	Kaempferol 3-*O*-(feruloyl)-soph	760.2 ± 1.1	533.1 ± 42.0
14	Kaempferol 3-*O*-soph	3093.6 ± 3.5	2003.9 ± 121.6
	∑	19567.7	9455.4

[a] Results are expressed as mean ± standard deviation of three determinations. ∑, sum of the determined phenolic compounds. nd: not detected. sophtr: sophorotriose; soph: sophorose; gluc: glucose.

Table 2
Quantification of phenolic compounds in *Brassica rapa* var. rapa inflorescences

	Compound	mg/kg (dry basis)[a]
1	3-*p*-Coumaroyl quinic acid	1084.8 ± 10.5
4	Kaempferol 3-*O*-soph-7-*O*-gluc	479.8 ± 24.4
6	Kaempferol 3-*O*-soph-7-*O*-soph	2098.5 ± 82.7
15	Caffeic acid	422.2 ± 10.5
9	Kaempferol 3-*O*-(feruloyl/caffeoyl)-soph-7-*O*-gluc	2109.7 ± 165.4
16	Kaempferol 3,7-*O*-digluc	1208.9 ± 94.8
17	Isorhamnetin 3,7-*O*-digluc	3483.8 ± 23.1
18	Ferulic acid	2189.8 ± 58.3
19	Sinapic acid	790.4 ± 7.8
14	Kaempferol 3-*O*-soph	2127.8 ± 97.9
20	1,2-Disinapoyl-gentiobiose	136.3 ± 14.1
21	1,2′-Disinapoyl-2-feruloyl-gentiobiose	103.3 ± 2.3
22	Kaempferol 3-*O*-gluc	734.0 ± 18.7
23	Isorhamnetin 3-*O*-gluc	1414.3 ± 8.3
	∑	18383.8

[a] Results are expressed as mean ± standard deviation of three determinations. ∑, sum of the determined phenolic compounds; nd: not detected; soph: sophorose and gluc: glucose.

and kaempferol 3-*O*-sophorotrioside-7-*O*-glucoside are the less abundant, each one corresponding to ca. 2%. In these varieties the phenolic acids contribution is very small, ca. 2% of total phenolics in each variety, being clearly distinct from the ca. 26% exhibited by the inflorescences of *B. rapa* var. *rapa* (Tables 1 and 2). In this latter species, isorhamnetin 3,7-*O*-diglucoside is the major compound, accounting for 19% of total phenolics, and 1,2′-disinapoyl-2-feruloylgentiobiose is the one present in lowest amounts (ca. 1%).

3.2. Organic acids in the inflorescences

An identical qualitative profile was found for the three analyzed *Brassica* varieties, which was composed by six organic acids: aconitic, citric, pyruvic, malic, shikimic and fumaric acids (Fig. 3). All these compounds were already described to occur in both *B. oleracea* var. *costata* (Ferreres et al., 2007, 2006; Sousa et al., 2005) and *B. rapa* var. *rapa* (Fernandes et al., 2007), with the exception of pyruvic acid that is identified for the first time in these varieties. Additionally, ascorbic acid that was present in leaves and seeds of *B. oleracea* var. *costata* (Ferreres et al., 2007, 2006; Sousa et al., 2005) was not detected in its inflorescences. Regarding *B. oleracea* var. *acephala*, only citric and malic acids were previously reported in the leaves (Ayaz et al., 2006).

From a quantitative point of view, *B. oleracea* var. *acephala* inflorescences showed the highest organic acids content (ca. 163 g/kg), corresponding to about three and four times the amount found for those of *costata* variety and *B. rapa* var. *rapa*, respectively (Table 3). *B. oleracea* var. *costata* exhibited a profile in which citric acid was the main compound (ca. 57% of total organic acids) and shikimic acid the minor one (less than 0.5%). In *B. oleracea* var. *acephala* and *B. rapa* var. *rapa* malic acid was the major organic acid, corre-

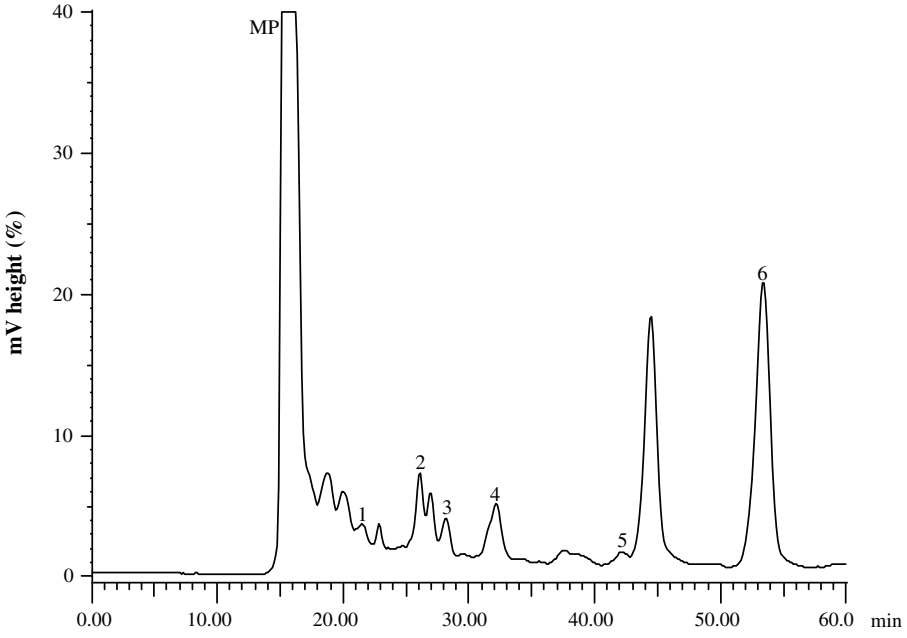

Fig. 3. HPLC–UV organic acid profile of *Brassica rapa* var. *rapa* inflorescences aqueous lyophilized extract. Detection at 214 nm. Peaks: (MP) mobile phase; (1) aconitic acid; (2) citric acid; (3) pyruvic acid; (4) malic acid; (5) shikimic acid and (6) fumaric acid.

Table 3
Quantification of organic acids in *Brassica* inflorescences (mg/kg, dry basis)[a]

Compound	*B. oleracea* var. *costata*	*B. oleracea* var. *acephala*	*B. rapa* var. *rapa*
Aconitic acid	426.7 ± 7.7	97.0 ± 2.6	42.3 ± 0.2
Citric acid	27925.7 ± 166.9	48373.3 ± 1846.1	13177.3 ± 75.4
Pyruvic acid	2684.0 ± 5.6	5686.7 ± 77.3	1123.2 ± 1.0
Malic acid	16734.2 ± 78.9	108158.7 ± 445.9	22349.6 ± 2.1
Shikimic acid	137.4 ± 0.6	764.7 ± 6.4	68.7 ± 0.8
Fumaric acid	1115.9 ± 4.4	17.5 ± 0.3	1260.8 ± 2.0
∑	49023.9	163097.9	38022.0

[a] Results are expressed as mean ± standard deviation of three determinations. ∑, sum of the determined organic acids.

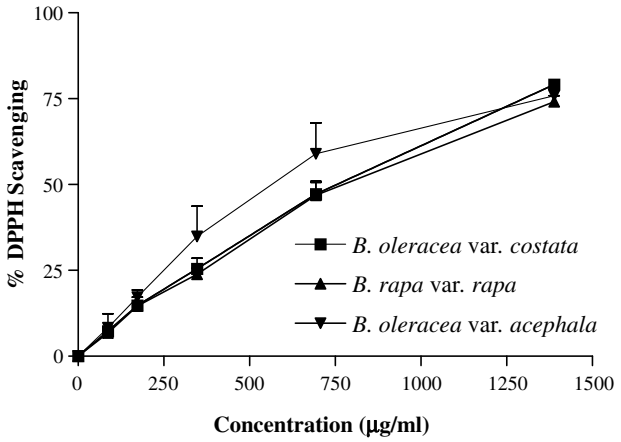

Fig. 4. Effect of inflorescences aqueous lyophilized extracts on DPPH· reduction. Values show mean ± SE from three experiments performed in triplicate.

Table 4
Antioxidant activity of the inflorescences' aqueous extracts (μg/ml)

Assay	*B. oleracea* var. *costata*	*B. oleracea* var. *acephala*	*B. rapa* var. *rapa*
DPPH[a]	754	565	774
Superoxide radical (X/XO)[b]	507	405	244
Superoxide radical (NADH/PMS)[b]	349	281	363
Hydroxyl radical[b]	172	10	12
HOCl[c]	639	1186	770

[a] Data correspond to IC_{50} values.
[b] Data correspond to IC_{25} values.
[c] Data correspond to IC_{10} values.

sponding to ca. 66 and 59% of total compounds, respectively. In both cases aconitic acid was the compound present in lowest amount, representing ca. 0.1% of total acids. Despite this coincidence, it can be noticed that, comparing with *B. oleracea* var. *acephala*, *B. rapa* var. *rapa* has a higher relative content of citric acid. On the other hand, its malic acid amount is inferior to that of *B. oleracea* var. *acephala*. Nevertheless, and according to the obtained results, it is evident that both citric and malic acids are the most important compounds: the sum of their amounts in the three analyzed *Brassica* inflorescences varies between ca. 91% and 94% of total organic acids (Table 3).

3.3. Antioxidant activity

The *Brassica* inflorescences were screened by the DPPH· assay, which provides basic information about their capacity to scavenge free radicals. In this assay the three varieties displayed a concentration dependent antioxidant potential. *B. oleracea* var. *acephala* was revealed to have a stronger capacity than *B. oleracea* var. *costata* and *B. rapa* var. *rapa*, which exhibited a similar behavior (Fig. 4, Table 4).

The three varieties exhibited a concentration dependent superoxide radical-scavenging activity, using the enzymatic system, and *B. rapa* var. *rapa* was the most effective one (Fig. 5A, Table 4). The effect of the aqueous lyophilized extracts on XO activity was also checked; once in this assay the inhibitory effect on the enzyme itself could also lead to a decrease of NBT reduction (Valentão et al., 2001). Thus, a control experiment monitoring the metabolic conversion of xanthine to uric acid was performed, revealing that for

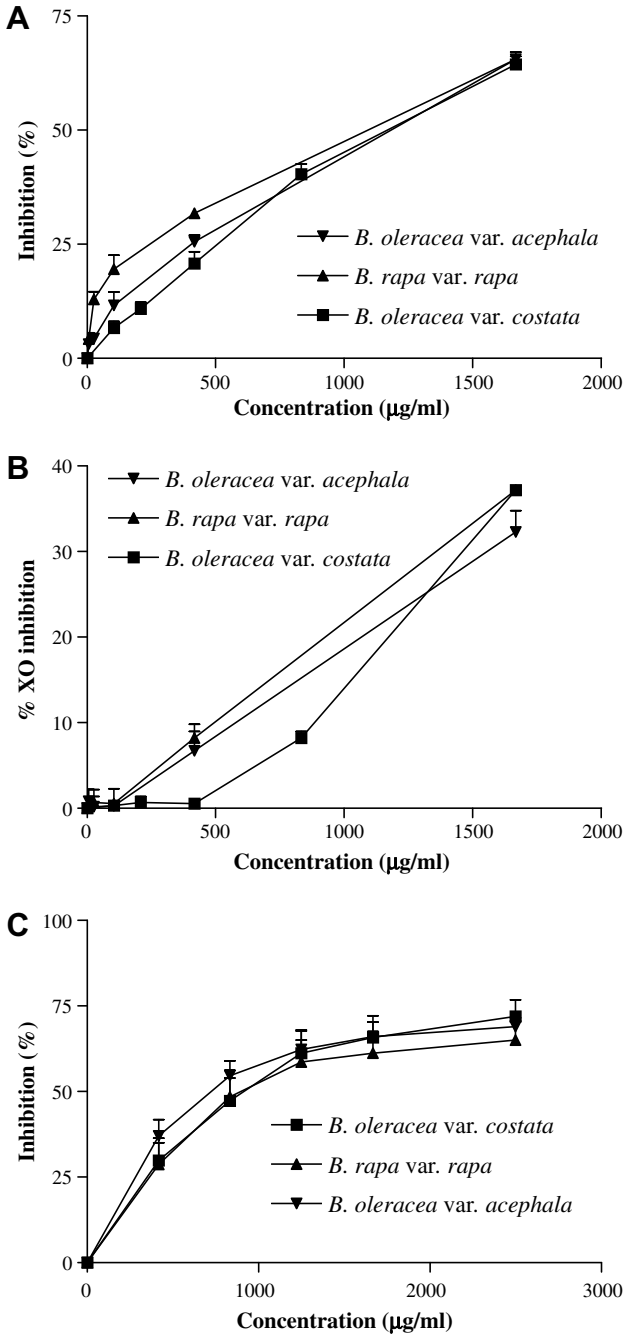

Fig. 5. Effect of inflorescences aqueous lyophilized extracts against superoxide radical generated in X/XO system (A), on XO activity (B), and against superoxide radical generated in NADH/PMS system (C). Values show mean ± SE from three experiments performed in triplicate.

concentrations above 104 μg/ml both *B. oleracea* var. *acephala* and *B. rapa* var. *rapa* have XO inhibitory capacity. *B. oleracea* var. *costata* was also able to inhibit this enzyme, but only for concentrations higher than 417 μg/ml (Fig. 5B). Considering these results it was not possible to show a clear-cut scavenging effect on superoxide radical. To confirm the scavenging capacity we also determined the effect of the extracts on superoxide radical generated in a chemical system, and a concentration dependent effect was observed, with *B. oleracea* var. *acephala* displaying the stronger capacity (Fig. 5C, Table 4).

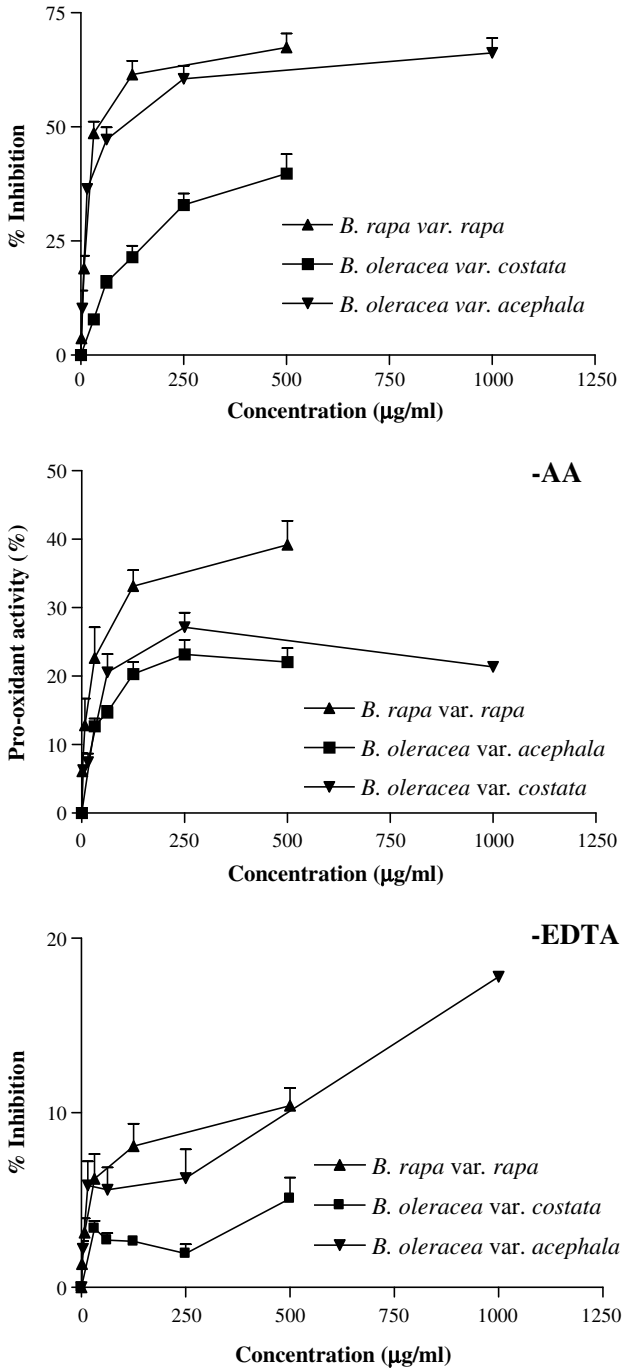

Fig. 6. Inflorescences aqueous lyophilized extracts non-specific hydroxyl radical-scavenging activity, pro-oxidant activity (-AA) and specific hydroxyl radical-scavenging (-EDTA). Values show mean ± SE from three experiments performed in triplicate.

B. oleracea var. *acephala* and *B. rapa* var. *rapa* lyophilized extracts also exhibited a similar potent scavenging activity for hydroxyl radical, in a concentration dependent manner, which was more pronounced than that of *B. oleracea* var. *costata* (Fig. 6, Table 4). If we omit ascorbate from the reaction mixture, and if pro-oxidant compounds are present, they will be able to redox cycle the metal ion required for hydroxyl generation, thus increasing the radical production (Valentão et al., 2002). In order to evaluate the pro-oxidant potential of the three inflorescences, we omitted ascorbic acid, and we found that they were effective substitutes for ascorbate, although *B. oleracea* var. *costata* presented pro-oxidant capacity only for concentrations below 250 μg/ml (Fig. 6). So, it seems that, at the tested concentrations, the three inflorescences have both anti-oxidant and pro-oxidant effects, with the first being more pronounced than the latter. Some compounds prevent deoxyribose damage in this assay, not by reacting with hydroxyl radicals, but because they present ion-binding capacity and can withdraw the iron ions rendering them inactive or poorly active in Fenton reactions (Valentão et al., 2002). The assay performed in the absence of EDTA showed that the three *Brassica* varieties have some capacity to chelate iron ions, being *B. oleracea* var. *acephala* the most effective (Fig. 6).

The oxidizing properties of HOCl induce the conversion of TNB to DTNB, which is inhibited by a HOCl scavenger (Valentão et al., 2002). The analyzed inflorescences displayed protective activity against damage by HOCl, which was concentration dependent. Among the distinct varieties *B. oleracea* var. *costata* and *B. rapa* var. *rapa* revealed to have higher scavenging ability, as shown in Fig. 7 and Table 4.

3.4. Antimicrobial activity

The aqueous extracts of the inflorescences were screened for their antimicrobial properties against *B. cereus*, *B. subtilis*, *S. aureus*, *E. coli*, *P. aeruginosa*, *K. pneumoniae*, *C. albicans* and *C. neoformans*. The minimal inhibitory concentration (MIC) values found for the tested bacteria and fungi (Table 5) were determined as an evaluation of the antimicrobial activity of the samples.

All the extracts presented antimicrobial capacity, inhibiting only Gram-positive bacteria and in the order *S. aureus* > *B. cereus* >> *B. subtilis*. Despite this, the response of each *Brassica* variety against the assayed microorganisms was different. *B. rapa* var. *rapa* revealed the highest activity against *B. cereus*, followed by *B. oleracea* var. *costata* and *B. oleracea* var. *acephala*. Only *B. rapa* var. *rapa* and *B. oleracea* var. *costata* showed some activity against *B. subtilis*. *S. aureus* was the most susceptible microorganism, presenting

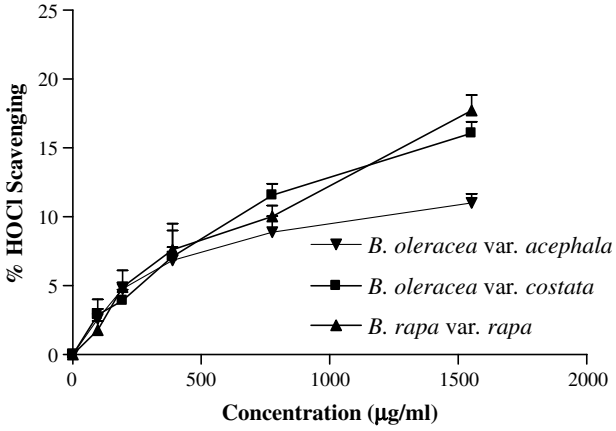

Fig. 7. Effect of inflorescences aqueous lyophilized extracts on the oxidation of TNB by HOCl. Values show mean ± SE from three experiments performed in triplicate.

Table 5
Antimicrobial activity of the inflorescences' aqueous extracts[a]

Samples	MIC (mg/ml)							
	B. cereus	*B. subtilis*	*S. aureus*	*P. aeruginosa*	*E. coli*	*K. peumoniae*	*C. albicans*	*C. neoformans*
B. rapa	0.1	10.0	0.1	50.0	50.0	50.0	50.0	50.0
var. *rapa*	(+ + +)	(+)	(+ + +)	(−)	(−)	(−)	(−)	(−)
B. oleracea	0.1	10.0	0.1	50.0	50.0	50.0	50.0	50.0
var. *costata*	(+ +)	(+)	(+ + + +)	(−)	(−)	(−)	(−)	(−)
B. oleracea	0.1	50.0	0.1	50.0	50.0	50.0	50.0	50.0
var. *acephala*	(+)	(−)	(+ + +)	(−)	(−)	(−)	(−)	(−)

[a] No antimicrobial activity (−), inhibition zone < 1 mm. Slight antimicrobial activity (+), inhibition zone 2–3 mm. Moderate antimicrobial activity (+ +), inhibition zone 4–5 mm. High antimicrobial activity (+ + +), inhibition zone 6–9 mm. Strong antimicrobial activity (+ + + +), inhibition zone > 9 mm. Standard deviation ± 0.5 mm.

MICs of 0.1 mg/ml for the three inflorescences tested, with *B. oleracea* var. *costata* displaying the best antimicrobial capacity (Table 5). The tested Gram-negative bacteria (*E. coli*, *P. aeruginosa* and *K. peumoniae*) and fungi (*C. albicans* and *C. neoformans*) species were resistant to the inflorescences extracts (Table 5).

The chemical composition of the analyzed extracts can obviously be very complex and may contain several classes of hydrophilic compounds, besides the phenolics and organic acids indicated above. Consequently, it seems important to evaluate the activity of the inflorescences aqueous lyophilized extracts as a whole, because interactions may occur among the different compounds present. Although no correlation was found between the phenolics or organic acids contents and the observed activities, the detected compounds are, most probably, contributing to them. In fact, hydroxycinnamic acids and their derivatives (Fukumoto & Mazza, 2000; Plumb, Price, Rhodes, & Williamson, 1997), flavonol glycosides, including acylated derivatives (Braca et al., 2003; Tang, Lou, Wang, Li, & Zhuang, 2001), or organic acids (Madhavi, Singhal, & Kulkarni, 1996; Silva et al., 2004) have been reported to possess antioxidative properties, assessed in different systems. Additionally, the antimicrobial capacity of these phytochemicals against several microorganisms was also demonstrated before (Alakomi et al., 2007; Binutu, Adesogan, & Okogun, 1996; Bloor, 1995; Lee, Thrupp, Owens, Cesario, & Shanbrom, 2001; Mokbel & Suganuma, 2006; Ou & Kwok, 2004; Pomilio, Buschi, Tomes, & Viale, 1992; Rigano et al., 2007). The obtained results are important, considering that the studied reactive oxygen species are produced in the organism or come from exogenous sources, being involved in several diseases (Aruoma, Halliwell, Hoey, & Butler, 1989; Bast, Haenen, & Doelman, 1991; Halliwell, 1991; Halliwell, Aeschbach, Löliger, & Aruoma, 1995; Puppo, 1992). Furthermore, the dietary intake of these inflorescences may lower the risk of bacterial infections, namely of the gastrointestinal tract, being also useful in food industry as preservative (Frazier & Westhoff, 1988).

In conclusion, the work herein indicates that the inflorescences of the three analyzed *Brassica* varieties are an appreciable source of protective compounds, like phenolics and organic acids. In addition, it points to the need of a diverse diet to get the most complete protection, through overlapping or complementary effects, as it is not possible to suggest one variety as being the best in terms of antioxidant or antimicrobial capacity.

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