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# Antioxidative properties of tronchuda cabbage (*Brassica oleracea* L. var. *costata* DC) external leaves against DPPH, superoxide radical, hydroxyl radical and hypochlorous acid

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## Abstract

The ability of the aqueous extract of tronchuda cabbage (*Brassica oleracea* L. var. *costata* DC) external leaves to act as a scavenger of DPPH<sup>•</sup> and reactive oxygen species (superoxide radical, hydroxyl radical and hypochlorous acid) was investigated. A phytochemical study was also undertaken, and thirteen phenolic compounds and five organic acids were identified and quantified. Tronchuda cabbage extracts exhibited antioxidant capacity in a concentration-dependent manner in all assays, although some pro-oxidant effect was also noticed. The samples with higher phenolic and organic acid contents displayed the major antioxidant potentials.

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**Keywords:** Tronchuda cabbage; *Brassica oleracea* L. var. *costata* DC; DPPH; Superoxide radical; Hydroxyl radical; Hypochlorous acid; Phenolics; Organic acids

## 1. Introduction

Reactive oxygen species (ROS) are generated by many redox processes that normally occur in the metabolism of aerobic cells. If not eliminated, ROS can attack important biological molecules, such as lipids, proteins, enzymes, DNA and RNA (Jung, Park, Chung, Kim, & Choi, 1999; Pietta, Simonetti, & Mauri, 1998; Yen & Chen, 1995). Although the human body possesses many defence mechanisms against oxidative stress, including antioxidant enzymes and non-enzymatic compounds,

an excess of free radicals can go out of control, the organism being unable to scavenge all ROS (Halliwell, Aeschbach, Löliger, & Aruoma, 1995; Miyake et al., 2000; Sies, 1993; Tseng et al., 1997). Their excess has been implicated in the development of chronic diseases, such as cancer, arteriosclerosis, nephritis, diabetes mellitus, rheumatism, ischemic and cardiovascular diseases and also in the ageing process (Behl & Moosmann, 2002; Gyamfi, Yonamine, & Aniya, 1999; Pulido, Bravo, & Saura-Calixto, 2000). Oxidative stress can also play an important role in the development of neurodegenerative disorders, such as Alzheimer's and Parkinson's diseases (Behl & Moosmann, 2002; Coulson, Siobhan, Cathal, Passmore, & Johnston, 2004). There is convincing epidemiological evidence that the

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consumption of fruits and vegetables is, in general, beneficial for health, due to the protection provided by the antioxidant phytonutrients contained in them (Guthrie & Kurowska, 2001). Especially vitamin C, vitamin E, carotenoids and dietary flavonoids can play important roles in human nutrition, and foods rich in these compounds should be involved in an optimal diet (du Toit, Volsteadt, & Apostolides, 2001).

Brassicaceae vegetables are reported to reduce the risks of some cancers, especially due to their contents of glucosinolates and derived products (Beecher, 1994; Park & Pezzuto, 2002; Stoewsand, 1995). Flavonoids and other phenolics are also considered to contribute to this capacity (Galati & O'Brien, 2004; Hertog, Hollman, & Van de Putte, 1993; Hollman, Hertog, & Katan, 1996). The antioxidant activity of some *Brassica oleracea* varieties has already been investigated. Cauliflower has been assayed for the ability to scavenge DPPH<sup>•</sup> and ABTS<sup>•+</sup>, as well as for ferric reducing efficiency and ability to inhibit lipid peroxidation (Llorach, Espín, Tomás-Barberán, & Ferreres, 2003). The oxygen radical absorbance capacity values have been measured in broccoli (Kurilich, Jeffery, Juvik, Wallig, & Klein, 2002; Ninfali & Bacchiocca, 2003). To find the antiradical activity of white cabbage, hydroxyl radical was used (Racchi et al., 2002).

Tronchuda cabbage (*B. oleracea* L. var. *costata* DC) is consumed throughout the world, being widely used in the preparation of soups. Its external leaves are rather bitter and tough and are usually prepared by boiling. The analysis of phenolics in tronchuda cabbage external leaves has already been done and fourteen phenolic compounds were identified and quantified (Ferreres et al., 2005). However, as far as we know, nothing has been reported about the antioxidant properties of tronchuda cabbage.

The aim of the present study was to evaluate the antioxidant potential of four tronchuda cabbage external leaves aqueous extracts, since this is the most common use of the species. Therefore, the capacity to act as scavenger of DPPH<sup>•</sup> and reactive oxygen species (superoxide radical, hydroxyl radical and hypochlorous acid) was investigated. In order to define the phenolic and organic acid compositions of the extracts, we carried out, respectively, HPLC/diode array (HPLC/DAD) and HPLC/UV analysis. A possible relationship between chemical composition and antioxidant potential was established.

## 2. Materials and methods

### 2.1. Standards and reagents

The standards were from Sigma (St. Louis, MO, USA) and from Extrasynthèse (Genay, France). Methanol and formic acid were obtained from Merck (Darms-

tadt, 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),  $\beta$ -nicotinamide adenine dinucleotide (NADH), phenazine methosulfate (PMS), nitroblue tetrazolium chloride (NBT), anhydrous ferric chloride (FeCl<sub>3</sub>), ethylenediaminetetraacetic acid disodium salt (EDTA), ascorbic acid, trichloroacetic acid, thiobarbituric acid, deoxyribose, sodium hypochlorite solution with 4% available chlorine (NaOCl) and 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) were obtained from Sigma Chemical Co. (St. Louis, USA).

### 2.2. Plant material and sampling

Tronchuda cabbages grown by different agronomic practices and collected in two periods (Table 1) were studied. These samples were chosen among previously studied ones (Ferreres et al., 2005) because of their distinct phenolic composition. After harvesting, plants were immediately transported to the laboratory, where external and internal leaves were separated. Samples were stored in a freezer at  $-20^{\circ}\text{C}$ .

### 2.3. Sample preparation

Tronchuda cabbage external leaves extracts were prepared by pouring 600 ml of boiling water on to 30 g of plant material. The mixture was boiled for one hour and then filtered through a Büchner funnel. The resulting extract was then lyophilised in a Labconco 4.5 apparatus (Kansas City, MO). The yields of the lyophilised extracts of samples A, B, C and D were 1.19, 1.07, 1.91 and 0.98 g, respectively. The lyophilised extracts were kept in a desiccator, in the dark.

### 2.4. HPLC analysis of organic acids

The separation was carried out as previously reported (Silva, Andrade, Mendes, Seabra, & Ferreira, 2002) with an analytical HPLC unit (Gilson), using an ion exclusion column Nucleogel<sup>®</sup> Ion 300 OA (300  $\times$  7.7 mm) column. Detection was performed with a UV detector set at 214 nm.

Table 1  
Characterization of tronchuda cabbage samples

Date of collection	Sample	Cultivation procedure
November 2002	A	Organic
	B	Conventional
December 2002	C	Organic
	D	Conventional

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

## 2.5. HPLC analysis of phenolics

Twenty microlitres of each extract were analysed using a HPLC unit (Gilson) and a Spherisorb ODS2 reversed-phase (Waters, Milford, USA) column (250 × 4.6 mm, 5 μm particle size) (Ferreeres et al., 2005). The solvent system was a mixture of formic acid 5% (A) and methanol (B), with a flow rate of 1 ml min<sup>-1</sup>, and the gradient was 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. 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 a 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. With the exception of kaempferol 3-*O*-glucoside, which was quantified as itself, the identified compounds were quantified as kaempferol 3-*O*-rutinoside, if not commercially available.

## 2.6. DPPH scavenging activity

The antiradical activity of the extracts was determined spectrophotometrically in an ELX808 IU Ultra Microplate Reader (Bio-Tek Instruments, Inc.), by monitoring the disappearance of DPPH<sup>•</sup> at 515 nm, according to a described procedure (Silva et al., 2004). For each extract, a dilution series (five different concentrations) was prepared in a 96 well plate. The reaction mixtures in the sample wells consisted of extract and DPPH radical dissolved in methanol. Three experiments were performed in triplicate.

## 2.7. Evaluation of superoxide radical scavenging activity

### 2.7.1. General

Antiradical activity was determined spectrophotometrically in an ELX808 IU Ultra Microplate Reader (Bio-Tek Instruments, Inc), by monitoring the effect of the lyophilised extracts on the O<sub>2</sub><sup>-</sup>-induced reduction of NBT at 562 nm.

### 2.7.2. 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 phos-

phate 19 mM buffer, pH 7.4. Three experiments were performed in triplicate.

### 2.7.3. Enzymatic assay

Superoxide radicals were generated by the xanthine/xanthine oxidase (X/XO) system, following a described procedure (Valentão et al., 2001). Xanthine was dissolved in 1 μM NaOH and subsequently in phosphate 50 mM buffer with 0.1 mM EDTA, pH 7.8, xanthine oxidase in 0.1 mM EDTA and the other components in 50 mM phosphate buffer with 0.1 mM EDTA, pH 7.8. Three experiments were performed in triplicate.

## 2.8. Effect on xanthine oxidase activity

The effect of the lyophilised extracts on xanthine oxidase activity was evaluated by measuring the formation of uric acid from xanthine in a double beam spectrophotometer (Helios α, Unicam), at room temperature. 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 600 μl. The absorbance was measured at 295 nm for 2 min. Three experiments were performed in triplicate.

## 2.9. Hydroxyl radical assay

The deoxyribose method for determining the scavenging effect of the aqueous extracts on hydroxyl radicals was performed according to a described procedure (Valentão et al., 2002) in a double beam spectrophotometer (Helios α, Unicam). Reaction mixtures contained ascorbic acid, FeCl<sub>3</sub>, EDTA, H<sub>2</sub>O<sub>2</sub>, deoxyribose and lyophilised extracts. All components were dissolved in 10 mM KH<sub>2</sub>PO<sub>4</sub>-KOH buffer, 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 potentials, respectively. Three experiments were performed in triplicate.

## 2.10. 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 (Helios α, Unicam). Hypochlorous acid and TNB were prepared immediately before use. Scavenging of hypochlorous acid was ascertained by using lipoic acid as a reference scavenger, which inhibited TNB oxidation in a concentration-dependent manner. Three experiments were performed in triplicate.

### 3. Results and discussion

#### 3.1. Identification and quantification of phenolic compounds by HPLC/DAD

The aqueous lyophilised extracts of tronchuda cabbage external leaves presented different phenolic compositions. In sample D, only three compounds were detected: kaempferol 3-*O*-sophorotrioside, kaempferol 3-*O*-(sinapoyl)-sophoroside and kaempferol 3-*O*-(feruloyl)-sophorotrioside (Table 2). In sample C, besides kaempferol 3-*O*-(feruloyl)-sophorotrioside, we identified kaempferol 3-*O*-sophoroside-7-*O*-glucoside, kaempferol 3-*O*-sophorotrioside-7-*O*-sophoroside, kaempferol 3-*O*-(feruloyl)-sophoroside, kaempferol 3-*O*-sophoroside and kaempferol 3-*O*-glucoside (Table 2). Sample B showed all the compounds described above and kaempferol 3-*O*-sophorotrioside-7-*O*-glucoside, kaempferol 3-*O*-(methoxycaffeoyl/caffeoyl)-sophoroside-7-*O*-glucoside, kaempferol 3-*O*-(sinapoyl/caffeoyl)-sophoroside-7-*O*-glucoside and kaempferol 3-*O*-(feruloyl/caffeoyl)-sophoroside-7-*O*-glucoside (Table 2). Sample A exhibited a chemical profile composed of the 12 above-mentioned kaempferol derivatives and kaempferol 3-*O*-sophoroside-7-*O*-sophoroside (Fig. 1 and Table 2). These compounds have already been identified in tronchuda cabbage external leaves methanolic extracts (Ferrerres et al., 2005).

Sample A was the one with the highest amount of phenolics (ca. 1231 mg/kg) (Table 2), kaempferol 3-*O*-(feruloyl/caffeoyl)-sophoroside-7-*O*-glucoside being the main compound (26% of total identified compounds). Sample B had a lower phenolics content (ca. 32 mg/kg) (Table 2), in which kaempferol 3-*O*-sophoroside was the compound present in the highest amount (32%). In sample D, kaempferol 3-*O*-(feruloyl)-sophoro-

trioside was the major kaempferol derivative, while sample C exhibited only vestigial amounts of phenolics (Table 2).

As previously observed (Ferrerres et al., 2005), when considering the evolution during winter, a decrease of the total phenolics content was noticed between November and December, which is more evident in samples from organic culture. The lowest level of total phenolics, observed in December in a sample from organic production, may be explained by the commitment of tronchuda cabbage cells to morphogenic developmental pathways, since those cabbages presented more developed leaves than those of conventional culture in the same period. In addition, samples from organic culture exhibited higher total phenolics content than those from conventional practices, collected in the same period, with the exception of the samples from December. This might be due to the interference of the mineral fertilizers and/or pesticides, used in conventional practices, in the biosynthetic pathway of flavonoids, decreasing phenolic amounts (Ferrerres et al., 2005).

#### 3.2. Identification and quantification of organic acids by HPLC/UV

Tronchuda cabbage external leaves had a chemical profile composed of five identified organic acids: citric, ascorbic, malic, shikimic and fumaric acids (Fig. 2). These compounds are reported for the first time in tronchuda cabbage. As observed for phenolic compounds, sample A exhibited the highest content of organic acids (ca. 55 g/kg) and sample C the lowest one (ca. 20 g/kg) (Table 3). In samples A, B and C, malic acid was the main compound, ranging from 37% to 78% of total identified organic acids, followed by citric acid (16–36%) (Table 3). Sample D showed a distinct profile, in

Table 2  
Phenolic composition of tronchuda cabbage external leaves (mg of phenolic compound kg<sup>-1</sup> of lyophilised extract)<sup>a</sup>

Compounds <sup>b</sup>	Samples							
	A		B		C		D	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
1 + 2 (RT 24.0, 24.2 min)	73.1	2.1	nq	–	nd	–	nd	–
3 + 4 (Rt 25.8, 26.0 min)	237	5.2	8.2	0.5	nq	–	nd	–
5 (RT 27.8 min)	46.3	6.9	nd	–	nd	–	nd	–
6 (RT 30.7 min)	119	5.9	nq	–	nd	–	nd	–
7 (RT 31.2 min)	323	7.0	2.5	0.1	nd	–	nd	–
8 (RT 36.5 min)	26.1	0.1	nq	–	nd	–	nq	–
9 (RT 37.4 min)	nq	–	2.6	0.0	nd	–	nq	–
10 (RT 38.5 min)	85.8	1.5	7.4	0.0	nq	–	5.9	0.5
11 (RT 39.3 min)	99.8	1.8	1.3	0.1	nq	–	nd	–
12 (RT 39.7 min)	221	5.2	10.6	0.1	nq	–	nd	–
13 (RT 44.1 min)	nq	–	nq	–	nq	–	nd	–
Σ	1231.2		32.6		–		5.9	

<sup>a</sup> Results are expressed as means of three determinations. SD, standard deviation, Σ, sum of the determined phenolic compounds.

<sup>b</sup> Identity of compounds as in Fig. 1.

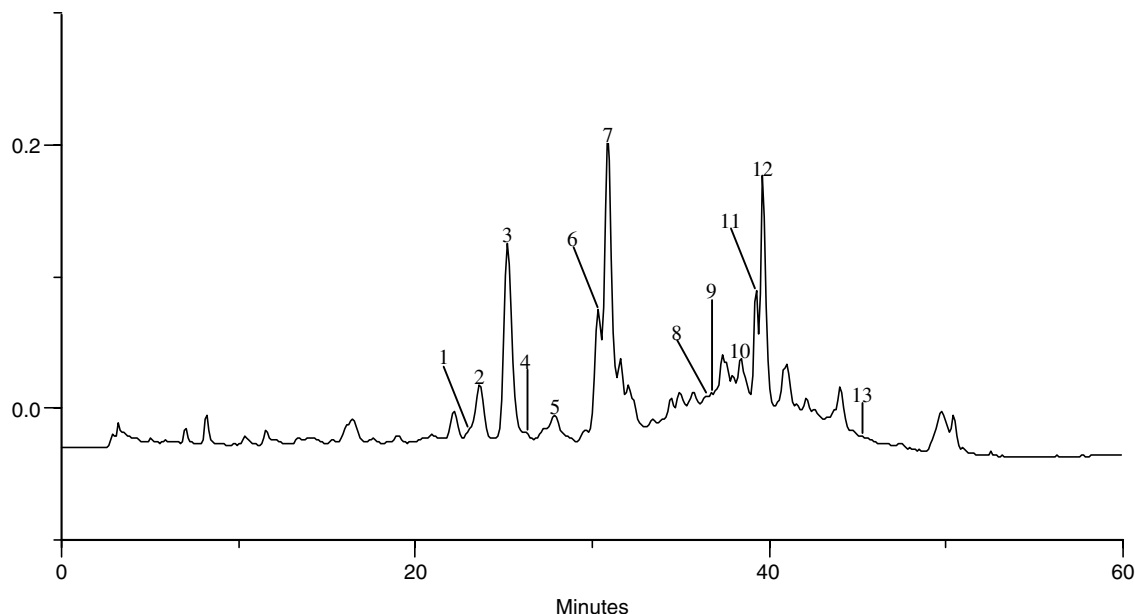


Fig. 1. HPLC phenolic profile of tronchuda cabbage external leaves. Detection at 330 nm. Peaks: (1) kaempferol 3-*O*-sophorotrioside-7-*O*-glucoside; (2) kaempferol 3-*O*-(methoxycaffeoyl/caffeoyl)-sophoroside-7-*O*-glucoside; (3) kaempferol 3-*O*-sophoroside-7-*O*-glucoside; (4) kaempferol 3-*O*-sophorotrioside-7-*O*-sophoroside; (5) kaempferol 3-*O*-sophoroside-7-*O*-sophoroside; (6) kaempferol 3-*O*-(sinapoyl/caffeoyl)-sophoroside-7-*O*-glucoside; (7) kaempferol 3-*O*-(feruloyl/caffeoyl)-sophoroside-7-*O*-glucoside; (8) kaempferol 3-*O*-sophorotrioside; (9) kaempferol 3-*O*-(sinapoyl)-sophoroside; (10) kaempferol 3-*O*-(feruloyl)-sophorotrioside; (11) kaempferol 3-*O*-(feruloyl)-sophoroside; (12) kaempferol 3-*O*-sophoroside; (13) kaempferol 3-*O*-glucoside.

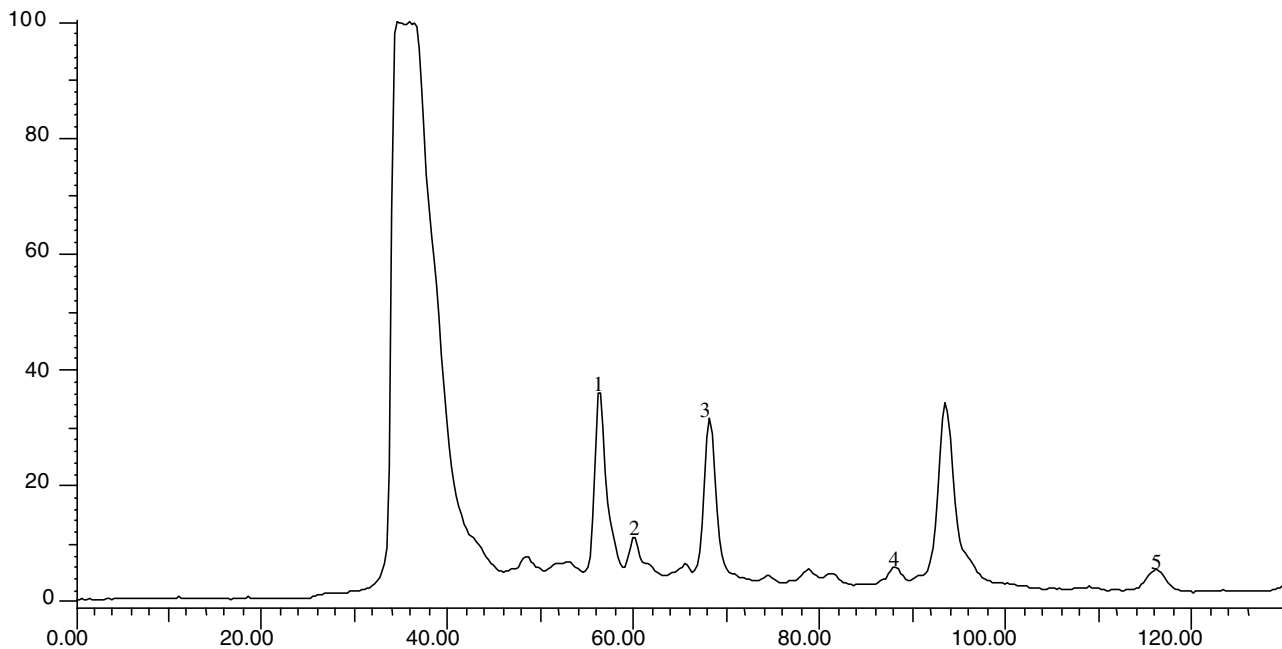


Fig. 2. HPLC organic acid profile of tronchuda cabbage external leaves. Detection at 214 nm. Peaks: (1) citric acid; (2) ascorbic acid; (3) malic acid; (4) shikimic acid; (5) fumaric acid.

which ascorbic acid was the acid present in highest amounts representing 44% of total compounds, followed by malic acid (39%) (Table 3). In all samples, shikimic and fumaric acids were minor compounds (Table 3).

### 3.3. Antioxidant activity

Antioxidant capacities of tronchuda cabbage external leaves aqueous lyophilised extracts are shown in Table 4

Table 3  
Organic acids in tronchuda cabbage external leaves (mg of organic acid kg<sup>-1</sup> of lyophilised extract)<sup>a</sup>

Compound <sup>b</sup>	Samples							
	A		B		C		D	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
1 (RT 56.7 min)	18464	52.2	9101	172	3312	185	6770	3.0
2 (RT 60.1 min)	10609	12.1	2937	2.8	1028	146.3	17343	206
3 (RT 68.6 min)	20506	57.3	13385	171	15472	286.9	15274	222
4 (RT 88.9 min)	24.1	0.2	17.3	2.0	31.7	1.3	34.0	3.8
5 (RT 116.8 min)	5336	115	27.7	0.1	111	0.0	58.0	1.0
Σ	54939		25468		19954		39479	

<sup>a</sup> Results are expressed as means of three determinations: SD, standard deviation, Σ, sum of the determined organic acids.

<sup>b</sup> Identity of compounds as in Fig. 2.

Table 4  
IC<sub>25</sub> values (mg ml<sup>-1</sup>) of tronchuda cabbage external leaves in antiradical assays

Samples	DPPH IC <sub>25</sub>	O <sub>2</sub> <sup>-</sup> (non-enzymatic) IC <sub>25</sub>	O <sub>2</sub> <sup>-</sup> (enzymatic) IC <sub>25</sub>	·OH IC <sub>25</sub>
A	0.681	0.102	0.047	0.019
B	1.66	0.045	0.139	0.045
C	2.32	0.362	0.311	0.223
D	1.14	0.146	0.101	0.018

and Figs. 3–6. The DPPH· test is a non-enzymatic method currently used to provide basic information on the ability of extracts to scavenge free radicals. Reduction of DPPH· by an antioxidant results in a loss of absorbance at 515 nm (Fukumoto & Mazza, 2000). Our results indicated that sample A was the one with the highest antioxidant potential, measured by the DPPH assay, followed by samples D and B, respectively. Sample C exhibited the lowest scavenging capacity (Table 4 and Fig. 3). The effects observed were concentration-dependent.

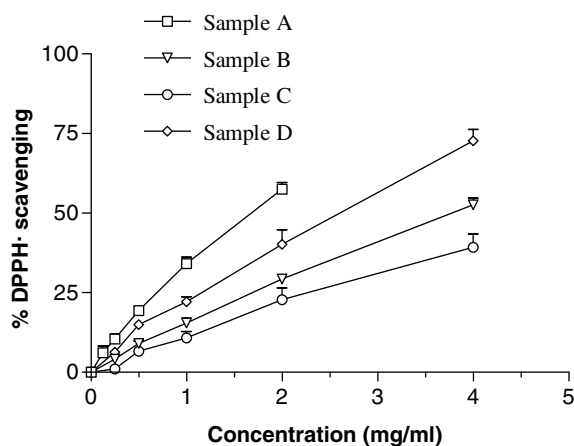


Fig. 3. Effect of tronchuda cabbage external leaves on DPPH· reduction. Values show means ± SE from 3 experiments performed in triplicate.

Superoxide radical is produced in vivo by activated phagocytes, by electron leakage from the mitochondrial electron transport chain (Halliwell, 1991) and in the conversion of xanthine to uric acid (Bast, Haenen, & Doelman, 1991). The reactivity of this radical is limited but it is considered to be toxic. In fact, much of the molecular damage that can be done by superoxide radical is due to its conversion into much more reactive species, namely hydroxyl radical and peroxynitrite (Halliwell et al., 1995).

In the present work, the lyophilised extracts of tronchuda cabbage external leaves exhibited superoxide radical-scavenging activity using the X/XO system in a concentration-dependent manner (Fig. 4(a)), sample A being the most active (Table 4). Bearing in mind that an inhibitory effect on the enzyme itself would also lead to a decrease of NBT reduction (Halliwell et al., 1995), the effect of the extracts on XO activity was checked. For this purpose a control experiment was performed evaluating the effect of tronchuda cabbage lyophilised extracts on the metabolic conversion of xanthine to uric acid. The results revealed that samples A, C and D had a weak inhibitory effect on XO, while sample B presented higher inhibitory activity (IC<sub>20</sub> at 184 µg/ml) (Fig. 4(b)), so it was not possible to show a clear-cut scavenging effect on superoxide radical. In view of clarification, the effect of the aqueous extracts on superoxide radical, generated by a chemical system composed of PMS, NADH and oxygen, was also evaluated. Under the assay conditions, all the samples were able to scavenge O<sub>2</sub><sup>-</sup> in a concentration-dependent way (Fig. 4(c)), in which sample B showed the strongest scavenging activity (Table 4).

Hydroxyl radical is the most reactive radical known: it can attack and damage almost every molecule found in living cells. Reactions of ·OH include its ability to interact with the purine and pyrimidine bases of DNA. It can also abstract hydrogen atoms from biological molecules, including thiols, leading to the formation of sulfur radicals able to combine with oxygen to generate oxysulfur radicals, a number of which damage biological molecules (Halliwell, 1991). The best-characterised

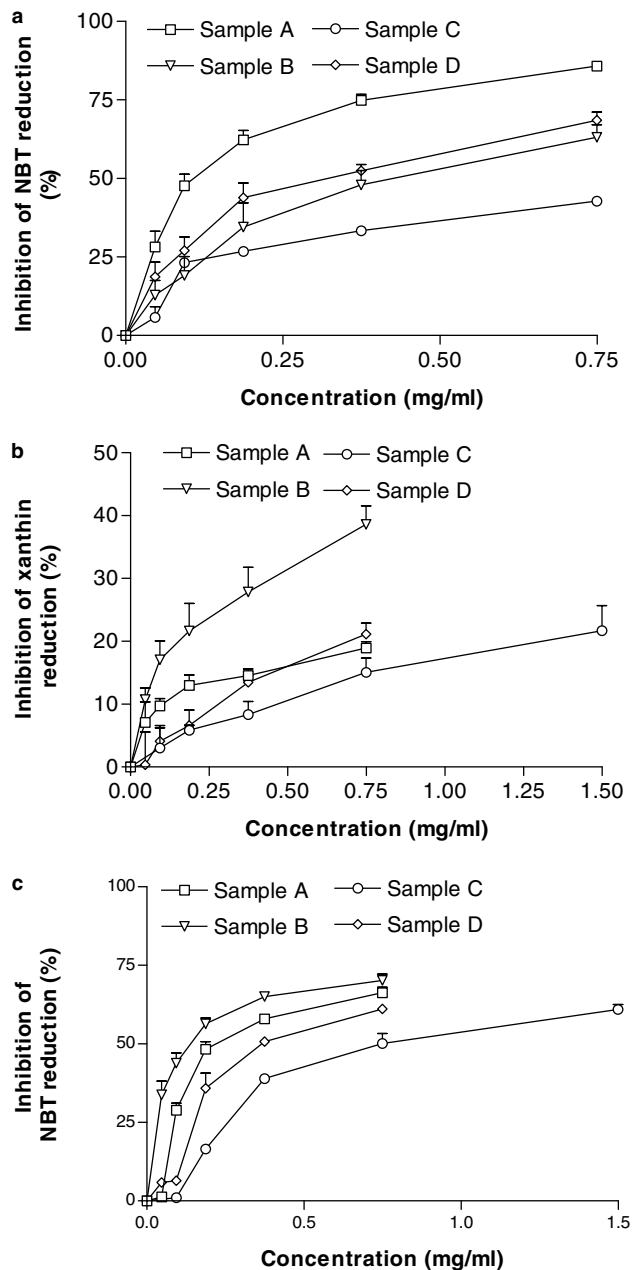


Fig. 4. Effect of tronchuda cabbage external leaves on: (a) NBT reduction induced by superoxide radical generated in a X/XO system; (b) on XO activity; (c) NBT reduction induced by superoxide radical generated in a NADH/PMS system. Values show means  $\pm$  SE from 3 experiments performed in triplicate.

biological damage caused by hydroxyl radical is its capacity to stimulate lipid peroxidation, which occurs when  $\cdot\text{OH}$  is generated close to membranes and attacks the fatty acid side chains of the membrane phospholipids (Halliwell, 1991).

Hydroxyl radicals are produced *in vivo* by Fenton-type reactions, in which transition metals (e.g., iron) reduce hydrogen peroxide. Reducing agents, such as ascorbic acid, can accelerate  $\cdot\text{OH}$  formation by reducing  $\text{Fe}^{3+}$  ions to  $\text{Fe}^{2+}$  (Puppo, 1992). Deoxyribose is de-

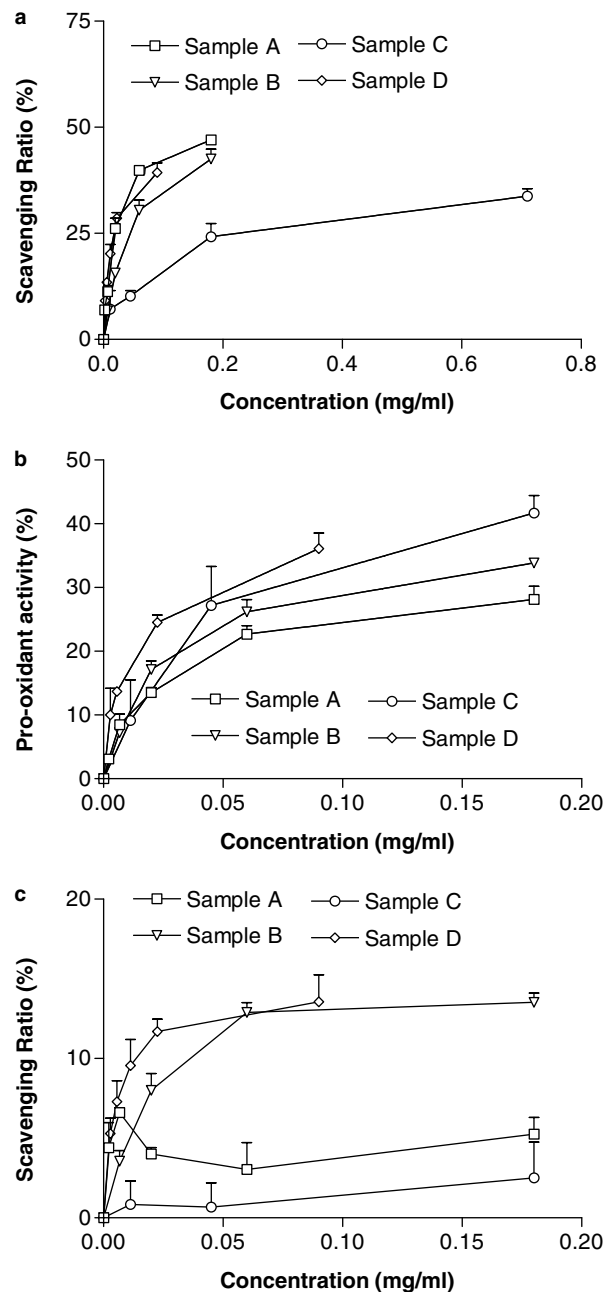


Fig. 5. Effect of tronchuda cabbage external leaves: (a) non-specific hydroxyl radical-scavenging activity; (b) pro-oxidant activity (-AA); (c) specific hydroxyl radical-scavenging (-EDTA). Values show means  $\pm$  SE from 3 experiments performed in triplicate.

graded into malonaldehyde on exposure to hydroxyl radicals generated by Fenton systems. If the resulting mixture is heated under acid conditions, malonaldehyde may be detected by its ability to react with thiobarbituric acid to form a pink chromogen (Halliwell, Gutteridge, & Aruoma, 1987). In the work herein, tronchuda cabbage lyophilised extracts exhibited scavenging activity for hydroxyl radical in a concentration dependent manner (Fig. 5(a)), with samples A and D presenting the highest capacity (Table 4). Sample C exhibited minor ability.

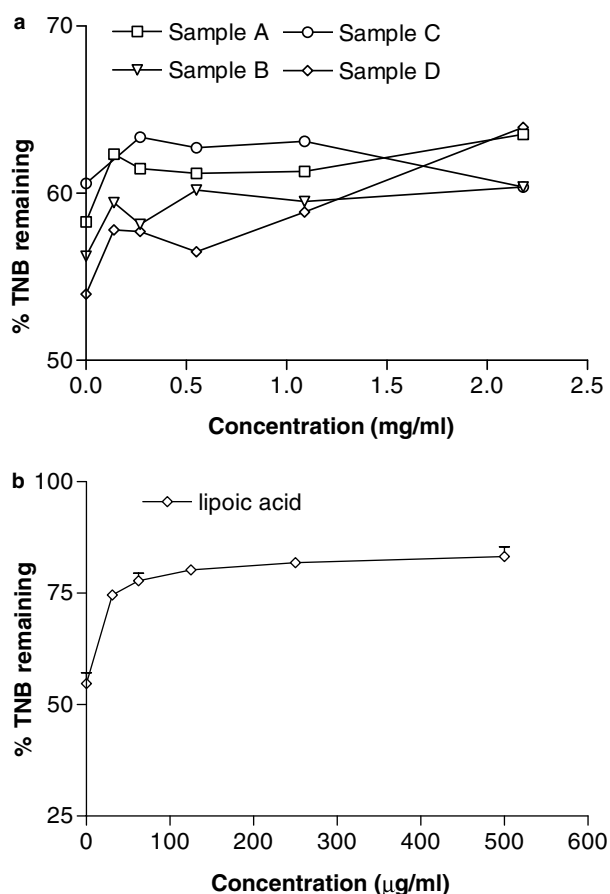


Fig. 6. Effects of tronchuda cabbage external leaves (a) and lipoic acid (b) on the oxidation of TNB by HOCl. The amount of TNB unchanged after incubation is calculated and expressed as percentage of the initial value. Values show means  $\pm$  SE from 3 experiments performed in triplicate.

However, some compounds have the ability to cause redox cycling of the metal ion required for hydroxyl generation, thus increasing the radical production, exhibiting pro-oxidant activity (Li & Xie, 2000). In order to evaluate the pro-oxidant potential of the extracts, we omitted ascorbic acid. As can be seen in Fig. 5(b), all samples were a very effective substitute for ascorbic acid. Thus, these extracts may act as concentration-dependent pro-oxidants, that from sample D being the most active.

Damage to deoxyribose also occurs if the  $\text{Fe}^{3+}$ -ascorbate- $\text{H}_2\text{O}_2$ -induced generation of hydroxyl radicals is performed in the absence of EDTA, since omission of the chelator allows iron ions to bind directly to the sugar. Under such conditions, compounds inhibit deoxyribose degradation, not by reacting with hydroxyl radicals, but because they present ion-binding capacity and can withdraw the iron ions and render them inactive or poorly active in Fenton reactions (Payá, Halliwell, & Hout, 1992). The assay performed in the absence of EDTA showed that extracts from samples B and D were, to some extent, able to chelate iron ions in a con-

centration-dependent manner, while those from samples A and C were practically ineffective (Fig. 5(c)).

Reactive oxygen species produced in vivo by activated phagocytic cells include HOCl. Hypochlorous acid is produced in the organism at sites of inflammation by the oxidation of  $\text{Cl}^-$  ions, catalysed by neutrophil-derived myeloperoxidase, in the presence of  $\text{H}_2\text{O}_2$  (Aruoma, Halliwell, Hoey, & Butler, 1989). HOCl is a powerful oxidant, which reacts readily with many biologically important molecules. Thiol groups are easily oxidized by HOCl (Ching, De Jong, & Bast, 1994). HOCl damages and induces target cell lysis, caused by sulfhydryl oxidation in plasma membrane proteins (Cochrane, 1991), inactivates  $\alpha_1$ -antiprotease, activates collagenase and gelatinase, depletes antioxidant vitamins, such as ascorbic acid, and inactivates antioxidant enzymes such as catalase (Payá et al., 1992; Visioli, Belomo, & Galli, 1998).

In the present assay, HOCl induces the oxidation of TNB ( $\lambda_{\text{max}} = 412 \text{ nm}$ ) into DTNB ( $\lambda_{\text{max}} = 325 \text{ nm}$ ) (Künzel, Zee, & Ijzerman, 1996). An HOCl scavenger inhibits the oxidation of TNB by this species. When the antioxidant protection against damage by HOCl was evaluated, tronchuda cabbage lyophilised extracts exhibited a very weak antioxidant protective activity, with the exception of sample D, which showed some concentration dependent capacity (Fig. 6(a)). In the assayed conditions, lipoic acid was used as a reference compound, which scavenged HOCl effectively in a concentration-dependent manner, presenting a protective effect of 83% at 500  $\mu\text{g/ml}$  (Fig. 6(b)).

In general terms, and according to the results obtained in all assays, sample A proved to be the one with highest antioxidant potential. This is not surprising, considering that it is the sample with the highest content of both phenolics and organic acids (Tables 2 and 3), which are known to have antioxidant activity (Madhavi, Singhal, & Kulkarni, 1996). In addition, sample A has a high amount of acylated flavonols, namely caffeoyl derivatives (Table 2). These compounds are reported to have high scavenging ability due to the presence of an *O*-dihydroxy structure in the caffeoyl moiety, which confers great stability to the radical form and participates in the electron delocalisation (Braca et al., 2003). The presence of high contents of caffeic acid derivatives may also contribute to its lower pro-oxidant effect, because of the presence of a catechol ring (Galati, Sabzevari, Wilson, & O' Brien, 2002).

In opposition, sample C displayed the lowest antioxidant capacity, which can be ascribed to the presence of only vestigial amounts of phenolic compounds and to the minor organic acid content. Furthermore, this sample exhibited the lowest ascorbic acid content, when compared with the other samples. In spite of this, sample C showed a pro-oxidant effect, which might be related to the presence of other compounds in the extract.



The great antioxidant activity presented by sample D may be attributed to its major content of ascorbic acid. However, the high amount of this organic acid can also explain the pro-oxidant effect observed in the deoxyribose assay performed in the absence of ascorbic acid.

Although the antiradical activity of phenolic compounds and organic acids is already known, it remains uncertain how a complex mixture, obtained from plant extracts, functions against reactive oxygen species, because other compounds of the mixture may potentiate or prevent the expected activity. In fact, this study indicates that phenolics and organic acids were not correlated with antioxidant capacity of the aqueous lyophilised extracts.

In conclusion, considering the results obtained and regarding the presence of several phenolics and organic acids in the aqueous lyophilised extracts of tronchuda cabbage external leaves, the scavenging activities observed against DPPH<sup>•</sup>, superoxide radical, hydroxyl radical and hypochlorous acid are most probably due to the presence of these compounds, which contribute to the protective effects observed in the work herein. As far as we know, this is the first study concerning the antioxidant activity of tronchuda cabbage, and indicates that its external leaves can be used as good sources of antioxidants in our diet. This may be relevant in the prevention of diseases in which free radicals are involved.

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