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# Tronchuda Cabbage (*Brassica oleracea* L. var. *costata* DC): Scavenger of Reactive Nitrogen Species

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The ability of tronchuda cabbage (*Brassica oleracea* L. var. *costata* DC) to act as a scavenger of the reactive nitrogen species nitric oxide and peroxynitrite was investigated. The aqueous extracts obtained from tronchuda cabbage seeds and from its external and internal leaves exhibited a concentration dependent scavenging capacity. The antioxidant potential observed against the two reactive species was as follows: seeds > external leaves > internal leaves. In order to establish a possible correlation with the chemical composition of the extracts, the activity of ascorbic and sinapic acids and kaempferol 3-*O*-rutinoside was also studied. Among the compounds tested, sinapic acid showed the strongest antioxidant activity against both species.

**KEYWORDS:** ***Brassica oleracea* L. var. *costata* DC; Brassicaceae; nitric oxide; peroxynitrite; phenolic compounds; ascorbic acid**

## INTRODUCTION

Nitric oxide (NO) is an important molecule involved in several physiological processes, which include blood pressure control, neural signal transduction, platelet function, and antimicrobial defense, among others (*1–4*). NO is produced in various cell types by nitric oxide synthases and reacts rapidly with superoxide to form peroxynitrite ($ONOO^-$), essential in the defensive process against invading microorganisms *in vivo* (*5*).

Despite their beneficial effects, an overproduction of these reactive nitrogen species (RNS) is associated with several types of biological damage. Deleterious effects include lipid peroxidation, protein oxidation and nitration, enzyme inactivation, and DNA damage (*6*), which lead to chronic inflammation, cardiovascular diseases, Parkinson's and Alzheimer's diseases, and several types of cancer (*7, 8*). Scavengers of RNS, especially those from exogenous sources, may play a pivotal role in preventing/controlling degenerative diseases. Antioxidants must react with reactive species faster than biological substrates, thus protecting biological targets from oxidative damage (*9*).

With this respect, it has been shown that fruits and vegetables can be a constant source of health-promoting compounds (*8, 10*). One group of vegetables that has been regarded as potentially cancer protective is the Brassicaceae family. The consumption of *Brassica* vegetables throughout the world is enormous and they constitute an important part of a well balanced diet. They are reported to reduce the risks of some cancers especially due to its glucosinolates and their derived products (*11, 12*), although phenolic compounds are also found to contribute to this ability (*13*).

Among *Brassica* species, tronchuda cabbage (*Brassica oleracea* L. var. *costata* DC) is commonly consumed, being widely used in the preparation of soups or just boiled. Additionally, it also constitutes an ingredient of salads, particularly the internal leaves which are tender and sweeter than the external ones. Previous work on the chemical characterization of this species revealed distinct compositions of its external and internal leaves, seeds, and sprouts, in terms of phenolic compounds and organic acids (*14–19*). Tronchuda cabbage leaves and seeds were also shown to be effective scavengers of reactive oxygen species, namely superoxide and hydroxyl radicals and hypochloride (*14, 15, 18*), but, as far as we know, its activity against RNS has not been reported.

In this paper tronchuda cabbage aqueous extracts from internal and external leaves and from seeds were studied for their antioxidant ability against nitric oxide and peroxynitrite. With this purpose the capacity of tronchuda cabbage extracts to scavenge NO was evaluated by monitoring nitrite accumulation in the presence of nitroprusside. The protection against damage by $ONOO^-$ was assessed by their abilities to inhibit tyrosine nitration. In order to establish some correlation with the chemical composition of the extracts, phenolic compounds and organic acids were determined by HPLC-DAD and HPLC-UV, respectively, and the antioxidant capacity of ascorbic and sinapic acids and kaempferol 3-*O*-rutinoside was also evaluated.

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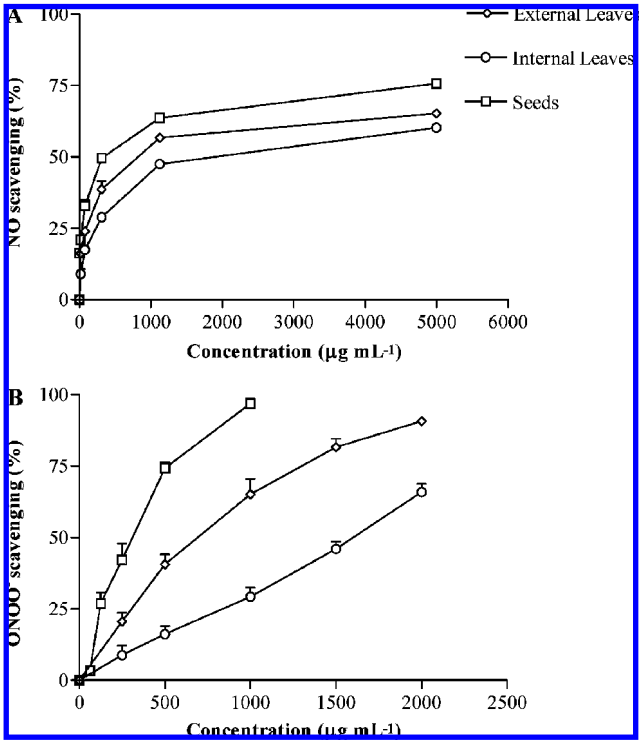


**Figure 1.** Effect of tronchuda cabbage extracts against (**A**) nitric oxide and (**B**) peroxynitrite. Values show mean + SE from three experiments, performed in triplicate.

**Table 1.** $IC_{50}$ Values ± SE ($\mu$g $mL^{-1}$) of Tronchuda Cabbage against Reactive Nitrogen Species

| sample | NO | ONOO |
|---|---|---|
| seeds | 356 ± 58 | 302 ± 34 |
| internal leaves | 2228 ± 214 | 1641 ± 90 |
| external leaves | 884 ± 100 | 707 ± 92 |

## MATERIALS AND METHODS

**Standards and Reagents.** Sinapic acid was from Sigma (St. Louis, MO), kaempferol 3-*O*-rutinoside and rutin from Extrasynthése (Genay, France), and L-(+)-ascorbic acid from Merck (Darmstadt, Germany). Methanol, hydrogen peroxide 30%, potassium dihydrogen phosphate, *N*-(1-naphthyl)ethylenediamine dihydrochloride, and phosphoric acid were obtained from Merck. Sodium nitroprussiate dihydrate was from Riedel-de Haën (St. Louis, MO), magnesium peroxide was from Aldrich (St. Louis, MO), DL-penicillamine, sulfanilamide, potassium nitrite, L-tyrosine, and 3-nitro-L-tyrosine were from Sigma. The water was treated in a Milli-Q water purification system (Millipore, Bedford, MA).

**Plant Material.** Tronchuda cabbage (*Brassica oleracea* L. var. *costata* DC) was produced under organic practices, as described previously (*15*). Three plants were randomly collected, and their external and internal leaves were separated, frozen, and lyophilized. External and internal leaf dried material was powdered and kept in a desiccator, in the dark. Tronchuda cabbage seeds were obtained from local farmers in Bragança, Northeast Portugal, which produce and commercialize tronchuda cabbage. Identification was performed by José A. Pereira, Ph.D. (CIMO/Escola Superior Agrária, Instituto Politécnico de Bragança). Voucher specimens (20041027E, 20041027I, and TCS) were deposited at Pharmacognosy Service, Faculty of Pharmacy, Porto University.

**Extracts Preparation.** Tronchuda cabbage leaf and seed extracts were prepared as previously reported (*14, 15*). Briefly, 3.0 g of lyophilized plant leaves or ground seeds were boiled for 1 h in 600 mL of water. The resulting extracts were filtered over a filtration funnel and then lyophilized in a Labconco FreeZone 4.5 apparatus. The lyophilized extracts were kept in a desiccator, in the dark. For phenolic compounds and organic acids analyses, they were redissolved in water and 0.01 N sulfuric acid, respectively.

**Nitric Oxide Scavenging Activity.** The antiradical activity was determined spectrophotometrically in an ELX808 IU Ultra Microplate Reader (Bio-Tek Instruments, Inc.), according to a described procedure (*20*), with modifications. A 100 $\mu$L amount of 20 mM sodium nitroprusside was incubated with 100 $\mu$L of sample (five different concentrations) for 60 min, at room temperature, under light. All solutions were prepared in 0.1 M phosphate buffer (pH 7.4). After incubation, 100 $\mu$L of Greiss reagent, containing 1% sulfanilamide and 0.1% naphthylethylenediamine in 2% phosphoric acid, was added to each well. The mixture was incubated at room temperature for 10 min, and the absorbance of the chromophore formed during the diazotization of nitrite with sulfanilamide and subsequent coupling with naphthylethylenediamine was read at 562 nm. Rutin was used as positive control (*21*).

**Peroxynitrite Scavenging Activity.** *Peroxynitrite Synthesis.* $ONOO^-$ was synthesized as previously reported (*22*). Briefly, 20 mL of an acidic solution (0.6 M HCl) of $H_2O_2$ (0.7 M) was mixed with 20 mL of $KNO_2$ (0.6 M), on ice, for 1 s, and the reaction was quenched with 20 mL of ice-cold NaOH (1.2 M). Residual $H_2O_2$ was removed by adding 10−15 mg of $MnO_2$. The solution was then filtered and frozen overnight at −20 °C. The yellow top layer of $ONOO^-$ formed by freeze fractionation was scraped, and the concentration of stock $ONOO^-$ was determined at 302 nm, using a molar extinction coefficient of 1670 $cm^{-1}$ $M^{-1}$.

*Nitration of Tyrosine by $ONOO^-$.* For each extract, a dilution series (five different concentrations) and a control solution without sample was prepared. Tyrosine 10 mM was prepared by dissolving 18.1 mg of pure compound in 8 mL of water with 250 $\mu$L (10% w/v) of KOH, followed by neutralization with 250 $\mu$L of 5% phosphoric acid and addition of 1.5 mL of water. A 100 $\mu$L amount of tyrosine solution and 100 $\mu$L of sample were added to 795 $\mu$L of buffer (100 mM $KH_2PO_4$, pH 7.4) and incubated in a water bath at 37 °C, for 15 min. After this time, 5 $\mu$L of 200 mM $ONOO^-$ was added under vigorous stirring (15 s), and the resulting mixture (pH 7.4−7.5) was incubated at 37 °C for another15 min. DL-penicillamine was used as positive control (*23*).

*HPLC Determination of 3-Nitrotyrosine.* Measurement of 3-nitrotyrosine was performed with a HPLC unit (Gilson Medical Electronics, France), using a reversed phase Spherisorb ODS2 column (250 × 4.6 mm i.d., 5 $\mu$m; Waters, Milford, MA). The injection volume was 20 $\mu$L. The eluent was 500 mM $KH_2PO_4$−$H_3PO_4$, pH 3.0, with 20% methanol (v/v) at a flow rate of 0.6 mL $min^{-1}$. Detection was achieved with a Gilson UV detector set at 274 nm, and the peak areas were calculated using the 712 HPLC System Controller Software (Gilson).

**HPLC-DAD Analysis of Phenolic Compounds.** Twenty microliter amounts of lyophilized extracts were analyzed using an HPLC unit (Gilson) and a RP-18 LiChroCART (Merck, Darmstadt, Germany) column (250 × 4 mm, 5 $\mu$m particle size). Chromatographic separations of seed and internal leaf extracts were accomplished under the conditions described previously (*14, 15*), using acetic acid 1% (A) and methanol (B) as solvents. Elution was performed at a flow rate of 1 mL $min^{-1}$, starting with 20% B and using a gradient to obtain 50% B at 30 min and 80% B at 37 min.

For the external leaf extract, the solvent system was a mixture of formic acid 5% in water (A) and methanol (B), with a flow rate of 1 mL $min^{-1}$, and the gradient was as follows: 10% B at 0 min, 20% B at 25 min, 50% B at 40 min, 50% B at 45 min, 90% B at 46 min, 90% B at 50 min, 100% B at 55 min, 100% B at 58 min, and 10% B at 60 min (*15*).

In all cases 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 Software system (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. As standards correspondent to the compounds identified in the lyophilized

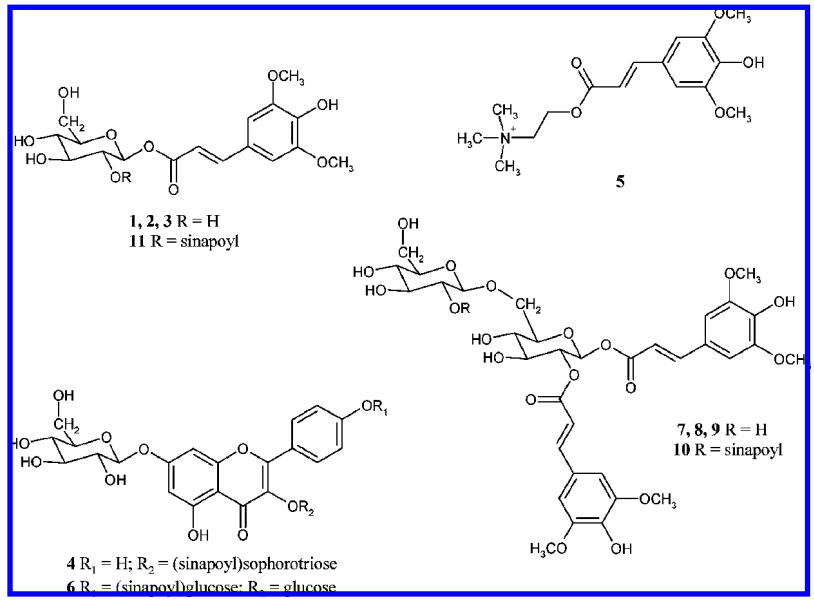


**Figure 2.** Chemical structures of several phenolic compounds present in tronchuda cabbage seeds. 1-Sinapoylglucose isomer (**1**); 1-sinapoylglucose isomer (**2**); 1-sinapoylglucose (**3**); kaempferol-3-*O*-(sinapoyl)sophorotrioside-7-*O*-glucoside (**4**); sinapoylcholine (**5**); kaempferol-3,7-*O*-diglucoside-4′-*O*-(sinapoyl)glucoside (**6**); 1,2-disinapoylgentiobiose isomer (**7**); 1,2-disinapoylgentiobiose isomer (**8**); 1,2-disinapoylgentiobiose (**9**); 1,2,2′-trisinapoylgentiobiose (**10**); 1,2-disinapoylglucose (**11**).

extracts were not commercially available, 3- and 4-*p*-coumaroylquinic acids were quantified as *p*-coumaric acid, sinapic acid derivatives as sinapic acid, and the kaempferol derivatives as kaempferol-3-*O*-rutinoside. Sinapic acid was quantified as itself.

**HPLC-UV Analysis of Organic Acids.** The separation of the organic acids present in the lyophilized extracts was carried out as previously reported (*14, 15*), 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 $min^{-1}$, with 0.01 N sulfuric acid. The detection was performed with a UV detector set at 214 nm. Organic acid quantification was achieved by the absorbance recorded in the chromatograms relative to external standards. Malic and quinic acids were quantified together as malic acid.

## RESULTS AND DISCUSSION

RNS have several roles in mammals, but their unregulated production can cause adverse effects through reaction with biological target molecules. Our recent studies have demonstrated that tronchuda cabbage can provide protection against some reactive oxygen species (*14, 15, 18*). In the present work the study of the antioxidant capacity of tronchuda cabbage was extended to the RNS nitric oxide and peroxynitrite.

Nitric oxide is a short-lived free radical, which possesses a small dipole moment because of the similar electronegativity of oxygen and nitrogen, turning it essentially hydrophobic and freely diffusible across membranes (*24*). NO was generated from sodium nitroprusside, an inorganic complex used as NO donor: in aqueous solution at physiological pH, and under light irradiation, NO is spontaneously released from sodium nitroprusside (*25*). As NO is relatively unstable in the presence of molecular oxygen, it rapidly autoxidizes to yield a variety of nitrogen oxides, namely nitrogen dioxide, dinitrogen trioxide, and nitrite (*26*). Nitrite is the only stable product and can be estimated by use of Greiss reagent (*26*). In this assay scavengers of NO compete with oxygen, leading to reduced nitrite production (*20*). Tronchuda cabbage extracts showed a concentration-dependent protective effect against NO (**Figure 1A**), with the seed extract being the one with the major scavenging potential (**Table 1**). The external leaves were shown to have higher NO scavenging capacity than the internal ones (**Table 1**). Recently we have reported tronchuda cabbage antioxidant ability against superoxide radical (*14, 15, 18*). Now a scavenging activity towards nitric oxide was demonstrated, which may be important in preventing peroxynitrite formation by its reaction with superoxide radical.

Peroxynitrite is a major damaging oxidant produced *in vivo* that reacts slowly enough with most biological molecules to be partially selective in the types of molecules that it attacks (*27*). It is a potent nitrating agent that converts free and protein-bound tyrosine to the corresponding 3-nitro derivative (*28*), a stable adduct involving a carbon−nitrogen bond that is difficult to remove chemically (*27*). 3-Nitrotyrosine is considered to be a marker of $ONOO^-$-dependent damage *in vivo*: tyrosine nitration affects protein structure and function, contributing to further dysfunctional changes (*28, 29*). In this work 3-nitrotyrosine was formed from the reaction between free tyrosine and peroxynitrite, at physiological pH, and determined by HPLC-UV analysis. Thus, in the presence of peroxynitrite scavengers there will be an inhibition of tyrosine nitration. Qualitative analyses were previously performed to verify that there was no coelution with 3-nitrotyrosine. Tronchuda cabbage extracts effectively inhibited the formation of 3-nitrotyrosine, in a concentration dependent manner (**Figure 1B**). Seed extract displayed the most potent $ONOO^-$ scavenging capacity, followed by the external leaves (**Table 1**).

When comparing the results obtained here with those previously reported for reactive oxygen species (*14, 15, 18*), the same order of antioxidant capacity could be observed: tronchuda cabbage seeds exhibit the highest protective activity and the internal leaves the lowest one. The different results obtained with the three tested tronchuda cabbage extracts may be explained by their distinct chemical composition. In addition to the vitamins and minerals of fruits and vegetables, phytochemicals, such as phenolic compounds and organic acids, may contribute to their antioxidant capacity. The phenolic and organic acid composition of the tested extracts was determined by HPLC-DAD and HPLC-UV, respectively.

**Table 2.** Quantification of Phenolic Compounds (mg/kg, dry basis)[a]

| compounds | seeds | internal leaves | external leaves |
|---|---|---|---|
| | Sinapic Acid Derivatives[b] | | |
| SnGt | 309 ± 0.3 | | |
| 1-SnGl isomer | 368 ± 11.7 | | |
| SnGt isomer | 270 ± 1.7 | | |
| 1-SnGl isomer | 417 ± 9.8 | | |
| 1-SnGl | 703 ± 11.5 | | |
| SnCholine | 376 ± 7.1 | | |
| 1,2-diSnGt isomer | 152 ± 3.2 | | |
| 1,2-diSnGt isomer | 345 ± 2.0 | | |
| 1,2-diSnGt | 1023 ± 37.1 | 52 ± 0.3 | |
| 1,2,2′-triSnGt | 448 ± 2.2 | 63 ± 0.4 | |
| 1,2-diSnGl | 368 ± 2.4 | | |
| SnGl acid | | 26 ± 0.2 | |
| Sinapic acid | | 180 ± 1.1 | |
| 1,2′-diSn-2-FrGt | | 11 ± 0.1 | |
| | *p*-Coumaric Acid Derivatives[c] | | |
| 3-*p*-CmQn | | 189 ± 1.4 | 482 ± 8.6 |
| 4-*p*-CmQn | | 126 ± 0.9 | |
| | Kaempferol Derivatives[d] | | |
| K-3-(Sn)Sphtri-7-Gl | 911 ± 17.7 | | |
| K-3,7-diGl-4′-(Sn)Gl | 267 ± 17.6 | | |
| K-3-Sph-7-Gl | | 288 ± 8.8 | 852 ± 14.0 |
| K-3-(Cf)Sph-7-Gl | | 121 ± 3.7 | |
| K-3-(Sn)Sph-7-Gl | | 181 ± 5.5 | |
| K-3-(Fr)Sph-7-Gl | | 54 ± 1.6 | |
| K-3-Sph | | 100 ± 3.1 | 797 ± 31.4 |
| K-3-Sphtri-7-Gl + K-3-(MtCf/Cf)Sph-7-Gl | | | 453 ± 12.3 |
| K-3-Sphtri-7-Sph | | | 445 ± 6.1 |
| K-3-Sph-7-Sph | | | 2788 ± 48.9 |
| K-3-(Sn/Cf)Sph-7-Gl | | | 1682 ± 21.2 |
| K-3-(Fr/Cf)Sph-7-Gl | | | 1886 ± 45.1 |
| K-3-Sphtri | | | 719 ± 7.2 |
| K-3-(Sn)Sph | | | 1244 ± 62.8 |
| K-3-(Fr)Sphtri + K-3-(Fr)Sph | | | 1989 ± 33.1 |
| Σ | 5974 | 1390 | 13337 |

[a] Results are expressed as mean ± standard deviation of three determinations. Σ, sum of the determined phenolic compounds. [b] Sinapic acid equivalents. [c] *p*-Coumaric acid equivalents. [d] Kaempferol-3-*O*-rutinoside equivalents. Cf, caffeoyl; Cm, coumaroyl; Fr, feruloyl; Gl, glucose; Gt, gentiobiose; K, kaempferol; Mt, methoxy; Qn, quinic; Sn, sinapoyl; Sph, sophorose; Sphtri, sophorotriose.

Tronchuda cabbage seed extract was characterized by the presence of two sinapoylgentiobiose isomers, three sinapoylglucose isomers, kaempferol-3-*O*-(sinapoyl)sophorotrioside-7-*O*-glucoside, sinapoylcholine, kaempferol-3,7-*O*-diglucoside-4′-*O*-(sinapoyl)glucoside, three disinapoylgentiobiose isomers, 1,2,2′-trisinapoylgentiobiose, and 1,2-disinapoylglucose (**Figure 2**). All these compounds were already reported to occur in this matrix (*14*). These phenolics were present in high content in the analyzed extract (ca. 6.0 g/kg), from which ca. 80% corresponded to the nonflavonoid sinapic acid derivatives (**Table 2**).

The external leaf extract exhibited 3-*p*-coumaroylquinic acid, kaempferol 3-*O*-sophoroside-7-*O*-glucoside, kaempferol 3-*O*-sophoroside, kaempferol 3-*O*-sophorotrioside-7-*O*-glucoside, kaempferol 3-*O*-(methoxycaffeoyl/caffeoyl)sophoroside-7-*O*-glucoside, kaempferol 3-*O*-sophorotrioside-7-*O*-sophoroside, kaempferol 3-*O*-sophoroside-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, and kaempferol 3-*O*-(feruloyl)-sophoroside (**Figure 3**), which were already found previously in this material (*15, 16, 18*). This extract showed the highest phenolics amount (ca. 13 g/kg), in which the kaempferol

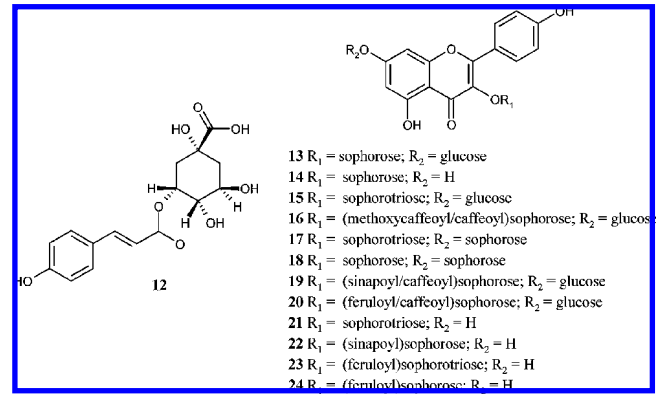


**Figure 3.** Chemical structures of the phenolic compounds present in tronchuda cabbage external leaves. (**12**) 3-*p*-coumaroylquinic acid; (**13**) kaempferol 3-*O*-sophoroside-7-*O*-glucoside; (**14**) kaempferol 3-*O*-sophoroside; (**15**) kaempferol 3-*O*-sophorotrioside-7-*O*-glucoside; (**16**) kaempferol 3-*O*-(methoxycaffeoyl/caffeoyl)sophoroside-7-*O*-glucoside; (**17**) kaempferol 3-*O*-sophorotrioside-7-*O*-sophoroside; (**18**) kaempferol 3-*O*-sophoroside-7-*O*-sophoroside; (**19**) kaempferol 3-*O*-(sinapoyl/caffeoyl)sophoroside-7-*O*-glucoside; (**20**) kaempferol 3-*O*- (feruloyl/caffeoyl)sophoroside-7-*O*-glucoside; (**21**) kaempferol 3-*O*-sophorotrioside; (**22**) kaempferol 3-*O*-(sinapoyl)sophoroside; (**23**) kaempferol 3-*O*-(feruloyl)sophorotrioside; (**24**); kaempferol 3-*O*-(feruloyl)sophoroside.

derivatives were predominant, being *p*-coumaric acid derivative a minor compound (ca. 4%) (**Table 2**).

The internal leaf extract presented both flavonol glycosides and hydroxycinnamic acid esters: 3-*p*-coumaroylquinic acid, kaempferol 3-*O*-sophoroside-7-*O*-glucoside, kaempferol 3-*O*-(caffeoyl)sophoroside-7-*O*-glucoside, sinapoyl glucoside acid, kaempferol 3-*O*-(sinapoyl)sophoroside-7-*O*-glucoside, kaempferol 3-*O*-(feruloyl)sophoroside-7-*O*-glucoside, 4-*p*-coumaroylquinic acid, sinapic acid, kaempferol 3-*O*-sophoroside, 1,2-disinapoylgentiobiose, 1,2,2′-trisinapoylgentiobiose and 1,2′-disinapoyl-2-feruloylgentiobiose (**Figure 4**). These compounds were also described previously in tronchuda cabbage internal leaves (*15, 17*). This extract exhibited the lowest phenolics content (ca. 1.4 g/kg), with kaempferol derivatives representing ca. 54% of total compounds (**Table 2**).

Concerning organic acids, the qualitative profile of the different plant material was identical, being composed of aconitic, citric, ascorbic, malic, quinic, shikimic, and fumaric acids (**Figure 5**). All these compounds were already reported in tronchuda cabbage (*14–19*). Seed extract consisted of ca. 16 g/kg of total identified compounds, while internal and external leaves showed a slightly higher amount (ca. 23 g/kg and 25 g/kg, respectively). Despite this, ascorbic acid was the major compound in both seed (ca. 52%) and external leaf (ca. 34%) extracts and an important one in the internal leaf extract (ca. 26%) (**Table 3**).

In order to establish a possible correlation between the chemical constitution of tronchuda cabbage extracts and the protective effects observed against NO and $ONOO^-$, sinapic acid and kaempferol 3-*O*-rutinoside were tested, since none of the identified phenolic acids or kaempferol derivatives were commercially available. Ascorbic acid was also assayed because of its predominance in the extracts. For the three tested compounds, a concentration dependent scavenging activity was observed against the two RNS (**Figure 6**), sinapic acid being the most effective one in both cases (**Table 4**). In fact, this compound exhibited a NO scavenging effect comparable to that of rutin and an activity against $ONOO^-$ stronger than that of DL-penicillamine, the two positive controls used in the assays.

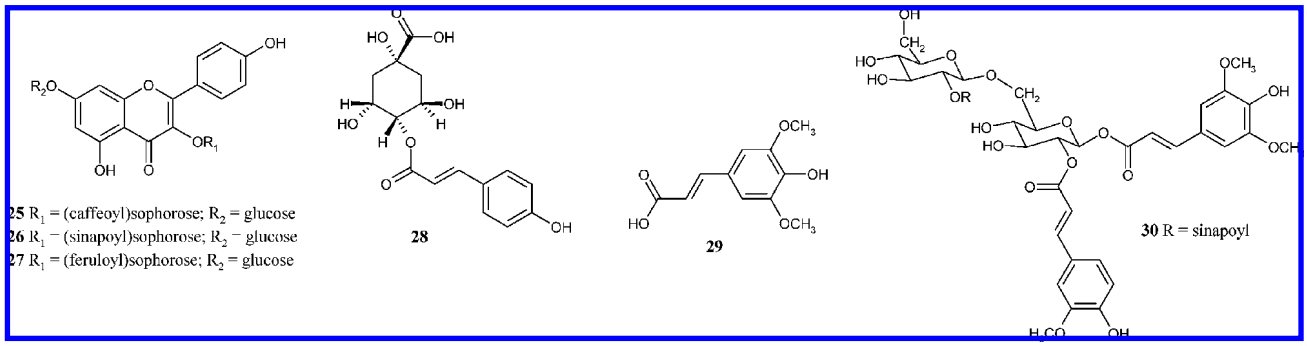


**Figure 4.** Chemical structures of several phenolic compounds present in tronchuda cabbage internal leaves. Kaempferol 3-*O*-(caffeoyl)sophoroside-7-*O*-glucoside (**25**); kaempferol 3-*O*-(sinapoyl)sophoroside-7-*O*-glucoside (**26**); kaempferol 3-*O*-(feruloyl)sophoroside-7-*O*-glucoside (**27**); 4-*p*-coumaroylquinic acid (**28**); sinapic acid (**29**); 1,2′-disinapoyl-2-feruloylgentiobiose (**30**).

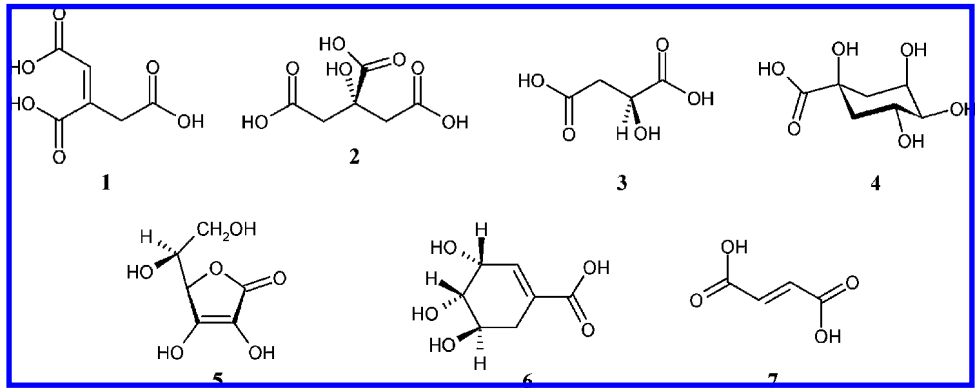


**Figure 5.** Chemical structures of the organic acids present in tronchuda cabbage materials. Aconitic acid (**1**); citric acid (**2**); malic acid (**3**); quinic acid (**4**); ascorbic acid (**5**); shikimic acid (**6**); fumaric acid (**7**).

**Table 3.** Quantification of Organic Acids (mg/kg, dry basis)[a]

| | compound | | | | | | |
|---|---|---|---|---|---|---|---|
| sample | aconitic | citric | malic + quinic | ascorbic | shikimic | fumaric | Σ |
| seeds | 17 ± 2.5 | 4685 ± 196.9 | 3049 ± 221.6 | 8546 ± 438.4 | 18 ± 0.4 | 39 ± 0.5 | 16507 |
| internal leaves | 191 ± 3.7 | 9975 ± 68.2 | 6626 ± 164.8 | 6020 ± 143.4 | 35 ± 1.0 | 408 ± 1.8 | 23255 |
| external leaves | 22 ± 8.2 | 8131 ± 421.2 | 8605 ± 974.6 | 8754 ± 517.4 | 20 ± 0.5 | 14 ± 0.1 | 25545 |

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

The strong protective effect of sinapic acid against NO and $ONOO^-$ has already been observed in other systems and an electron donation mechanism is involved (*5, 30*). These findings are in good agreement with those obtained with tronchuda cabbage seed extract once this is the extract with the highest sinapic acid derivatives content (**Table 2**).

Ascorbic acid exhibited the weakest NO scavenging ability, but a higher antioxidant potential was obtained against $ONOO^-$-induced tyrosine nitration (**Figure 6**, **Table 4**). In fact, the reactivity of ascorbic acid toward peroxynitrite through two intermediate forms is well documented in kinetic studies (*31–33*). Kaempferol 3-*O*-rutinoside showed the lowest capacity to inhibit 3-nitrotyrosine formation, but its scavenging activity against NO was higher than that from ascorbic acid (**Figure 6**, **Table 4**). These results suggest that flavonol glycosides contribute to the effects observed with tronchuda cabbage external leaves, in which this class of phenolic compounds is predominant, its content being higher than that found in the internal leaf extract (**Table 2**). As far as we know, this is the first time that a kaempferol derivative is studied for its RNS scavenging capacity.

Although the scavenging activity of phenolic compounds and organic acids was demonstrated, it is difficult to predict how a complex mixture as that of a plant extract functions against RNS, because interactions between these and other compounds present in the extract may potentiate or prevent the expected activity. Consequently, it seems important and most realistic to evaluate the activity of the extracts as a whole, as the antioxidant capacity exhibited is the resulting sum effect of the several compounds present, belonging to different chemical classes. It should be noticed that, as mentioned above, tronchuda cabbage is widely used in soups, which requires a preparation similar to that of the extracts used in the present work. So, those soups would be expected to have the same effect as that of the analyzed extracts.

In conclusion, the results obtained in the present study show that tronchuda cabbage can effectively scavenge reactive nitrogen species, and these antioxidant properties are concentration dependent. These findings, in addition to those found previously for reactive oxygen species, may explain, at least in part, the protective effect of tronchuda cabbage against free radical-mediated diseases, namely

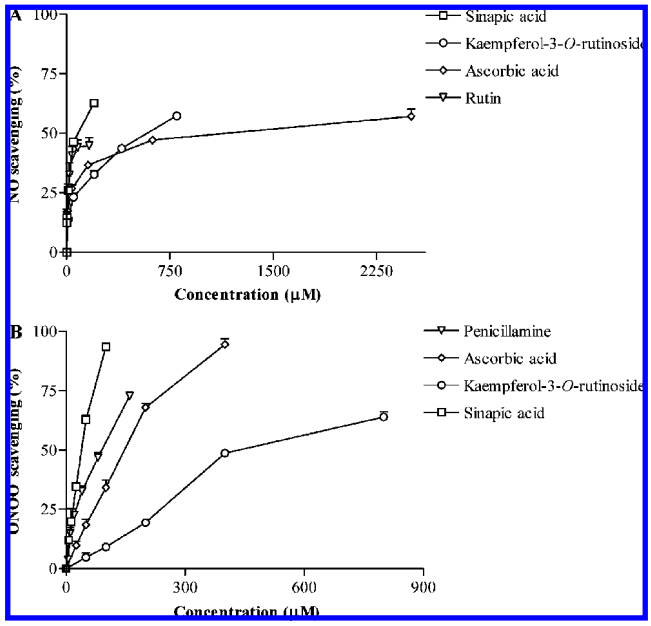


**Figure 6.** Effect of standards and reference compounds against (**A**) nitric oxide and (**B**) peroxynitrite. Values show mean + SE from three experiments, performed in triplicate.

**Table 4.** $IC_{50}$ Values ± SE ($\mu$M) of Standard and Reference Compounds against Reactive Nitrogen Species

| compound | NO | ONOO |
|---|---|---|
| ascorbic acid | 1301 ± 412 | 146 ± 6 |
| sinapic acid | 79 ± 11 | 39 ± 1 |
| kaempferol 3-*O*-rutinoside | 567 ± 25 | 430 ± 20 |
| rutin | 14.0 ± 4[a] | - |
| DL-penicillamine | - | 89 ± 7 |

[a] $IC_{25}$ value.

cancer. It should also be taken into account that especially tronchuda cabbage seed extracts could be used as natural antioxidants or to functionalize foodstuffs, since this material is not eaten and displayed the strongest antioxidant potential. If an extract does not show antioxidant activity *in vitro*, then it will certainly not exhibit this capacity *in vivo*. Further studies concerning their *in vivo* activity and bioavailability are necessary before incorporating seed extracts as a dietary complement.

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