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Pieris brassicae Inhibits Xanthine Oxidase

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The antioxidant potential of an aqueous extract obtained from *Pieris brassicae* larvae reared on *Brassica oleracea* L. var. *costata* DC was evaluated against 2,2-diphenyl-1-picrylhydrazyl radical and several reactive oxygen species. The results revealed an effective concentration-dependent protective activity against superoxide and hydroxyl radicals, being superior to that of the host plant. In addition, the larvae extract also exhibited a strong inhibitory effect on xanthine oxidase that was not observed for *B. oleracea* var. *costata*. A weak scavenging ability was noticed for hypochlorous acid. Several phenolic compounds with complex chemical structures that are hard to synthesize in the laboratory were found in *P. brassicae* extract. This is the first time that an insect has been tested for its xanthine oxidase inhibitory capacity, which proved to be very high. These findings are interesting considering that they can be used by food or pharmaceutical industries to prevent the oxidation of their products, to increase the dietary supply of antioxidants, or for prevention of free radical-mediated diseases, namely, gout.

KEYWORDS: *Pieris brassicae*; *Brassica oleracea* var. *costata*; phenolic compounds; antioxidant activity; xanthine oxidase

INTRODUCTION

Oxidants relevant to human diseases come from normal intracellular biological functions, inflammatory processes, and exposure to xenobiotics, either because they have pro-oxidant activity or because they induce the formation of other oxidative agents in cell (*1*). Because of the facility of those agents to accept electrons from target molecules, they are able to modify their structure or function. Thus, oxidants can interact with the membranes, genetic material, and enzymatic processes. Additionally, they can change extracellular media, modifying tissue architectures, defense molecules, and cellular mediators. In fact, oxidants are able to change the structure and/or function of biological important molecules, like nucleic acids, lipids, proteins, and carbohydrates (*1*−*3*).

Although essential to aerobic organisms, oxygen exhibits undesirable effects due to the formation of reactive oxygen species (ROS) in every cell with aerobic metabolic activity. To maintain the homeostasis, oxidants are inactivated by an array of intra- and extracellular antioxidants. Disequilibrium in the oxidant/antioxidant status causes oxidative stress, resulting in many pathophysiologic conditions (*2*, *3*).

The importance of natural antioxidants is well-established, being of great interest for health, nutritional, and food purposes. To deal with ROS, several antioxidant defenses have arisen from insects. Their role is to keep low steady-state levels of ROS and other radicals in the cell. The enzymes superoxide dismutase, catalase, glutathione reductase, selenium-dependent glutathione peroxidase, selenium-independent glutathione peroxidase, and the glutathione-*S*-transferases constitute an essential defense line against radicals (*4*−*6*).

The insects also possess low molecular weight antioxidants. The glutathione, besides its role as a substrate for glutathione reductase, glutathione peroxidase, and the glutathione-*S*-transferases, is a scavenger of hydroxyl and singlet oxygen, can reactivate some enzymes inhibited under oxidizing conditions, and is implicated in vitamin E regeneration (*5*). The role of glutathione as an antioxidant defense mechanism was assessed in several insect species, namely, in *Epiblema scudderiana* (*5*), *Mayetiola destructor* (*6*), *Melanoplus sanguinipes*, and *Aulocara ellioti* (*7*). Within phytochemicals, phenolic compounds, namely, flavonoids, are recognized for their antioxidative capacity (*8*). The presence of flavonoids in insects is positively associated with their existence in the feeding vegetal material once insects

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are unable to synthesize flavonoids or their precursors de novo (*9, 10*).

Larvae of *Pieris brassicae* L. (Lepidoptera: Pieridae) insects are specialists on crucifers, feeding on a variety of Brassicaceae species. The phenolic profiles of *P. brassicae* larvae reared on the leaves of *Brassica oleracea* L. var. *costata* DC and of *Brassica rapa* var. *rapa* L. have been determined before by high-performance liquid chromatography−diode array detection−mass spectrometry/mass spectrometry−electrospray ionization (HPLC-DAD-MS/MS-ESI) (*11, 12*). Several flavonoids, with complex structures that are hard to synthesize, were found in the larvae extracts. In addition to the determinant role displayed by the feeding material composition, those studies made it possible to determine that the larvae has the ability to sequester and metabolize the phenolic compounds present in the two host plants.

Previous works concerning *B. oleracea* var. *costata* revealed the antioxidant capacity of this species, for which its phenolic compounds contributed (*13*−*15*). Despite the occurrence of a high content of flavonoids not detected in *B. oleracea* var. *costata*, the antioxidant potential of *P. brassicae* reared on this matrix has not so far been assessed. Only the antiradical capacity of *P. brassicae* fed with *B. rapa* var. *rapa* (with 12 h food privation) against 2,2-diphenyl-1-picrylhydrazyl (DPPH), superoxide radical, and nitric oxide was evaluated in chemical systems (*16*).

The aim of the work herein was to achieve further knowledge on the antioxidant capacity of *P. brassicae*, a frequent pest of some *Brassica* species. For this purpose, aqueous extracts of the larvae with 1 h food privation and of *B. oleracea* var. *costata* host plant were studied for their capacity to act as scavengers of several ROS (superoxide radical, hydroxyl radical, and hypochlorous acid), chemically and enzymatically generated.

MATERIALS AND METHODS

Reagents. Kaempferol 3-*O*-rutinoside was from Extrasynthése (Genay, France). Methanol and formic were purchased from Merck (Darmstadt, Germany). The water was treated in a Milli-Q water purification system (Millipore, Bedford, MA). DPPH, xanthine (X), xanthine oxidase (XO) grade I from buttermilk (EC 1.1.3.22), β-nicotinamide adenine dinucleotide reduced form (NADH), phenazine methosulfate (PMS), nitroblue tetrazolium chloride (NBT), anhydrous ferric chloride ($FeCl_3$), 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), β-nicotinamide adenine dinucleotide phosphate reduced form (NADPH), glutathione (GSH), glutathione disulfide (GSSG), glutathione reductase (EC 1.6.4.2), and L-cysteine were obtained from Sigma Chemical Co. (St. Louis, MO).

Larvae and Host Plant Material. Wild *P. brassicae* larvae at the fourth instar and respective *B. oleracea* var. *costata* host plant external leaves (with 45 days old) were collected in Bragança (northeastern Portugal). Larvae and plant material were immediately transported to the laboratory. *B. oleracea* var. *costata* leaves were frozen at −20 °C. The larvae were deprived from food for 1 h and then frozen. The frozen larvae and plant material were then freeze-dried.

Extracts Preparation. For the antioxidant capacity screening, aqueous extracts were prepared as follows: 0.4 g of dried larvae and 2 g of dried *B. oleracea* var. *costata* leaves were boiled for 30 min in 400 mL of water and filtered over a Büchner funnel. The resulting extracts were then frozen and lyophilized. The lyophilized extracts were kept in an exsicator, in the dark.

HPLC-DAD Analysis. The determination of the phenolic compounds in the aqueous lyophilized extracts was performed as previously reported (*11*). Briefly, 20 μL of each lyophilized extract redissolved in water was analyzed in a HPLC unit (Gilson), using a Spherisorb ODS2 (250 mm × 4.6 mm, 5 μm particle size) and a flow rate of 1 mL/min. The solvent system was a mixture of formic acid 5% (A) and methanol (B), 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; and 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. Phenolic compounds were identified by comparing their UV spectra and retention times with data previously reported (*11*). 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. The compounds were quantified as kaempferol 3-*O*-rutinoside.

DPPH Scavenging Activity. The disappearance of DPPH$^{\bullet}$ was monitored spectrophotometrically at 515 nm on a Multiskan Ascent plate reader (Thermo Electron Corp.), following a described procedure (*15*). For each extract, a dilution series was prepared in a 96 well plate. The reaction mixtures in the sample wells consisted of 25 μL of lyophilized extract and 200 μL of 150 μM DPPH dissolved in methanol. The plate was incubated for 30 min at room temperature after addition of DPPH. Three experiments were performed in triplicate.

Superoxide Radical Scavenging Activity. The effect of the lyophilized extracts on the superoxide radical-induced reduction of NBT was monitored spectrophotometrically in a Multiskan Ascent plate reader (Thermo Electron Corp.), in kinetic function, at 562 nm.

Nonenzymatic Assay. Superoxide radicals were generated by the NADH/PMS system, as previously reported (*15*). All components were dissolved in phosphate buffer (19 mM, pH 7.4). For each extract, five different concentrations were tested. Three experiments were performed in triplicate.

Enzymatic Assay. Superoxide radicals were generated by the X/XO system following a described procedure (*15*). Briefly, X was dissolved in NaOH (1 μM) and subsequently in phosphate buffer (50 mM) with EDTA (0.1 mM, pH 7.8), XO in EDTA (0.1 mM), and the other components in phosphate buffer (50 mM) with EDTA (0.1 mM, pH 7.8). For each extract, a dilution series was assayed. Three experiments were performed in triplicate.

Effect on XO. The effect of the lyophilized extracts on XO activity was evaluated by measuring the formation of uric acid from X in a double beam spectrophotometer (Helios α, Unicam), at room temperature, as before (*15*). The reaction mixtures contained the same components of the enzymatic assay at the same proportion, except for NBT. The absorbance was measured at 295 nm for 2 min. Three experiments were performed in triplicate.

Effect of GSH. To check for the possibility of GSH from larvae extract or fragments resulting from its degradation to interfere with XO, an aqueous solution of GSH at the concentration corresponding to the IC_{25} value found in the evaluation of the effect of lyophilized larvae extract on the enzyme was prepared and submitted to boiling for 30 min, as it happened for the larvae.

The effect of boiled GSH solution against superoxide radical generated by the enxymatic system and on XO activity was evaluated as indicated above for larvae lyophilized extract. The GSH content in boiled GSH solution was determined by the DTNB-GSSG reductase recycling assay, as described before (*17, 18*), at 415 nm.

Hydroxyl Radical Scavenging Activity. The deoxyribose method for determining the scavenging effect of the aqueous extracts on hydroxyl radicals was performed according to a described procedure (*15*) in a double beam spectrophotometer (Helios α, Unicam). Reaction mixtures contained ascorbic acid, $FeCl_3$, EDTA, H_2O_2, deoxyribose, and lyophilized extracts. All components were dissolved in KH_2PO_4−KOH buffer (10 mM, pH 7.4). This assay was also performed without either ascorbic acid or EDTA, to evaluate the extracts prooxidant and metal chelation potential, respectively. For each extract, five different concentrations were tested. Three experiments were performed in triplicate.

Hypochlorous Acid Scavenging Activity. The inhibition of hypochlorous acid-induced TNB oxidation to DTNB was evaluated as previously reported (*15*), in a double beam spectrophotometer (Helios

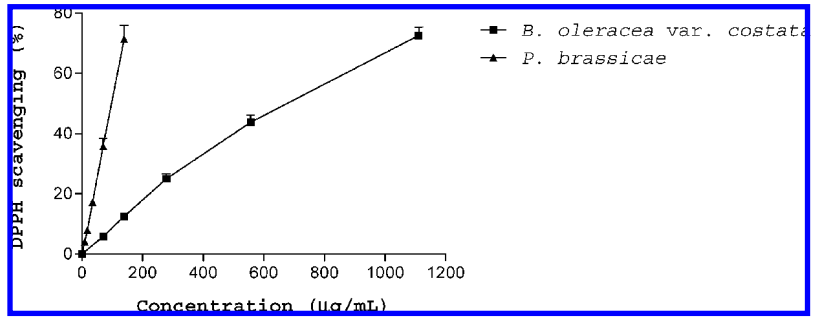

Figure 1. Effect of *P. brassicae* and *B. oleracea* var. *costata* on DPPH radical reduction. Values show means ± SEs from three experiments performed in triplicate.

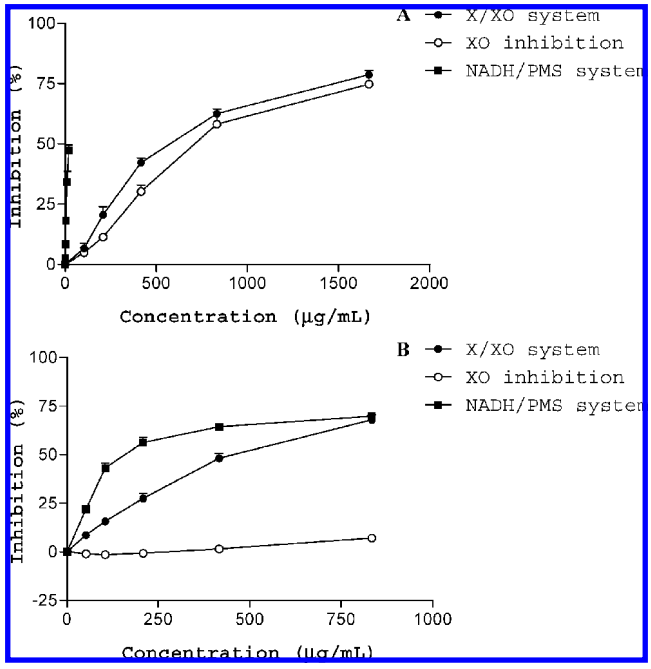

Figure 2. Effect of (**A**) *P. brassicae* and (**B**) *B. oleracea* var. *costata* against superoxide radical and on XO. Values show means ± SEs from three experiments performed in triplicate.

α, Unicam). Hypochlorous acid and TNB were prepared immediately before use. For each extract, a dilution series was prepared. Three experiments were performed in triplicate.

RESULTS AND DISCUSSION

DPPH is a stable free radical, able to accept one electron or hydrogen atom, turning it into a nonradical species, hardly oxidizable. The DPPH assay provides basic information on the antiradical activity of extracts (*19*). A microassay was performed in which the scavenging of DPPH was followed by monitoring the decrease in absorbance at 515 nm, which occurs due to the reduction by the antioxidant. The *P. brassicae* aqueous extract exhibited a strong antioxidant activity, in a concentration-dependent way (IC_{50} at 97 μg/mL), being much more effective than the host plant (IC_{50} at 678 μg/mL) (**Figure 1**).

Superoxide radical is the first product of oxygen univalent reduction. Activated phagocyte cells generate this ROS, which is important to allow killing of some of the bacterial strains that they engulf. Some superoxide radical is accidentally formed in vivo: Some of the electrons passing through the respiratory chain leak from the electron carriers and pass directly onto oxygen, reducing it (*20*). XO is also involved in the in vivo production of superoxide radical, by catalyzing the conversion of hypoxanthine in X and of X into uric acid (*21*). The biological

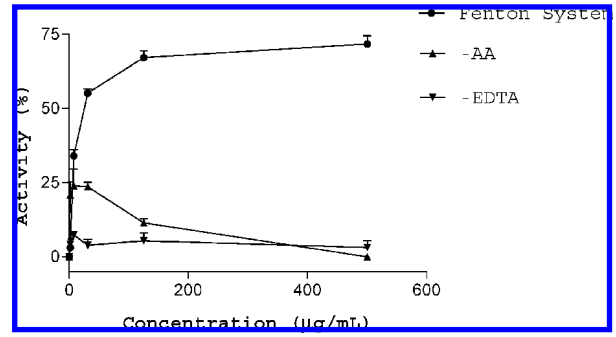

Figure 3. Effect of *P. brassicae* against hydroxyl radical, pro-oxidant activity (−AA), and metal chelating capacity (−EDTA). Values show means ± SEs from three experiments performed in triplicate.

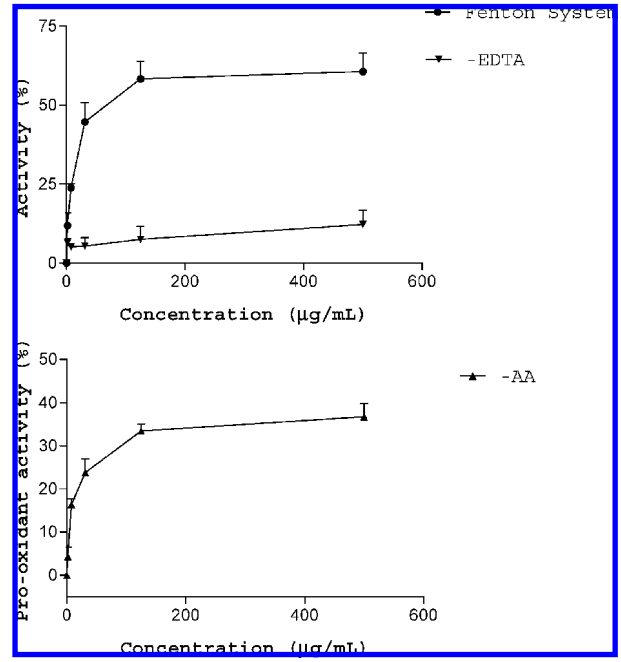

Figure 4. Effect of *B. oleracea* var. *costata* against hydroxyl radical, pro-oxidant activity (−AA), and metal chelating capacity (−EDTA). Values show means ± SEs from three experiments performed in triplicate.

significance of superoxide radical is mainly related with its conversion into much more reactive species, like hydroxyl radical and peroxynitrite (*22*).

Superoxide radical was generated by the enzymatic X/XO and by the chemical phenazine methosulphate/NADH (PMS/NADH) systems. The prevention of NBT reduction to the chromogen formazan, induced by superoxide radical, was used as the measured end point. Taking into account that an inhibitory effect on the enzyme itself would also lead to a decrease in

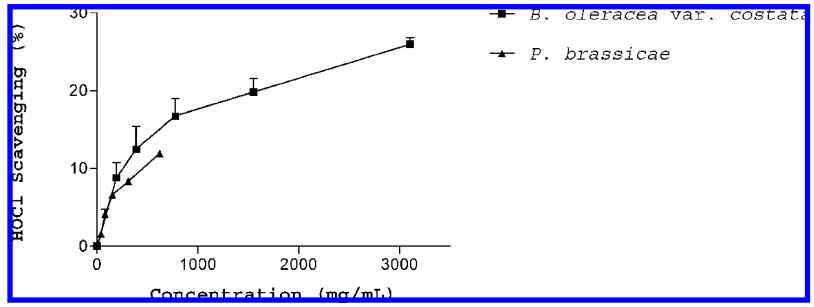

Figure 5. Effect of *P. brassicae* and *B. oleracea* var. *costata* against hypochlorous acid. Values show means ± SEs from three experiments performed in triplicate.

NBT reduction (*23*), the effect of the extracts on the metabolic conversion of X to uric acid was also studied.

An effective concentration-dependent antioxidant capacity was found for both *P. brassicae* and *B. oleracea* var. *costata* leaf lyophilized extracts, which exhibited superoxide radical scavenging activity using the X/XO system, with an IC_{25} at 251 and 186 μg/mL, respectively (**Figure 2**). However, the larvae displayed a potent inhibitory effect on XO, in a concentration-dependent manner (IC_{25} at 358 μg/mL), while *B. oleracea* var. *costata* had no effect on this enzyme (**Figure 2**). It is the first time that an insect is reported to possess XO inhibitory capacity. These findings can be of great relevance in the case of gout disease, in which high levels of uric acid are involved (*21*). As it was not possible to show a clear-cut scavenging effect on superoxide radical for the larvae, the effect of both extracts on superoxide radical generated in PMS/NADH system was determined. A concentration-dependent scavenging effect was observed, which was noticeably superior for the larvae extract: IC_{25} at 7.4 and 59 μg/mL, for larvae and *B. oleracea* var. *costata*, respectively (**Figure 2**). According to these results, it may be anticipated that *P. brassicae* exerts an effective protective role against superoxide radical by acting as both scavenger and XO inhibitor.

Within free radicals and other ROS, hydroxyl radical is the most unstable and reactive: It shows a great oxidative power and rapidly combines with almost all molecules in its immediate vicinity. The formation of hydroxyl radical requires traces of transition metal ions, among which iron and copper seem likely to be the most important in vivo (*20*, *22*). Exposure to ionizing radiation, peroxynitrite protonation, and decomposition and the reaction of hypochlorous acids with superoxide radical are also sources of hydroxyl radical (*22*).

Hydroxyl radical was generated by the Fe^{3+}-EDTA/ascorbate Fenton system and assayed by evaluating deoxyribose degradation into thiobarbituric acid-reactive substances. The lyophilized extract of *P. brassicae* revealed scavenging activity for hydroxyl radical, in a concentration-dependent way (IC_{25} at 6.1 μg/mL) (**Figure 3**), being superior to that exhibited by *B. oleracea* var. *costata* (IC_{25} at 9.2 μg/mL) (**Figure 4**). If we omit ascorbate in the reaction system and if pro-oxidant compounds exist, they will promote the formation of hydroxyl radical by redox cycling the metal ion required for its generation, causing deoxyribose degradation (*24*). In the assay performed under these conditions, some pro-oxidant effect was observed for concentrations of larvae extract higher than 7.8 μg/mL (**Figure 3**), while the cabbage was revealed to have pro-oxidant activity at all tested concentrations (**Figure 4**). Inhibition of iron-dependent deoxyribose degradation in the absence of EDTA depends not only on the ability of a scavenger to react with hydroxyl radicals but also on its capacity to form complexes with iron ions (*25*). Under these circumstances, either *P. brassicae* or *B. oleracea* var. *costata* extracts showed no metal chelating activity (**Figures 3** and **4**).

Hypochlorous acid is produced by the oxidation of Cl^- ions catalyzed by neutrophil-derived myeloperoxidase, in the presence of hydrogen peroxide. It damages and induces target cell lysis, caused by sulfydril oxidation, in plasma membrane proteins, inactivates α_1-antiprotease and antioxidants enzymes like catalase, and activates collagenase and gelatinase (*20*, *26*−*28*).

The hypochlorous acid scavenging activity was tested by measuring the inhibition of hypochlorous acid-induced 5-thio-2-nitrobenzoic acid (TNB) oxidation to DTNB. *P. brassicae* aqueous extract showed some concentration-dependent protective activity (IC_{10} at 453 μg/mL), although it was less effective than *B. oleracea* var. *costata* (IC_{10} at 257 μg/mL) (**Figure 5**).

In a general way, and according to the results obtained in all assays, *P. brassicae* was revealed to be a more effective antioxidant than its host plant. The phenolics profile of the two analyzed extracts was identical to that described before (*11*), being composed by flavonol derivatives glycosilated at 3 or simultaneously at 3 and 7 positions, with some of them acylated (**Figures 6**−**8**). Kaempferol 3-*O*-sophoroside was the main compound in *P. brassicae* aqueous lyophilized extract, while kaempferol 3-*O*-(feruloyl/caffeoyl)sophoroside-7-*O*-glucoside was the main compound in that of the host *B. oleracea* var. *costata* (**Tables 1** and **2**). Although the last presented higher phenolics content, the distinct qualitative composition seems to be determinant for the antioxidant potential exhibited by the two analyzed extracts. In fact, either *P. brassicae* or *B. oleracea* var. *costata* extracts contained several flavonol derivatives, but only kaempferol 3-*O*-sophoroside-7-*O*-glucoside, kaempferol 3-*O*-sophoroside-7-*O*-sophoroside, and kaempferol 3-*O*-sophoroside are common to both.

Flavonoids have been identified as fulfilling most of the criteria involved in the mechanisms of antioxidant action, which include suppressing ROS formation, either by inhibition of enzymes or chelation of trace elements involved in free radical production, scavenging ROS, and upregulating or protecting antioxidant defenses (*29*). Flavonol glycosides have already been demonstrated to possess antioxidant capacity (*30*, *31*). As observed in the previous work (*11*), despite the identical glycosylation pattern, the larva exhibited a higher relative amount of 3-*O*-glycosides (994 mg/kg as compared to 497 mg/kg found in tronchuda cabbage extract), probably by hydrolysis by the larva. This seems to suggest that flavonol 3-*O*-glucosides contribute to a greater extent to the strongest protective effects displayed by *P. brassicae*. In fact, it is known that the addition of a second glycoside residue decreases the activity due to steric hindrance by addition of sugar moieties (*19*). Additionally, the presence of higher amounts of quercetin derivatives in *P. brassicae* extract (around 360 mg/kg) can also, at least partially, explain its highest antioxidative properties once it is well-established that quercetin is a more potent antioxidant than kaempferol, due to the presence of a cathecol group in the B ring of the former (*29*). Indeed, as before (*11*), only trace

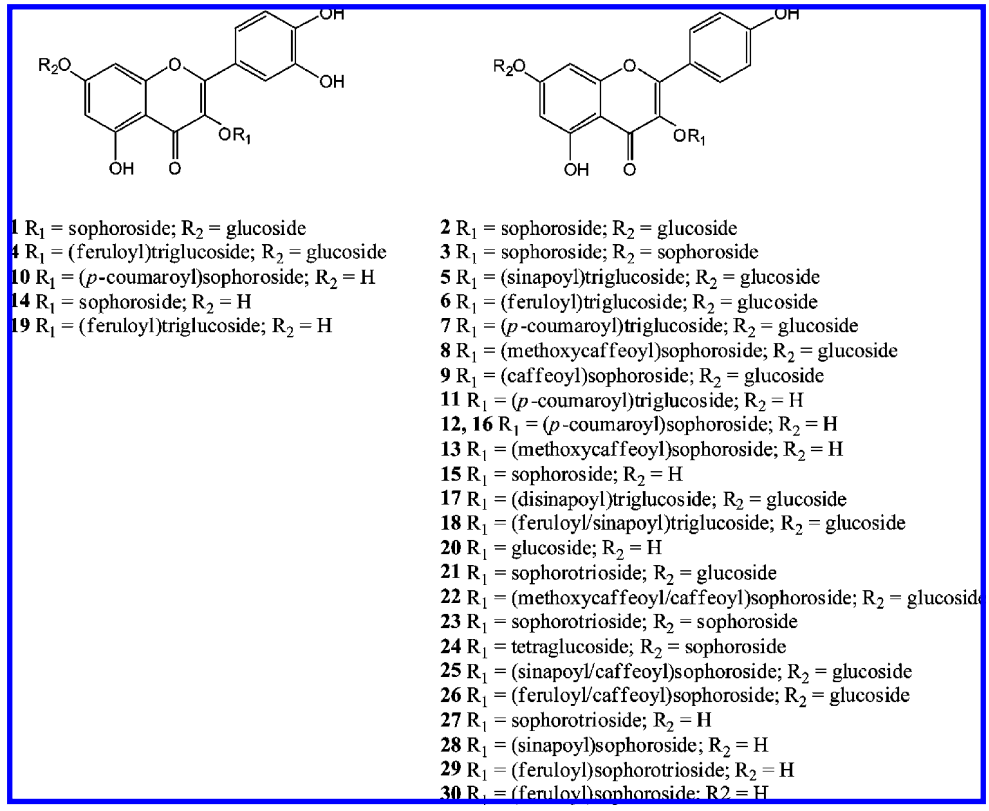

Figure 6. Structures of the phenolic compounds identified in *P. brassicae* and *B. oleracea* var. *costata*. The identities of compounds are as in **Tables 1** and **2**.

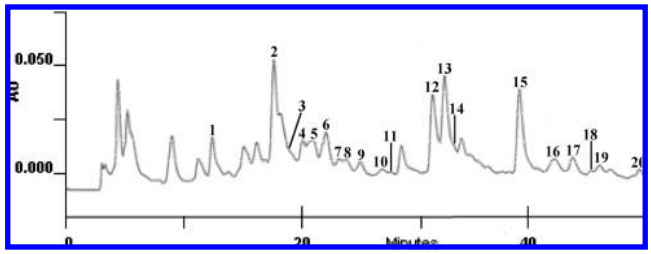

Figure 7. HPLC-DAD phenolic profile of larvae extract. Detection at 330 nm. The identities of compounds are as in **Table 1**.

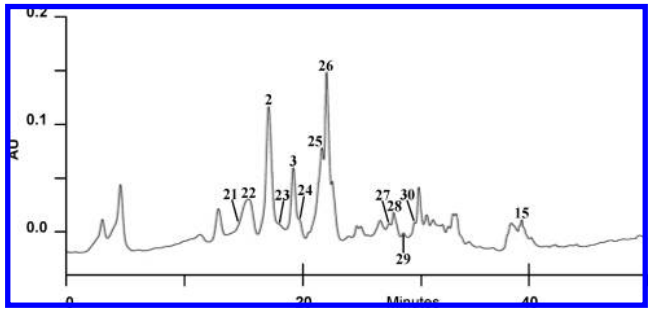

Figure 8. HPLC-DAD phenolic profile of tronchuda cabbage extract. Detection at 330 nm. The identities of compounds are as in **Table 2**.

amounts of quercetin glycosides were found in the host plant, suggesting that *P. brassicae* selectively sequesters these flavonoids or that the kaempferol derivatives are metabolized into quercetin glycosides by the larvae.

The presence of antioxidant enzymes in *Pieris* larvae extracts that may contribute to the antioxidative properties can be discarded, once they are denaturated by boiling at 100 °C for 30 min, turning them inactive. Concerning GSH, we verified that a boiled solution (358 μg/mL) was not able

Table 1. Phenolic Composition of *P. brassicae* Aqueous Lyophilized Extract

	compound	mg/kg
1	quercetin 3-*O*-sophoroside-7-*O*-glucoside	169
2	kaempferol 3-*O*-sophoroside-7-*O*-glucoside	195
3	kaempferol 3-*O*-sophoroside-7-*O*-sophoroside	128
4	quercetin 3-*O*-(feruloyl)triglucoside-7-*O*-glucoside	87
5	kaempferol 3-*O*-(sinapoyl)triglucoside-7-*O*-glucoside	97
6	kaempferol 3-*O*-(feruloyl)triglucoside-7-*O*-glucoside	108
7	kaempferol 3-*O*-(*p*-coumaroyl)triglucoside-7-*O*-glucoside	51
8	kaempferol 3-*O*-(methoxycaffeoyl)sophoroside-7-*O*-glucoside	10
9	kaempferol 3-*O*-(caffeoyl)sophoroside-7-*O*-glucoside	36
10	quercetin 3-*O*-(*p*-coumaroyl)sophoroside	67
11	kaempferol 3-*O*-(*p*-coumaroyl)triglucoside	62
12	kaempferol 3-*O*-(*p*-coumaroyl)sophoroside	261
13	kaempferol 3-*O*-(methoxycaffeoyl)sophoroside +	179
14	quercetin 3-*O*-sophoroside	
15	kaempferol 3-*O*-sophoroside	307
16	kaempferol 3-*O*-(*p*-coumaroyl)sophoroside (isomer)	46
17	kaempferol 3-*O*-(disinapoyl)triglucoside-7-*O*-glucoside	41
18	kaempferol 3-*O*-(feruloyl/sinapoyl)triglucoside-7-*O*-glucoside	26
19	quercetin 3-*O*-(feruloyl)triglucoside	36
20	kaempferol 3-*O*-glucoside	36

to inhibit uric acid formation by XO (0.45 ± 1.06% XO inhibition) but scavenged superoxide radical (51.70 ± 5.67% superoxide radical scavenging ability). Thus, it seems that the GSH protective effect against superoxide radical is related to its scavenging ability rather than its XO inhibitory capacity. In this solution, only ca. one-third of the initial total GSH was found, from which ca. 6% was in the oxidized form. This can be attributed to its alteration during boiling and also to its oxidation, since during this process it was not protected from light or oxygen exposure.

Although some GSH remained in the boiled solution, these data allow us to discard the possibility of the XO inhibition

Table 2. Phenolic Composition of *B. oleracea* var. *costata* Aqueous Lyophilized Extract

	compound	mg/kg
21	kaempferol 3-*O*-sophorotrioside-7-*O*-glucoside +	316
22	kaempferol 3-*O*-(methoxycaffeoyl/caffeoyl)sophoroside-7-*O*-glucoside	
2	kaempferol 3-*O*-sophoroside-7-*O*-glucoside	955
23	kaempferol 3-*O*-sophorotrioside-7-*O*-sophoroside	58
3	kaempferol 3-*O*-sophoroside-7-*O*-sophoroside +	477
24	kaempferol 3-*O*-tetraglucoside-7-*O*-sophoroside	
25	kaempferol 3-*O*-(sinapoyl/caffeoyl)–sophoroside-7-*O*-glucoside	711
26	kaempferol 3-*O*-(feruloyl/caffeoyl)–sophoroside-7-*O*-glucoside	1158
27	kaempferol 3-*O*-sophorotrioside +	214
28	kaempferol 3-*O*-(sinapoyl)sophoroside	
29	kaempferol 3-*O*-(feruloyl)sophorotrioside	18
30	kaempferol 3-*O*-(feruloyl)sophoroside	48
15	kaempferol 3-*O*-sophoroside	217

observed with the larvae extract to be related with GSH: Besides thermal degradation of the tripeptide, the oxidized GSH that can result from light or oxygen exposure cannot be recycled to the reduced form by glutathione reductase, due to the temperature used, which destroys the enzyme. Regeneration of glutathione reduced form can be achieved in vivo by glutathione reductase and NADPH (*32*), which is not present in our in vitro assay.

In conclusion, *P. brassicae* larvae aqueous extract provides powerful natural antioxidants, with complex chemical structures, impossible to be synthesized in the laboratory. This study provided evidence for the first time that the insect is able to inhibit XO and to prevent hydroxyl radical and hypochlorous acid-induced damage. Because of the potent activity exhibited, the food industry may employ it to prevent the oxidation of its products, maintaining their quality and safety and extending their shelf life, or to improve their nutritional value, by incorporating the extract in foodstuffs, thus increasing the dietary supply of antioxidants. It may also be used by the pharmaceutical industry in antioxidative formulations for prevention of free radical-mediated diseases, namely, gout, or even as a preservative of other oxidizable formulations. The same can be applied to the cosmetic industry, for which it can be further used in antiaging formulations. In addition, it may constitute an economical advantage for *B. oleracea* var. *costata* producers who have great losses caused by *P. brassicae* infestations.

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