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Targeted Metabolite Analysis and Biological Activity of *Pieris brassicae* Fed with *Brassica rapa* var. *rapa*

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For the first time, an insect−plant system, *Pieris brassicae* fed with *Brassica rapa* var. *rapa*, was tested for its biological capacity, namely, antioxidant (DPPH$^{\bullet}$, $^{\bullet}$NO, and $O_2^{\bullet-}$ radicals) and antimicrobial (bacteria and fungi) activities. Samples from the insect's life cycle (larvae, excrements, exuviae, and butterfly) were always found to be more efficient than the host plant. Also, *P. brassicae* materials, as well as its host plant, were screened for phenolics and organic acids. The host plant revealed higher amounts of both compounds. Two phenolic acids, ferulic and sinapic, as well as kaempferol 3-*O*-sophoroside, were common to insect (larvae and excrements) and plant materials, with excrements being considerably richer. Detection of sulfated compounds in excrements, absent in host plant, revealed that metabolic processes in this species involved sulfation. Additionally, deacylation and deglycosilation were observed. All matrices presented the same organic acids qualitative profile, with the exception of excrements.

KEYWORDS: *Brassica rapa* var. *rapa*; *Pieris brassicae*; plant−insect interactions; phenolic compounds; organic acids; antioxidant activity

INTRODUCTION

Pieris brassicae (Lepidoptera: Pieridae) is an oligophagous insect that has been thoroughly studied in association with its host plants as a model for oviposition and feeding modulation as a consequence of the plant's chemical composition (*1*). Its life cycle takes about 45 days, from egg to adult insect. The larvae feed exclusively on crucifers, whereas the adults feed on the nectar of several plants (*2*).

The role of the plant's chemical composition in shaping insect−plant interactions has been described for some species, such as *Shistocerca americana* (*3*), *Phyllotreta armoraciae* (*4*), *Spodoptera eridania* (*5*), and *Spodoptera littoralis* (*6*), among others. Among flavonoids, quercetin derivatives have been described as playing an important role in insect−plant interactions. For example, quercetin 3-*O*-glucoside was one of the first flavonoids whose importance in stimulating feeding behaviors in insects has been reported (*7*).

Some insects have the ability to differentiate even slight changes in flavonoid structures. As so, *Manduca sexta* can distinguish between quercetin 3-*O*-rutinoside, a phagostimulant, and quercetin 3-*O*-rhamnoside, an antifeedant, which lacks rutins's glucose in its structure. Also, *Bombyx mori* can differentiate among glycosides with different sugar moieties: While quercetin 3-*O*-glucoside was found to be a feeding stimulant, quercetin 3-*O*-rutinoside was inactive, and quercetin 3-*O*-rhamnoside was a deterrent (*7*).

In *P. brassicae*, host discrimination involves the sensilla on the tarsi of the midlegs, thus demonstrating the neural response involved in this process (*8*). However, the structure−activity relationships of phenolic compounds from which it feeds have yet to be determined.

Although insect−plant interactions, mainly sequestration, have been thoroughly studied in what concerns the glucosinolates (*1*), when it comes to flavonoids, the amount of information is rather scarce. The works of Burghardt et al. (*9*, *10*) and Schittko et al. (*11*) describe flavonoid sequestration from *Vicia villosa* by the butterfly *Polyommatus icarus*, and more recently, Ferreres et al. (*12*, *13*) have related the same phenomenon for *P. brassicae* reared with *Brassica oleracea* var. *costata* and *Brassica rapa* var. *rapa*, respectively, using high-performance

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liquid chromatography−diode array detection−mass spectrometry/mass spectrometry−electrospray ionization (HPLC-DAD-MS/MS-ESI). In fact, these last two works constitute, to our knowledge, the only available data concerning phenolics in *P. brassicae* fed with *Brassica* species. Several flavonoids, with complex structures that are hard to synthesize, have been found in the larvae extracts. In addition to the elucidation of the determinant role displayed by the feeding material in the insect's chemical composition, those studies have shown that the larva has the ability to sequester and metabolize the phenolic compounds present in the two host plants. As a matter of fact, Ferreres et al. (*13*) have described a new metabolization process in this species, namely, sulfation.

As *Brassica* constitutes one of the most widespread crops in the world, with *P. brassicae* being one of the most frequent pests, the study of this system is of major importance from both an agricultural and an economic point of view. The study of this complex, and still unknown, set of interactions between these organisms is particularly relevant in the search for new biologically active natural products for the pharmaceutical, agrochemical, and food industries. Additionally, the study of the biotransformation of compounds present in plants by a set of organisms, from insects to microbials, plays a very important role, as these interactions give rise to a variety of compounds rather unusual in nature and, in the particular case of flavonoids, very hard to synthesize in laboratory.

With this study, our intention is to see if *P. brassicae* material (larvae, butterfly, and exuviae) and its excrements present antimicrobial activity and antioxidant potential. If this is accomplished, it may constitute an economic advantage for *B. rapa* var. *rapa* producers who have great losses caused by *P. brassicae* infestations. So, chemical profiles and antioxidant and antimicrobial activities have been established for *P. brassicae* material and host plants (*B. rapa* var. *rapa*). The phenolics and organic acid compositions have also been determined. As far as we know, this is the first time that an insect−plant system has been tested for its biological activity.

MATERIALS AND METHODS

Standards and Reagents. Malic, shikimic, and fumaric acids were purchased from Sigma (St. Louis, MO); citric and sinapic acids, kaempferol 3-*O*-rutinoside, isorhamnetin 3-*O*-glucoside, and quercetin 3-*O*-glucoside were from Extrasynthése (Genay, France). Methanol, *N*-(1-naphthyl)ethylenediamine dihydrochloride, and phosphoric and formic acids were obtained from Merck (Darmstadt, Germany), and sulfuric acid was from Pronalab (Lisboa, Portugal). The water was treated in a Milli-Q water purification system (Millipore, Bedford, MA). 2,2-Diphenyl-1-picrylhydrazyl radical (DPPH$^{\bullet}$), β-nicotinamide adenine dinucleotide (NADH), phenazine methosulfate (PMS), nitroblue tetrazolium chloride (NBT), and sulfanilamide were obtained from Sigma Chemical Co. Sodium nitroprussiate dihydrate was from Riedel-de Haën (St. Louis, MO).

Samples. Wild *P. brassicae* larvae were captured in Bragança (northeastern Portugal) and taken to the laboratory to complete their life cycle and for oviposition in turnip (*B. rapa* var. *rapa* L.) leaves. New larvae developed, having only this species as a host plant. Fresh food was supplied every day, and larvae were free-fed. New larvae at the fourth instar and correspondent excrements were picked for analysis. Some of them were collected and kept without food for 12 h before freezing. Other larvae were left to develop until the butterfly stage, and 24 h maximum after eclosion (without food), they were also collected, together with the exuviae. *P. brassicae* (larvae, excrements, exuviae, and butterflies) and plant materials were freeze-dried and kept in a desiccator until analysis. Voucher specimens were deposited at Serviço de Farmacognosia from Faculdade de Farmácia, Universidade do Porto.

Sample Preparation. An aqueous extract was used for the phytochemical characterization and in the antioxidant activity assays: About 0.4 g of each *P. brassicae* dried material (larvae, excrements, exuviae, and butterfly) and 2 g of dried *B. rapa* var. *rapa* 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 a desiccator in the dark.

For the characterization and quantification of the phenolic compounds by HPLC-DAD, the lyophilized extract was redissolved in water (100 mg/mL) and filtered (0.45 μm). For organic acids determination, it was redissolved in 0.01 N sulfuric acid (100 mg/mL) prior to analysis by HPLC-UV.

HPLC/UV-DAD/MS^n-ESI Phenolics Qualitative Analysis. Analyses were developed as described before (*13*), using water−acetic acid (1%) (A) and methanol (B), at a flow rate of 1 mg/mL. A linear gradient, starting with 15% B, was installed to reach 40% B at 30 min, 60% B at 35 min, 80% B at 37 min, and 80% B at 40 min. Spectral data from all peaks were accumulated in the range 240/400 nm, and chromatograms were recorded at 330 nm. The HPLC/UV-DAD/ESI-MS^n analyses were carried out in an Agilent HPLC 1100 series equipped with a diode array detector and mass detector in series (Agilent Technologies, Waldbronn, Germany). The HPLC consisted of a binary pump (model G1312A), an autosampler (model G1313A), a degasser (model G1322A), and a photodiode array detector (model G1315B). The system was controlled by ChemStation software (Agilent, v. 08.03). The mass detector was an ion trap spectrometer (model G2445A) equipped with an ESI interface and was controlled by LCMSD software (Agilent, v. 4.1). The ionization conditions were adjusted to 350 °C and 4 kV for capillary temperature and voltage, respectively. The nebulizer pressure and flow rate of nitrogen were 65.0 psi and 11 L/min, respectively. The full scan mass covered the range from *m*/*z* 100 up to *m*/*z* 2000. Collision-induced fragmentation experiments were performed in the ion trap using helium as the collision gas, with voltage ramping cycles from 0.3 up to 2 V. Mass spectrometry data were acquired in the negative ionization mode. MS^n was carried out in the automatic mode on the more abundant fragment ion in MS$^{(n-1)}$.

HPLC-DAD Phenolics Quantitative Analysis. The determination of the phenolic compounds in the aqueous lyophilized extracts was performed as previously reported (*12*). Briefly, 20 μL of each lyophilized extract redissolved in water was analyzed in an 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. The data were processed on a Unipoint system Software (Gilson Medical Electronics, Villiers le Bel, France). The 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. Quercetin, isorhamnetin, and kaempferol derivatives were quantified as quercetin 3-*O*-glucoside, isorhamnetin 3-*O*-glucoside, and kaempferol 3-*O*-glucoside, respectively.

HPLC-UV Organic Acids Analysis. The separation of organic acids was carried out as previously reported (*14*) in a system consisting of an analytical HPLC-UV unit (Gilson) with an ion exclusion column, Nucleogel Ion 300 OA (300 mm × 7.7 mm) in conjunction with a column heating device set at 30 °C. Elution was performed in isocratic mode with 0.01 N sulfuric acid, under a flow rate of 0.2 mL/min. The detection was achieved with an UV detector set at 214 nm.

Each organic acid was quantified by the absorbance recorded in the chromatograms relative to commercially available external authentic standards. The peaks in the chromatograms were integrated using a default baseline construction technique.

DPPH$^{\bullet}$ 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

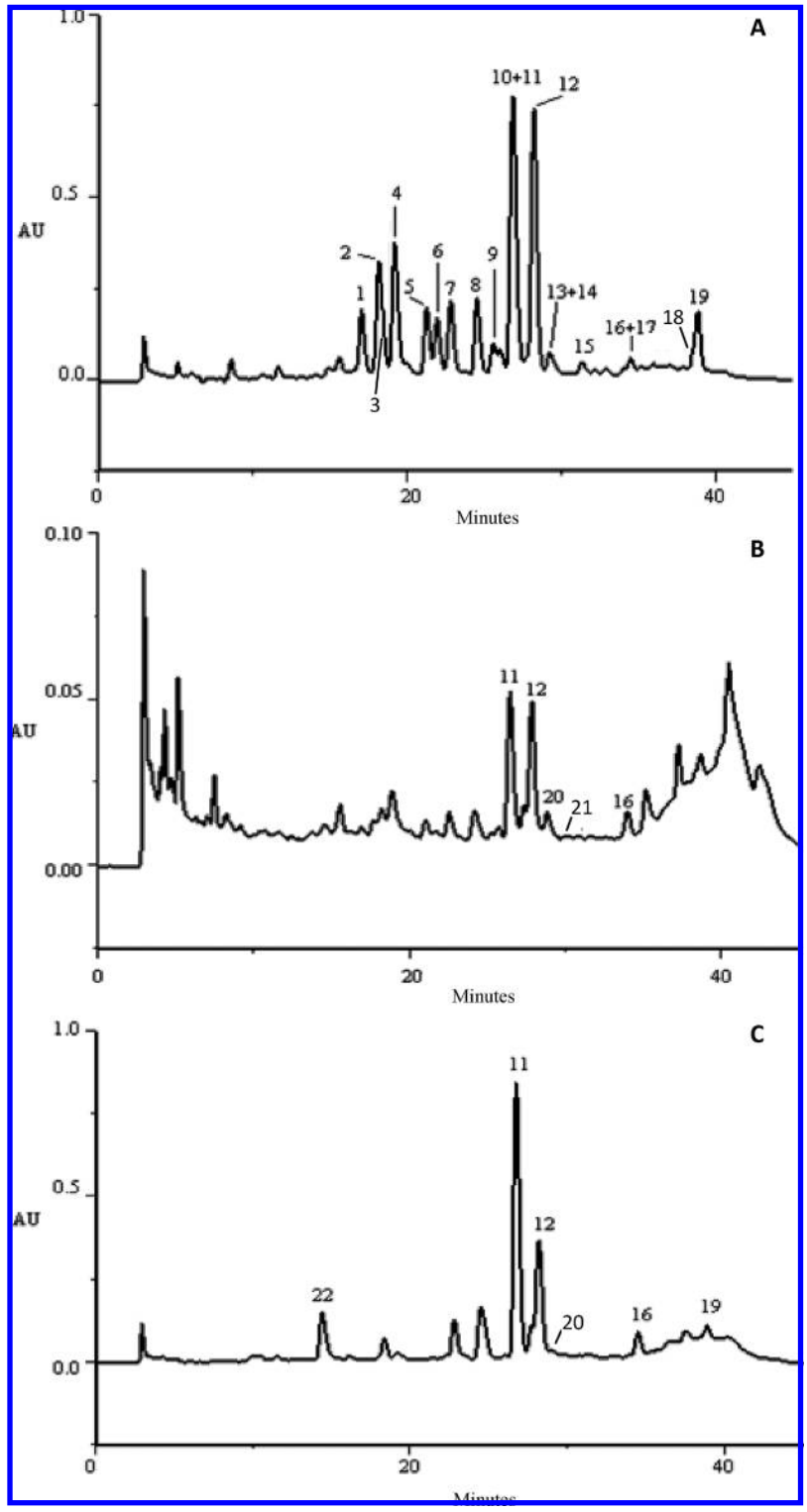

Figure 1. HPLC-DAD of phenolic compounds in *B. rapa* var. *rapa* leaves (**A**) and *P. brassicae* larvae (**B**) and excrements (**C**). Detection at 330 nm. Peaks: 1, kaempferol 3-*O*-(methoxycaffeoyl)sophoroside-7-*O*-glucoside; 2, quercetin 3-*O*-(sinapoyl)sophoroside-7-*O*-glucoside; 3, quercetin 3-*O*-(feruloyl)sophoroside-7-*O*-glucoside; 4, quercetin 3-*O*-(*p*-coumaroyl)sophoroside-7-*O*-glucoside; 5, kaempferol 3-*O*-(sinapoyl)sophoroside-7-*O*-glucoside; 6, kaempferol 3-*O*-(feruloyl)sophoroside-7-*O*-glucoside; 7, kaempferol 3-*O*-(*p*-coumaroyl)sophoroside-7-*O*-glucoside; 8, *p*-coumaric acid; 9, kaempferol 3,7-di-*O*-glucoside; 10, isorhamnetin 3,7-di-*O*-glucoside; 11, ferulic acid; 12, sinapic acid; 13, kaempferol 3-*O*-(feruloyl)sophoroside-7-*O*-glucoside (isomer); 14, kaempferol 3-*O*-(methoxycaffeoyl)sophoroside; 15, kaempferol 3-*O*-(caffeoyl)sophoroside; 16, kaempferol 3-*O*-sophoroside; 17, kaempferol 3-*O*-(feruloyl)sophoroside; 18, kaempferol 3-*O*-glucoside; 19, isorhamnetin 3-*O*-glucoside; 20, quercetin 3-*O*-sophoroside; 21, kaempferol 3-*O*-(*p*-coumaroyl)sophoroside (isomer); and 22, isorhamnetin 3,7-di-*O*-glucoside sulfate.

(*15*). 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 25 μL of lyophilized extract and 200 μL of 150 μM DPPH$^{\bullet}$ dissolved in methanol. The plate was incubated for 30 min at room temperature after the addition of DPPH$^{\bullet}$. Three experiments were performed in triplicate.

Table 1. Phenolic Compounds in *B. rapa* var. *rapa* and in *P. brassicae* Larvae and Excrement Samples (mg/kg, Dry Basis)[a]

compounds	sample		
	B. rapa var. *rapa*	larvae	excrements
1	872.2 (67.9)		
2	1693.9 (256.7)		
3	889.8 (108.9)		
4	3414.4 (315.5)		
5	924.8 (84.5)		
6	926.1 (98.9)		
7	1192.9 (177.4)		
8	565.7 (74.9)		
9	424.2 (74.6)		
10 + **11**	819.7 (62.0)	89.5 (7.1)[b]	3473.8 (187.5)[b]
12	4157.1 (323.5)	178.8 (17.0)	5193.4 (396.7)
13 + **14**	350 (68.5)		
15	2120.9 (24.5)		
16 + **17**	60.2 (4.7)	29.3 (2.6)[c]	1340.1 (188)[c]
18	296.2 (33.5)		
19	173.2 (26.8)		NQ
20		2.9 (0.1)	NQ
21		NQ	
22			219.4 (10.5)
total	18881.3	300.5	10226.7

[a] Results are expressed as means (standard deviations) of three determinations. The identity of phenolic compounds is as in **Figure 1**. [b] This is only compound **11**. [c] This is only compound **16**. NQ, not quantified.

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. 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.

Nitric Oxide Radical Scavenging Activity. The antiradical activity was determined spectrophotometrically in an ELX808 IU Ultra Microplate Reader (Bio-Tek Instruments, Inc.), according to a described procedure (*16*). Briefly, 100 μL of 20 mM sodium nitroprusside was incubated with 100 μL 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 μL of Greiss reagent (1% sulfanilamide and 0.1% naphthylethylenediamine, in 2% phosphoric acid) was added. 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.

Antimicrobial Activity. The antimicrobial activity of the lyophilized aqueous extracts was screened against sets of representative species of fungi and bacteria. The tested bacteria included *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, and *Pseudomonas aeruginosa* ATCC 27853, while the fungal strains were *Candida albicans* ATCC 10231, *Aspergillus fumigatus* ATCC 46645, and a clinical isolate of *Microsporum gypseum*. The antibacterial activity was performed using the latest update to the reference protocol M7-A7 published by the Clinical Laboratory Standards Institute (CLSI, formerly NCCLS; *17*). Five serial 2-fold dilutions of sterile distilled water solutions of the lyophilized extracts were tested, starting from maximum concentrations of at least 5 mg/mL of the lyophilized extract. Quality control was assured by testing the *E. coli* and the *P. aeruginosa* strains with gentamicin (Sigma-Aldrich, Germany), and the results were within the recommended limits. The antifungal activity of the water solutions of the lyophilized extracts was performed using methods based on the CLSI broth microdilution reference protocols M27-A2 and M38-A (*18, 19*) for the yeast and filamentous fungi, respectively. The reference antifungal compound fluconazole (Pfizer, United Kingdom) was used for quality control determinations with *Candida krusei* ATCC 6258. The results obtained were within the recommended limits. All determinations were performed in duplicate, and the results were confirmed in independent assays.

RESULTS AND DISCUSSION

Phytochemical Analysis. Phenolic compounds in the host plant, larvae, and excrements (**Figure 1**) were identified as before, by their retention times and MS and UV spectra (*13*). The HPLC-DAD-MS study of the *B. rapa* leaves extract revealed the existence of several hydroxycinnamic acid derivatives of quercetin 3-*O*-sophoroside-7-*O*-glucoside (compounds **2**−**4**), kaempferol 3-*O*-sophoroside-7-*O*-glucoside (compounds **1**, **5**−**7**, and **13**), and kaempferol 3-*O*-sophoroside (**16**) (compounds **14**, **15**, and **17**), *p*-coumaric (**8**), ferulic (**11**), and sinapic (**12**) acids, and the nonacylated flavonoid glycosides **9**, **10**, **18**, and **19**.

The analysis of *P. brassicae* larvae by HPLC-DAD-MS showed the presence of **11**, **12**, **16**, quercetin 3-*O*-sophoroside (**20**), and kaempferol 3-*O*-(*p*-coumaroyl)sophoroside (**21**). The excrements exhibited **11**, **12**, **16**, **20**, isorhamnetin 3-*O*-glucoside (**19**), and a sulfated derivative, identified by its characteristic loss of 80 u (isorhamnetin 3,7-di-*O*-glucoside sulfate, **22**).

Three compounds were found to be common to all matrices: two phenolic acids, ferulic (**11**) and sinapic (**12**), and one nonacylated flavonoid, kaempferol 3-*O*-sophoroside (**16**); all three were in higher amounts in excrements (**Table 1**). As expected, greater structural variety was found in the host plant (**Figure 1**), in which phenolics were distributed between flavonoids, namely, kaempferol, quercetin, and isorhamnetin derivatives and phenolic acids (*p*-coumaric, ferulic, and sinapic acids). The first group was clearly in major proportion.

Sinapic acid (**12**) was the main compound in matrices (22, 60, and 51% in *B. rapa*, larvae, and excrements, respectively) (**Table 1**). Quercetin 3-*O*-(*p*-coumaroyl)sophoroside-7-*O*-glucoside (**4**) (18%) and kaempferol 3-*O*-(caffeoyl)sophoroside (**15**) (11%) were the following ones in *B. rapa* var. *rapa*. On the other hand, in larvae and excrements, the second and third major compounds were ferulic acid (**11**) (30 and 34%, respectively) and kaempferol 3-*O*-sophoroside (**16**) (10 and 13%, respectively). Regarding sinapic acid, this compound was present in greater amounts in excrements. In larvae, the presence of these phenolic compounds may constitute a defense mechanism against external aggressions such as light, undesirable environmental conditions, oxidative processes, or microbian agents (*20*).

Butterfly and exuviae were also screened for phenolics detection; however, these compounds were not detected. These results indicate that during the metabolic process that occurs in *P. brassicae*, phenolic compounds are mainly excreted through the excrements, not being transferred to wings. These results are interesting, as flavonoids are often found as wing pigments (*21*), which was not the case of *P. brassicae* reared with *B. rapa* var. *rapa*.

It was possible to quantify one sulfated flavonoid in excrements, isorhamnetin 3,7-di-*O*-glucoside sulfate (**22**), not present in the host plant. Other sulfated compounds described by Ferreres et al. (*13*) were found in vestigial amounts. These findings point to a metabolization process that involves sulfation, described for the first time by Ferreres et al. (*13*). In fact, the resulting compounds have enhanced hydrophily, which may contribute to easier excretion, as it yields passage through the cell membrane more difficult.

Some compounds not found in host plants, such as quercetin 3-*O*-sophoroside (**20**) and kaempferol 3-*O*-(*p*-coumaroyl)sophoroside (**21**), were identified and quantified in *P. brassicae* fed with this matrix. As insects are unable to synthesize flavonoids or their precursors (*22*), these compounds can only arise from feeding on the host plant. Possibly, these compounds exist in the plant in quantities below the detection limit and, by a process of accumulation in the insect, ascend to detectable

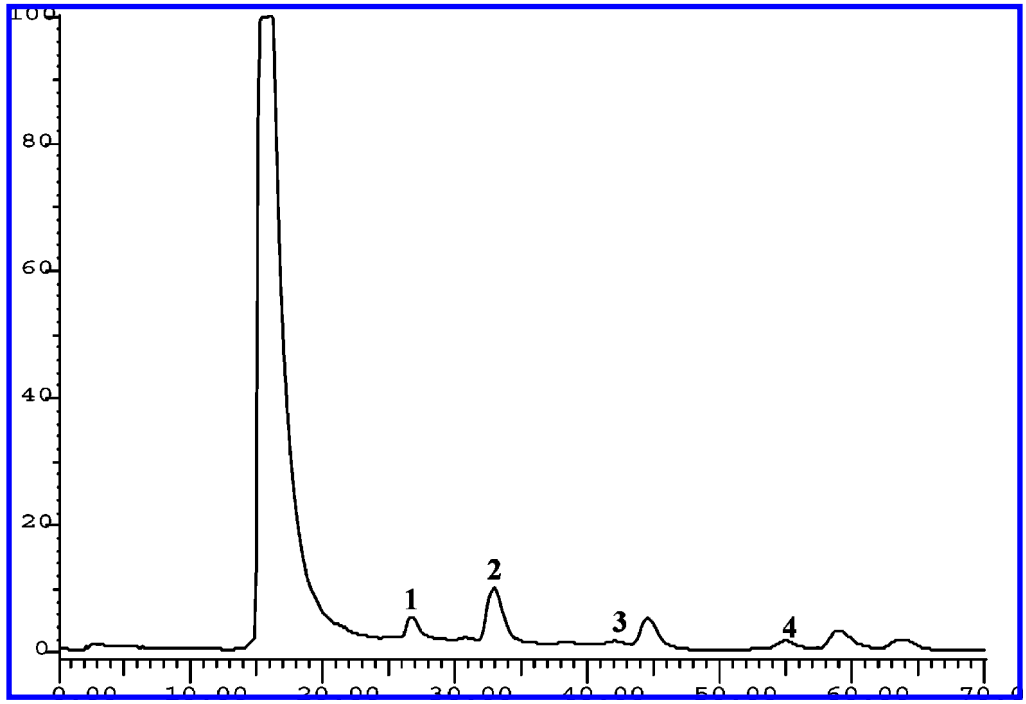

Figure 2. HPLC-UV profile of organic acids of *B. rapa* var. *rapa* leaves. Detection at 214 nm. Peaks: 1, citric acid; 2, malic acid; 3, shikimic acid; and 4, fumaric acid.

Table 2. Organic Acids in *B. rapa* var. *rapa* and *P. brassicae* Larvae, Butterfly, and Excrement Samples (mg/kg Dry Basis)[a]

	organic acid				
sample	citric	malic	shikimic	fumaric	total
B. rapa var. *rapa*	15485.0 (209.6)	84882.8 (3540.9)	78.0 (1.0)	79.9 (2.2)	100525.7
larvae	5070.2 (17.3)	16661.6 (512.7)	15.7 (1.1)	935.9 (8.0)	22683.4
excrements		NQ		411.6 (61.0)	411.6
butterfly	3093.9 (7.9)	9970 (60.9)	48.8 (1.6)	231.0 (1.1)	13343.8

[a] Results are expressed as means (standard deviations) of three determinations. [b] N.Q., not quantified.

concentrations. Additionally, during the metabolic process in *P. brassicae*, reactions like deacylation and deglycosilation of flavonoids may occur, leading to the appearance of compounds such as quercetin 3-*O*-sophoroside and kaempferol 3-*O*-(*p*-coumaroyl)sophoroside. In fact, these metabolization processes were confirmed in this species by Ferreres et al. (*13*).

Some considerations can also be made about the chemistry that attracts the insect. Kaempferol, quercetin, and isorhamnetin derivatives were found in the host plant; the larvae, however, only accumulated representatives of the first two [kaempferol 3-*O*-sophoroside (**16**), quercetin 3-*O*-sophoroside (**20**), and kaempferol 3-*O*-(*p*-coumaroyl)sophoroside (**21**)] in their bodies, thus eliminating isorhamnetin derivatives, which were found in excrements. In fact, this preference for kaempferol and quercetin derivatives by the insect, in deterrence of others, has been described in the common blue butterfly *P. icarus* (*10*). In part, these results may be due to the chemical structure of isorhamnetin, which is a methoxylated compound, while kaempferol and quercetin are not. The work of van Loon (*23*) described how the responsiveness of gustatory neurons of *P. brassicae* decreases with increasing methoxylation.

Besides the aglycone of these flavonoids, the sugar moiety also seems to play a role in the profile of the compounds sequestered by the insect. In the host plant, different glycosides, which include 3-*O*-glucosides, 3,7-di-*O*-glucosides, and 3-*O*-sophorosides, exist. Despite this fact, only 3-*O*-sophorose derivatives, such as kaempferol 3-*O*-sophoroside (**16**), quercetin 3-*O*-sophoroside (**20**), and kaempferol 3-*O*-(*p*-coumaroyl)sophoroside (**21**), were sequestered and accumulated in the larva's body, while the remaining two were excreted.

The screening of organic acids belonging to glycolysis, tricarboxylic acid, and glyoxylate cycles showed that *B. rapa* var. *rapa*, as well as *P. brassicae* larvae and butterfly, have the same qualitative composition, with citric (**1**), malic (**2**), shikimic (**3**), and fumaric (**4**) acids being found. In excrements, only fumaric (**4**) and malic (**2**) acids were noticed (**Figure 2** and **Table 2**). The sample with higher amounts of organic acids was *B. rapa* var. *rapa*, followed by *P. brassicae* larvae, and its excrements exhibited the lowest content (**Table 2**).

Quantitatively, malic and citric acids were found to be the most abundant compounds in *B. rapa* var. *rapa* (84 and 15% respectively), while citric acid was the major organic acid in larvae, accounting for 22% of total compounds. When detected, shikimic acid was the compound present in lower amounts. The absence of malic, shikimic, and citric acids in excrements may be due to their use in the larva's development cycle, since the majority of them are involved in Krebs cycle (*24*).

Antioxidant Activity. In the DPPH$^{\bullet}$ assay, *P. brassicae* butterfly was the sample that showed greater activity: about 10 times higher than *B. rapa* var. *rapa*, which was the least effective (**Figure 3A**). The order of activity found was butterfly > larvae > excrements > *B. rapa* var. *rapa*. Although all matrices were very different concerning scavenging activity against DPPH$^{\bullet}$, the effect was concentration-dependent.

When comparing the IC_{50} values of *B. rapa* var. *rapa* leaves, 557 μg/mL, with the one found for the inflorescences of this species, 774 μg/mL (*25*), it is possible to realize that the leaves exhibit greater antiradical capacity. When comparing the IC_{25} values obtained in this assay for the leaves of *B. rapa* var. *rapa*, 306 μg/mL, with the same parameter found for *B. oleracea* var. *costata* leaves, 440 μg/mL (*15*), it can be seen that *B. rapa* var. *rapa* is more active.

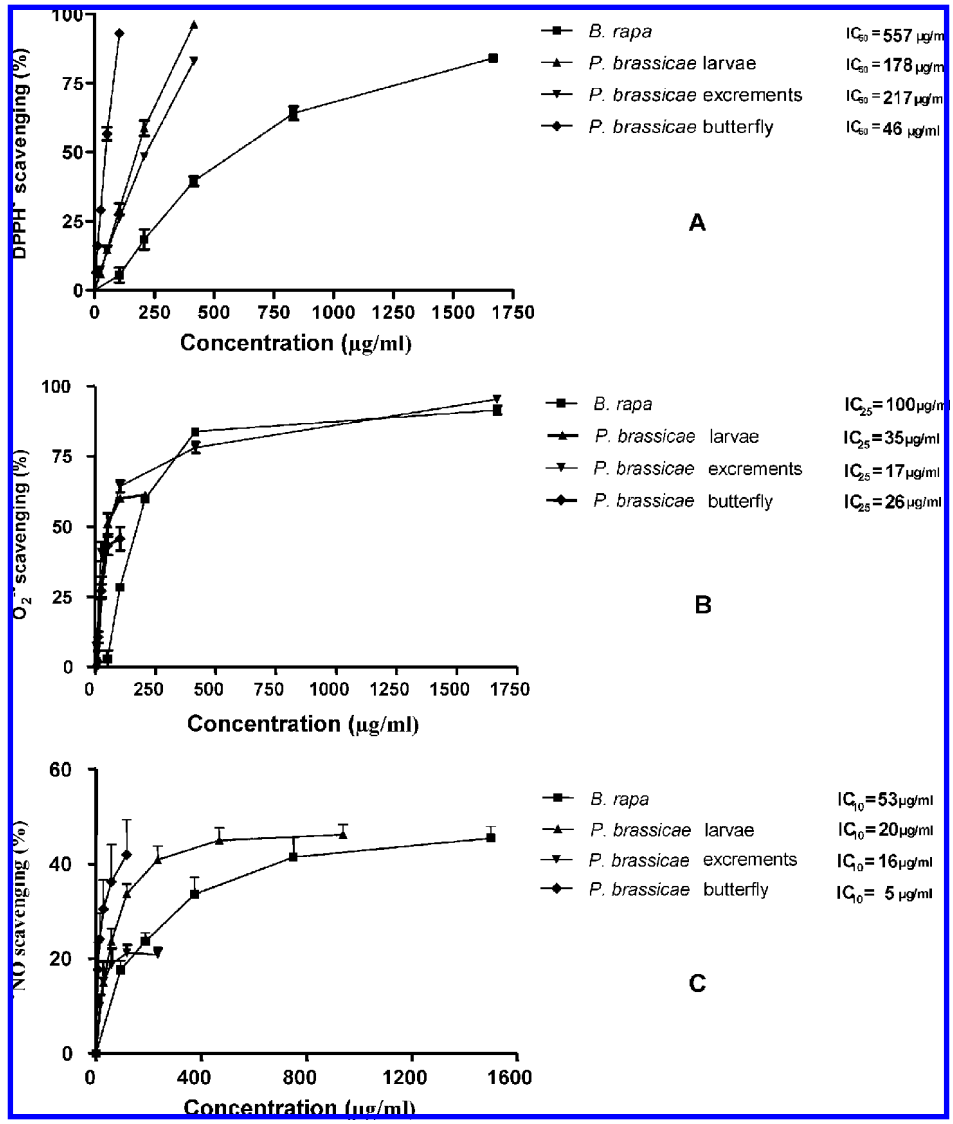

Figure 3. Effect of *B. rapa* var. *rapa* and *P. brassicae* material against DPPH˙ (**A**), $O_2^{\bullet-}$ (**B**), and ˙NO (**C**). Values show means ± SEs from three experiments, performed in triplicate.

Table 3. Antimicrobial Activity of *B. rapa* var. *rapa* and *P. brassicae* Larvae, Butterfly, And Excrement Samples: Minimum Inhibitory Concentrations (MIC) of Water Solutions of Lyophilized Aqueous Extracts of the Different Samples against Representative Bacterial and Fungal Species[a]

		MICs (μg/mL)		
strains	*B. rapa* var. *rapa*	*P. brassicae* larvae	*P. brassicae* excrements	*P. brassicae* butterfly
		bacteria		
E. coli	>10000	>6664	>6664	>5000
S. aureus	>10000	>6664	>6664	>5000
P. aeruginosa	>10000	>6664	>6664	>5000
		fungi		
C. albicans	>3654	>2435	>5000	>3750
A. fumigatus	>3654	>2435	>5000	>3750
M. gypseum	>3654	>2435	>5000	>3750

[a] Positive controls were within recommended values. MICs (μg/mL) for gentamicin were 0.5 (*E. coli* ATCC 25922) and 1.0 (*P. aeruginosa* ATCC 27853); for fluconazole, the MIC was 32 (*C. krusei* ATCC 6258).

In the assays for the evaluation of superoxide radical scavenging activity (**Figure 3B**), and differently from what was observed against DPPH˙ (**Figure 3A**), the samples under study revealed that they were quite equivalent in terms of activity. Excrements were the most active material (IC_{25} value of 17 μg/mL), and *B. rapa* var. *rapa* was the least one, with all samples exhibiting a concentration-dependent effect.

As noticed against DPPH˙, the leaves of *B. rapa* var. *rapa* were clearly more active against superoxide radical than its inflorescences, with an IC_{25} value of 100 vs 356 μg/mL (*25*), respectively. However, when compared to *B. oleracea* var. *costata*, the leaves of *B. rapa* var. *rapa* were less effective, since a IC_{25} value of 43 μg/mL was described for the first (*15*). The different activities certainly reflect the distinct chemical compositions of the matrices.

In the assay against nitric oxide (**Figure 3C**), *P. brassicae* butterfly was revealed as the most active sample (IC_{10} value of 5 μg/mL), similar to what had been verified against DPPH˙ (**Figure 3A**). Again, the activity was concentration-dependent. As observed in the two above-mentioned assays, *B. rapa* var. *rapa* was the least active sample, with an IC_{10} value at 53 μg/mL. The reason for the use of IC_{10} is related to the samples' poor solubility in the ˙NO assay's reagents and not due to lack of activity.

The results obtained allowed us to observe that *P. brassicae* material always exhibited higher activities than its host plant, which may be a consequence of selective sequester of antioxidant compounds for the insect's protection.

Antimicrobial Activity. No inhibition of the growth of the tested bacterial and fungal species was produced by the extracts; therefore, no antimicrobial activity was found (**Table 3**).

In conclusion, in this work, we established the unique set of compounds that results from the interaction of *P. brassicae* with one of its most common host plants, *B. rapa* var. *rapa*, whose chemical complexity renders it almost impossible to synthesize in the laboratory. The targeted metabolic analysis also allowed us to further understand some metabolic and sequestration processes that occur in *P. brassicae*.

In addition, although no antimicrobial activity was found, the high antioxidant activity of these materials was demonstrated. According to the results obtained in all assays, *P. brassicae* material was more active than *B. rapa* var. *rapa*, despite the fact that this last has the highest amount of phenolics and organic acids. This indicates that besides quantitative composition, the distinct qualitative composition of each extract seems to play a major role in the antioxidant activity.

The type of extraction involved herein, which applies high temperatures for a considerable period of time, allows exclusion of the presence of other constituents, like those involved in enzymatic defense pathways (both enzymes and substrates), as well as other compounds that do not withstand the experimental extraction conditions used.

Because of the potent activity exhibited by *P. brassicae* material, it may be used by the pharmaceutical industry in antioxidative formulations for the prevention of free radical-mediated diseases 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, or the food industry, where it can be used as an antioxidant or to improve the nutritional value of foodstuffs. In addition, it may constitute an economical advantage for *B. rapa* var. *rapa* producers who have great losses caused by *P. brassicae* infestations.

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