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ORIGINAL CONTRIBUTION

Neuroprotective effects of digested polyphenols from wild blackberry species

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Abstract
Purpose Blackberry ingestion has been demonstrated to attenuate brain degenerative processes with the benefits ascribed to the (poly)phenolic components. The aim of this work was to evaluate the neuroprotective potential of two wild blackberry species in a neurodegeneration cell model and compare them with a commercial variety.

Methods This work encompasses chemical characterization before and after an in vitro digestion and the assessment of neuroprotection by digested metabolites. Some studies targeting redox/cell death systems were also performed to assess possible neuroprotective molecular mechanisms.

Results The three blackberry extracts presented some quantitative differences in polyphenol composition that could be responsible for the different responses in the neurodegeneration cell model. Commercial blackberry extracts were ineffective but both wild blackberries, *Rubus brigantinus* and *Rubus vagabundus*, presented neuroprotective effects. It was verified that a diminishment of intracellular ROS levels, modulation of glutathione levels and activation of caspases occurred during treatment. The last effect suggests a preconditioning effect since caspase activation was not accompanied by diminution in cell death and loss of functionality.

Conclusions This is the first time that metabolites obtained from an in vitro digested food matrix, and tested at levels approaching the concentrations found in human plasma, have been described as inducing an adaptative response.

Keywords Caspase activity · Glutathione balance · In vitro digestion · Neurodegenerative diseases · Wild blackberries

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Introduction

In the developed world, the population lifespan is increasing with a concomitant increase in the incidence of age-related diseases such as neurodegeneration [1]. Due to the high impact at both financial and social levels

[2, 3], strategies to retard or reverse neuronal and behavioral deficits that occur in aging are urgently required. Indeed, these foci are areas of intense research effort, but the therapeutic strategies [4] and delivery of (pharma) products [5, 6] have been limited. Epidemiological evidence indicates that antioxidant supplementation may provide neuroprotection against age-related neurodegenerative disorders, including Parkinson's disease, amyotrophic lateral sclerosis and Alzheimer's disease [7–9]. Increased dietary intake of antioxidant fruits, in particular berry fruits, may cause positive and profound impacts on human health, performance and disease [10]. Their biological properties are attributed to the wide diversity and high levels of phenolic compounds, frequently associated with a high antioxidant capacity. Due to the multitude of phytochemicals found in these fruits, instead of a single compound, they can promote complementary, additive and/or synergistic effects [10, 11].

In vitro studies have revealed blackberries as possessing potent antioxidant, antiproliferative and anti-inflammatory activities [12, 13]. Moreover, in aged rats, these fruits were capable of improving performance on motor tests, which relied on balance and fine motor coordination, and on measures of spatial working memory [14]. However, these effects were not accompanied by an improvement in dopamine release [14] and consequently by an improvement of receptor sensitivity, events usually related with the observed effects [15, 16]. A large number of pathways and protein kinase cascades, such as protein kinase C, Nrf2/ARE antioxidant pathway, pro-survival MEK/ERK and PI3K/AKT pathways among others, have been reported as targets for phenolic compounds; nevertheless, the target pathways affected remain unknown [17].

The chemical diversity of plants constitutes an immense and relatively untapped reservoir of molecules with potential pharmacological/nutraceutical value. The diversity of Portuguese plants represents a reservoir of phytochemicals as yet poorly characterized and explored. In particular, in the north of Iberian Peninsula, there are endemic *Rubus* species, such as *Rubus brigantinus* Samp. and *Rubus vagabundus* Samp. [18], of which chemical diversity could be further explored.

The aim of this work is to evaluate the neuroprotective potential of two endemic blackberry species in a neurodegeneration cell model. This work encompasses an in vitro digestion (IVD) to mimic some alterations in metabolites that fruits are submitted to when ingested. Some studies targeting redox and cell death systems were performed to illustrate molecular mechanisms by which blackberry metabolites could exert beneficial effects.

Methods and materials

Plant material

Fruits of wild blackberry species (*R. brigantinus* Samp. and *R. vagabundus* Samp.) were collected in September 2009 in Bragança region (northeast region of Portugal) and frozen. Fruits were collected from several populations, growing in different locations in order to be representative of species. For both species, voucher samples were authenticated and deposited at the herbarium "João de Carvalho e Vasconcelos", Instituto Superior de Agronomia, Lisbon, Portugal (voucher number 716/2010 and 722/2010). For comparison purposes, the commercial blackberry cv. Apache (*Rubus* L. subgenus *Rubus* Watson) produced in Fataca experimental field (Odemira, Portugal) was also used. The samples were freeze-dried, ground without separation of seeds in an IKA M20 mill to pass a 0.5 mm sieve and stored at −80 °C prior to extraction.

Extract preparation

Fruit extracts were prepared using an hydroethanolic solution (ethanol 50% (v/v)) as previously described [19]. Briefly, 12 mL of ethanol 50% was added for each gram of blackberry freeze-dried powder. Homogenate was shaken for 30 min and filtered. Extracts obtained were dried under vacuum.

In vitro digestion (IVD)

Phytochemical alterations during digestion were mimicked using the IVD model already described [20]. Briefly, the undigested extract was submitted to conditions that mimic the gastric digestion such as adjusted to pH 1.7, the addition of pepsin and incubation at 37 °C with shaking at 100 rpm for 2 h. After, small intestine conditions were mimicked by the addition of pancreatin and bile salts, followed by dialysis with a cellulose tube containing $NaHCO_3$ to neutralize titratable acidity. After 2 h incubation at 37 °C, the solution inside the dialysis tubing (IN) and the solution outside the dialysis tubing (OUT) were taken.

Chemical characterization

Total phenolic quantification

Determination of total phenolic compounds was performed by the Folin–Ciocalteau method adapted to microplate reader [21]. Gallic acid was used as the standard and the results were expressed as mg of gallic acid equivalents (mg GAE).

Peroxyl radical scavenging capacity determination

Peroxyl radical scavenging capacity was determined by the ORAC (Oxygen Radical Absorbance Capacity) method as described by Tavares et al. [19]. The final results were calculated using the area differences under the fluorescence decay curves between the blank and the sample and were expressed as μM trolox equivalents (μM TE).

Determination of phenolic profile by LC–MS

Extracts and digested fractions, containing 500 mg GAE mL^{-1}, were applied to a C18 column (Synergi Hydro C18 column with polar end capping, 4.6 × 150 mm, Phenomonex Ltd.) and analyzed on a LCQ-DECA system controlled by the XCALIBUR software (2.0, Thermo-Finnigan), as reported by Tavares et al. [19]. The LCQ-Deca system comprised a Surveyor autosampler, pump and photodiode array (PDA) detector and a Thermo Finnigan iontrap mass spectrometer.

Animal cell culture

Human neuroblastoma SK-N-MC cells were obtained from the European Collection of Cell Cultures (ECACC) and cultured in EMEM (Eagle Minimum Essential medium, Sigma) supplemented with 2 mM L-glutamine (Sigma), 10% (v/v) heat-inactivated fetal bovine serum (FBS, Gibco), 1% (v/v) non-essential amino acids (Sigma), 1 mM sodium pyruvate (Sigma) and containing 50 U penicillin and 50 μg streptomycin per mL of medium. The cells were maintained at 37 °C in 5% CO_2 and split at sub-confluence of 70–80% using 0.05% trypsin/EDTA (Gibco).

Cytotoxicity evaluation

Digested fractions were dried under vacuum and dissolved in cell medium for the cytotoxicity tests by measuring cell viability as previously described [20]. Briefly, SK-N-MC neuroblastoma cells were seeded in a 96-well plate using 1.25×10^5 cells mL^{-1} and grown for 48 h prior to incubation with the IN fractions. Toxicity tests involved 24 h fractions incubation in the range 0–100 μg GAE mL^{-1} medium. Cell viability was assessed using the CellTiter-Blue® Cell Viability Assay (Promega), according to the manufacture instructions.

Neuroprotective evaluation

To evaluate the neuroprotective effect of fractions, a neurodegeneration cell model already described was used [20]. The model described the treatment of SK-N-MC neuroblastoma cells with H_2O_2 to induce cell death. Briefly, cells were seeded at 7.4×10^4 cells mL^{-1}, grown for 24 h and then after 24 h of pre-incubation with medium supplemented with non-toxic concentrations of blackberry fractions, the cells were treated with medium containing H_2O_2 (300 μM). After 24 h, the medium was removed and cells were washed with PBS and collected by trypsinization. Cells were then incubated with two fluorescent probes for 30 min at 37 °C. 3,3′-Dihexyloxacarbocyanine iodide ($DiOC_6(3)$, 20 nM, Invitrogen) was used to evaluate the mitochondrial transmembrane potential (ΔΨm), and propidium iodide (PI, 1 μg mL^{-1}, Invitrogen) was used to determine cell viability, based on plasma membrane integrity [22]. A flow cytometer (Partec) was used to analyze parameters. This cytometer contains a blue solid state laser (488 nm) with FL1 green fluorescence channel for $DiOC_6(3)$ at 530 nm and a FL3 red fluorescence channel for PI detection at 650 nm. The acquisition and analysis of the results were performed with FlowMax® (Partec) software.

Intracellular reactive oxygen species (ROS) production determination

To evaluate the ability of fractions to reduce ROS levels produced by cells, the conversion of 2′,7′-dichlorofluorescein diacetate (H_2DCFDA, Invitrogen) to fluorescent 2′,7′-dichlorofluorescein (DCF) was monitored [23, 24]. SK-N-MC neuroblastoma cells were seeded in a 96-well plate (1.25×10^5 cells mL^{-1}), grown for 24 h, then washed with PBS and pre-incubated with fractions prepared in medium (0.5% (v/v) FBS) for 2 or 24 h. After pre-incubation, cells were washed with PBS and incubated for 30 min at 37 °C with 25 μM H_2DCFDA prepared in PBS. Cells were washed with PBS and then H_2O_2 (200 or 300 μM) was added. Fluorescence was measured (λex: 485 nm, λem: 530 nm) using a FLx800 Fluorescence Microplate Reader (Biotek) over 1 h at 37 °C. ROS generation was calculated as an increase in fluorescent signal compared with cells not treated with H_2O_2.

Glutatione (GSH) and glutatione disulfide (GSSG) quantification

GSH and GSSG were quantified by HPLC after derivatization with orthophthalaldehyde, performed accordingly to Kand'ar [25] as already described in Tavares et al. [20].

Chromatographic analysis was accomplished using isocratic elution on a C_{18} analytical column (Supelcosil™ ABZ + Plus HPLC Column 15 cm × 4.6 mm, 3 μm (Supelco)) at 40 °C on an Acquity™ Ultra Performance LC system (Waters). The mobile phase was 15% (v/v) methanol in 25 mM sodium hydrogen phosphate, pH 6.0. The flow rate was kept constant at 0.7 mL min^{-1}. The excitation and emission wavelengths were set at 350 and

420 nm, respectively. The amount of GSH and GSSG was quantified from the corresponding peak areas using Empower® Pro 2.0 software. The concentration of GSH and GSSG in the samples was determined from standard curves with ranges 0–100 μM for GSH and 0–5 μM for GSSG. Values were normalized for total protein content, determined by Lowry method [26].

Caspase 3/7 activity determination

Caspase activity was determined using the Caspase-Glo™ 3/7 assay (Promega). SK-N-MC neuroblastoma cells were seeded in a 96-well plate (1×10^4 cells mL^{-1}). Cells were grown for 24 h, then washed with PBS and pre-incubated with fractions prepared in medium (0.5% (v/v) FBS) for 24 h. After this period, cells were washed again with PBS and medium containing 300 μM H_2O_2 was added. Cells were incubated for 24 h and then 100 μL of proluminescent caspase 3/7 substrate was added to each well. Cells were incubated at room temperature for 3 h and luminescent signal was recorded. Values were normalized for cell viability, determined by flow cytometry, as described above.

Statistical analysis

The results reported in this work are the averages of at least three independent experiments and are represented as the mean ± SD. Differences among treatments were detected by analysis of variance with Tukey HSD (Honest Significant Difference) multiple comparison test ($\alpha = 0.05$) using SigmaStat 3.10 (Systat).

Results

Characterization of the blackberry extracts

Characterization of the three blackberry extracts was performed, and although the total phenolic content (TPC) of the three blackberries was very similar, their antioxidant capacity (AC) was different (Table 1). The wild blackberries had a higher AC compared to the commercial variety, especially *R. brigantinus* that had 60% higher antioxidant capacity than the commercial blackberry.

The in vitro digestion model provided two fractions (IN and OUT) after pancreatic digestion. The fraction that passes through the dialysis membrane constitutes the IN fraction and contains metabolites that equate to those that should be able to reach serum by paracellular transport. The material that remains outside the dialysis tubing constitutes the OUT fraction and contains metabolites that equate to those that reach colon after digestion. After IVD, the TPC and AC were greatly changed (Table 1), and the TPC of the IN fractions was reduced to less than 10% of the original content. The AC values were also reduced in all samples, but since the reduction was lower than in the values of TPC, the ratio AC/TPC became higher in the IN fractions comparatively to the undigested extracts (Table 1). Concerning these ratios for the IN fraction, *R. brigantinus* was the most potent (222 μmol TE mg^{-1} GAE) followed by the commercial blackberry (100 μmol TE mg^{-1} GAE) and then by *R. vagabundus* (44 μmol TE mg^{-1} GAE; Table 1). Moreover, IN fractions had a higher ratio of AC/TPC than the respective OUT fractions (results not shown). Since IN fraction equates to the compounds that could potentially reach the serum through paracellular transport and at the same time, this fraction seems to be the most chemically reactive (higher AC/TPC), these factors led us to choose the IN fraction to be tested in neuroprotective studies.

LC–MS analysis (Fig. S1 and Table S1 on Supplementary material) showed that the polyphenol profiles of the wild blackberries before digestion were similar to that of the commercial blackberry (Fig. S1A on Supplementary material and Fig. 1a). The main differences were in the amount of anthocyanins (cyanidin-3-*O*-glucoside, -xyloside and -hydroxymethyl-glutaroyl glucoside), which were lower for the endemic species, and in the quantity of

Table 1 Chemical characterization of commercial blackberry and two wild blackberry species (*R. brigantinus* and *R. vagabundus*) extracts before and after in vitro digestion

	Undigested extract			IN fraction		
	TPC (mg GAE g^{-1} dw)	AC (μmol TE g^{-1} dw)	AC/TPC (μmol TE mg^{-1} GAE)	TPC (% of undigested extract)	AC (% of undigested extract)	AC/TPC (% of undigested extract)
Commercial blackberry	27.51 ± 0.98^a	221 ± 22^c	33 ± 2^a	5.5	17	307
R. brigantinus	28.17 ± 3.65^a	357 ± 10^a	21 ± 3^b	0.5	5	1,040
R. vagabundus	31.07 ± 1.47^a	274 ± 57^b	15 ± 3^b	1.7	5	292

For undigested extracts and IN fractions, the total phenolic content (TPC) and antioxidant capacity for peroxyl radical (AC) were determinate and the ratio AC/TPC was calculated. Values of TPC, AC and AC/TPC in the undigested extract were expressed as mg GAE g^{-1} dw, μmol TE g^{-1} dw and μmol TE mg^{-1} GAE, respectively. Values in the IN fraction were expressed as % of the values determined for the undigested extract. Statistical significant differences for $p < 0.05$ are denoted with different letters (a–c). All values are mean ± SD, $n = 3$

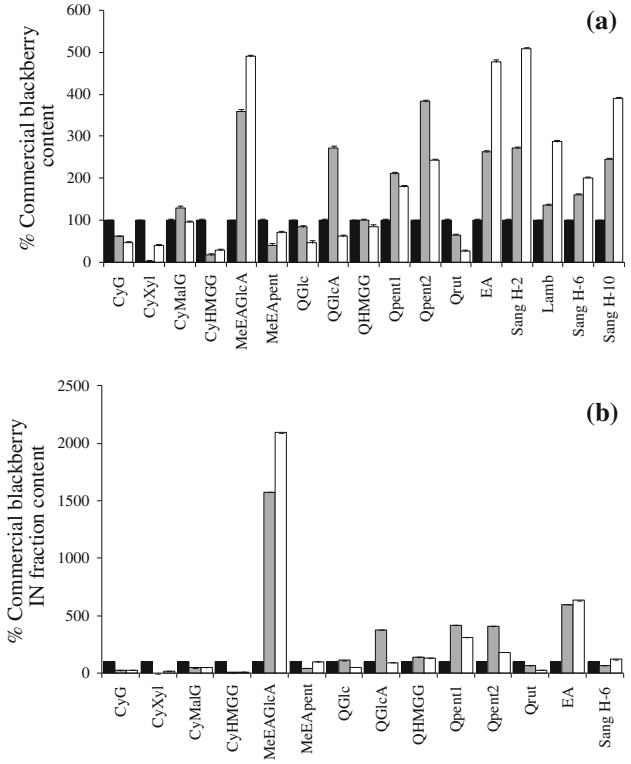


Fig. 1 Relative quantification of polyphenols in blackberries. **a** Undigested extract before in vitro digestion, **b** IN samples. All values are expressed as relative to the commercial blackberry variety (*black shaded bar*), *R. brigantinus* (*gray shaded bar*) and *R. vagabundus* (*open bar*). Values were calculated as peak area of the mass spectrometer response for the *m/z* characteristic of each component. Quantified metabolites: cyanidin-3-*O*-glucoside (CyG), cyanidin-3-*O*-xyloside (CyXyl), cyanidin-3-*O*-malonylglucoside (CyMalG), cyanidin-3-*O*-hydroxymethyl-glutaroyl glucoside (CyHMGG), methyl ellagic acid glucuronide (MeEAGlcA), methyl ellagic acid pentose (MeEApent), quercetin-glucoside (QGlc), quercetin-glucuronide (QGlcA), quercetin-HMG-glucoside (QHMGG), quercetin pentose 1 (Qpent1), quercetin pentose 2 (Qpent2), quercetin-rutinoside (Qrut), ellagic acid (EA), sanguiin H-2 (Sang H-2), lambertianin C (Lamb), sanguiin H-6 (Sang H-6), sanguiin H-10 (Sang H-10). Lambertianin C, sanguiin H-2 and sanguiin H-10 were not identified in IN samples

ellagitannins (sanguiins H-2, C, H-6 and H-10), which were particularly high in *R. vagabundus* (approx. 3.5-fold the amount detected in commercial blackberry). In addition, the endemic species had higher levels of certain flavonols (quercetin pentoside) and ellagic acid or ellagic acid derivatives (methyl ellagic acid glucuronide). After IVD (Fig. 1b), the amounts of anthocyanins and ellagitannins detected in the IN fractions were considerably reduced, while, conversely, quercetin derivatives were present in higher levels in fractions from the wild species than those from the commercial blackberry (specially quercetin pentoside; Fig. 2b). The most obvious difference between the *R. brigantinus*, *R. vagabundus* and the commercial blackberry IN samples was the presence of a component with properties that suggest a methyl ellagic glucuronide structure (compound 10 in Fig. S1A on Supplementary material).

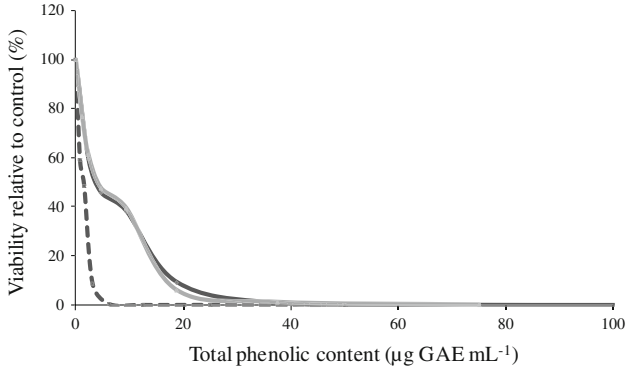


Fig. 2 Cytotoxicity evaluation of digested fractions of the three *Rubus* sp. SK-N-MC neuroblastoma cells were incubated for 24 h with digested fractions (0–100 μg GAE mL^{-1}), and the cytotoxicity was determined evaluating cell viability by CellTiter-Blue® Cell Viability Assay (Promega). Values are expressed as percentage relatively to control (without extract): Commercial blackberry (*black line*), *R. brigantinus* (*dashed line*), *R. vagabundus* (*gray line*)

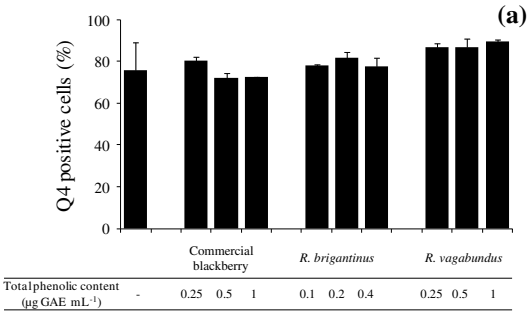
Cytotoxicity evaluation

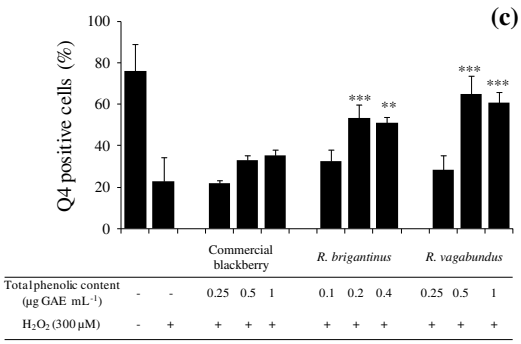
Cytotoxicity of digested blackberry metabolites was evaluated by conversion of resazurin into resorufin by viable cells (Fig. 2). *R. brigantinus* metabolites were more toxic than commercial blackberry and *R. vagabundus* (Fig. 2) which had similar toxicity profiles. Based on these profiles, ranges of non-toxic concentrations were selected as 0–1 μg mL^{-1} GAE for commercial blackberry and *R. vagabundus* and 0–0.4 μg mL^{-1} GAE for *R. brigantinus*.

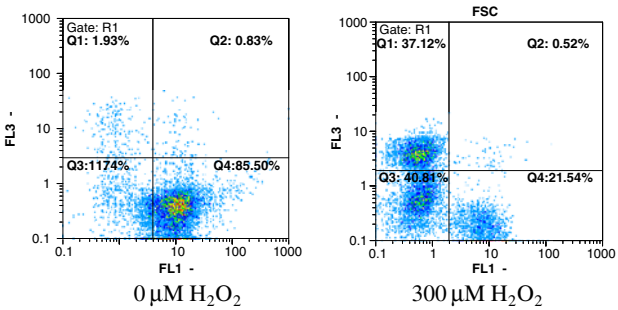
Neuroprotective evaluation

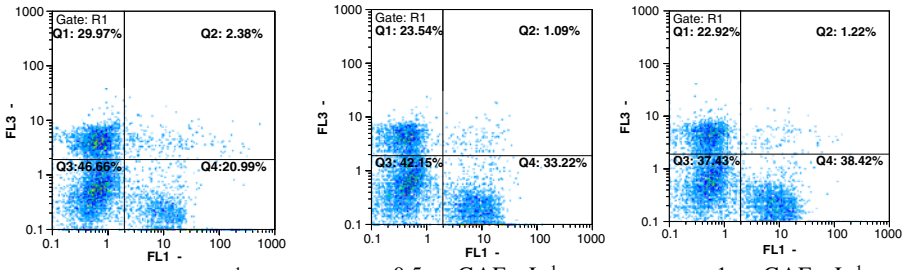
SK-N-MC neuroblastoma cells were pre-incubated for 24 h in the presence or absence of IN fractions and then challenged with H_2O_2 (300 μM, 24 h). Neuroprotection was then evaluated by monitoring cells that maintained both high ΔΨm and cell membrane integrity (Q4 positive cells; Fig. 3b). Incubation with all IN fractions in the absence of H_2O_2 injury did not alter cellular responses compared to the controls (Fig. 3a), which confirmed the absence of toxicity previously detected on neuroblastoma cells.

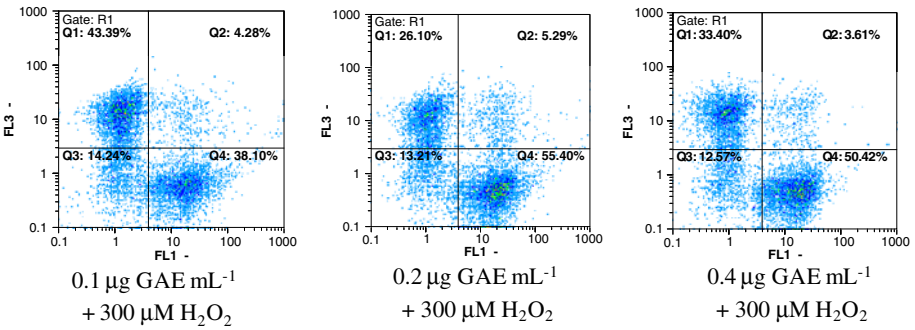
When cells were challenged with H_2O_2, pretreatment with the IN fraction from commercial blackberry did not cause a protective effect (Fig. 3b, c). However, IN fractions from wild species simultaneously increased ΔΨm and cell membrane integrity (Fig. 3b, c).

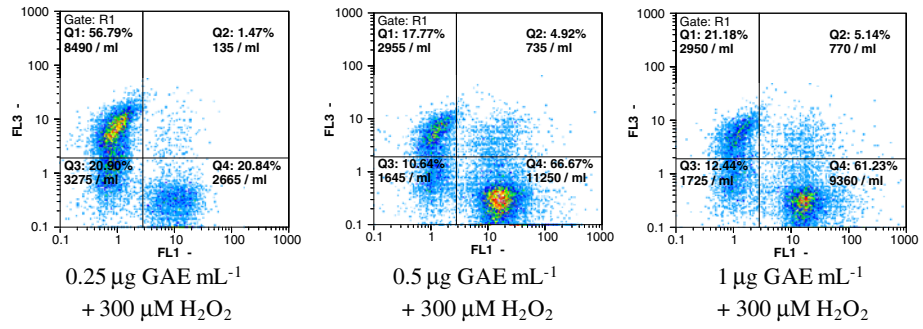

(a)
Q4 positive cells (%)
100
80
60
40
20
0
Commercial blackberry
R. brigantinus
R. vagabundus
Total phenolic content (μg GAE mL-1) - 0.25 0.5 1 0.1 0.2 0.4 0.25 0.5 1


(c)
Q4 positive cells (%)
100
80
60
40
20
0
***
**
***
***
Commercial blackberry
R. brigantinus
R. vagabundus
Total phenolic content (μg GAE mL-1) - - 0.25 0.5 1 0.1 0.2 0.4 0.25 0.5 1
H2O2 (300 μM) - + + + + + + + + + +


(b)
FSC
Gate: R1
Q1: 1.93%
Q2: 0.83%
Q3:11.74%
Q4:85.50%
Q1: 37.12%
Q2: 0.52%
Q3: 40.81%
Q4:21.54%
FL3 -
FL1 -
0 μM H2O2
300 μM H2O2


Commercial blackberry
Q1: 29.97%
Q2: 2.38%
Q3:46.66%
Q4:20.99%
Q1: 23.54%
Q2: 1.09%
Q3: 42.15%
Q4: 33.22%
Q1: 22.92%
Q2: 1.22%
Q3: 37.43%
Q4: 38.42%
0.25 μg GAE mL-1 + 300 μM H2O2
0.5 μg GAE mL-1 + 300 μM H2O2
1 μg GAE mL-1 + 300 μM H2O2


R. brigantinus
Q1: 43.39%
Q2: 4.28%
Q3: 14.24%
Q4: 38.10%
Q1: 26.10%
Q2: 5.29%
Q3: 13.21%
Q4: 55.40%
Q1: 33.40%
Q2: 3.61%
Q3: 12.57%
Q4: 50.42%
0.1 μg GAE mL-1 + 300 μM H2O2
0.2 μg GAE mL-1 + 300 μM H2O2
0.4 μg GAE mL-1 + 300 μM H2O2


R. vagabundus
Q1: 56.79%
8490 / ml
Q2: 1.47%
135 / ml
Q3: 20.90%
3275 / ml
Q4: 20.84%
2665 / ml
Q1: 17.77%
2955 / ml
Q2: 4.92%
735 / ml
Q3: 10.64%
1645 / ml
Q4: 66.67%
11250 / ml
Q1: 21.18%
2950 / ml
Q2: 5.14%
770 / ml
Q3: 12.44%
1725 / ml
Q4: 61.23%
9360 / ml
0.25 μg GAE mL-1 + 300 μM H2O2
0.5 μg GAE mL-1 + 300 μM H2O2
1 μg GAE mL-1 + 300 μM H2O2

◀ **Fig. 3** Cell viability assessed by flow cytometry. Cell viability was assessed in Q4, showing cells presenting membrane integrity (negative for PI) and high mitochondrial potential ($\Delta\psi m$; positive for $DiOC_6(3)$) and expressed as percentage. **a** Cell viability for cells incubated with IN digested fractions without oxidative stress (0 μM H_2O_2). Statistical differences of pre-incubated cells in comparison with cells not treated with H_2O_2 are denote as $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. All values are mean ± SD, $n >= 3$. **b** Flow cytometry outputs obtained with PI and $DiOC_6(3)$ allow the assessment of the percentage of viable cells, represented in quadrant Q4 (cells presenting membrane integrity and high mitochondrial potential). **c** Cell viability for cells incubated with IN digested fractions with oxidative stress (300 μM H_2O_2). Neuroblastoma cells were pre-incubated with digested fractions for 24 h and then injured by 300 μM H_2O_2 for 24 h. Graphic representation of flow cytometry panels and respective statistical evaluation. Statistical differences of pre-incubated cells in comparison with cells only treated with H_2O_2 are denote as $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. All values are mean ± SD, $n >= 3$

Intracellular ROS production

Intracellular ROS levels were monitored (Fig. 4) at two different times of pre-incubation, 2 and 24 h, to cover different time events. All blackberry digested extracts at 2 and/or 24 h pre-incubation (Fig. 4a) reduced basal ROS production. None of the IN fractions pre-incubations could maintain lower levels of ROS after the imposition of a non-lethal stress of 300 μM H_2O_2 for 1 h (results not shown). However, after a milder stress of 200 μM H_2O_2 for 1 h (Fig. 4b), 24 h pre-incubation with IN fraction from *R. brigantinus* could reduce 20% of ROS levels.

GSH and GSSG quantification

GSH and GSSG were measured after pre-incubation with digested metabolites and after application of H_2O_2 stress (Fig. 5). The commercial blackberry fraction caused a decrease in GSH content (Fig. 5a), contrarily to *R. brigantinus* and *R. vagabundus* fractions that augmented GSH with *R. vagabundus* being more effective (Fig. 5a). H_2O_2 insult (300 μM for 24 h) induced a GSH depletion (Fig. 5a). Pretreatment with commercial blackberry metabolites could not maintain GSH levels under H_2O_2 insult. Conversely, pre-incubation with the IN fractions from the wild blackberries prevented GSH depletion, maintaining GSH levels at around control levels. However, analysis of the GSH/GSSG did not highlight any significant alteration due to H_2O_2 stress or pre-incubation with digested metabolites (Fig. 5b).

Caspase 3/7 activity determination

Pre-incubation with IN metabolites in the absence of H_2O_2 injury enhanced caspase activity up to twofold (Fig. 6a). When cells were challenged with 300 μM H_2O_2, caspase activation was augmented by around fourfold compared to the control (Fig. 6b). Indeed, the same augmentation in caspase activity was verified when cells were pre-incubated with any of the blackberry IN samples and submitted to stress. Curiously, lower levels of endemic metabolites

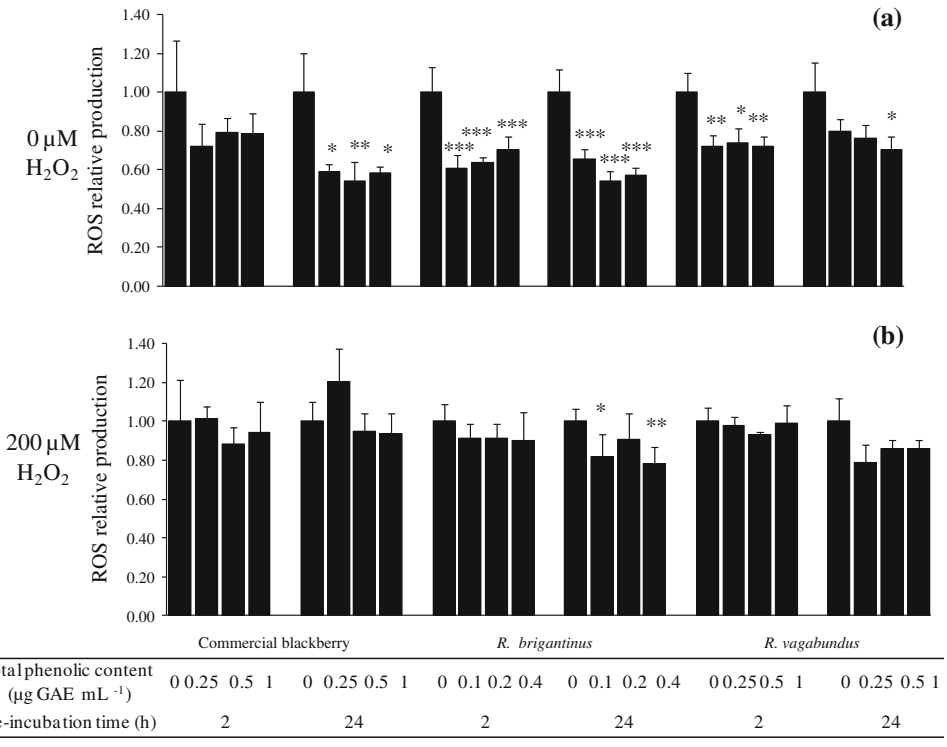


Fig. 4 Relative intracellular ROS production by SK-N-MC neuroblastoma cells pre-incubated with digested fractions for 2 or 24 h. **a** In absence of an oxidative stress **b** In the presence of an oxidative stress (200 μM H_2O_2 for 1 h). ROS were detected by fluorimetry using H_2DCFDA as probe. Statistical differences in relation to cells not treated with blackberries metabolites (0 μg GAE mL^{-1}) are denote as $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. All values are mean ± SD, $n = 3$

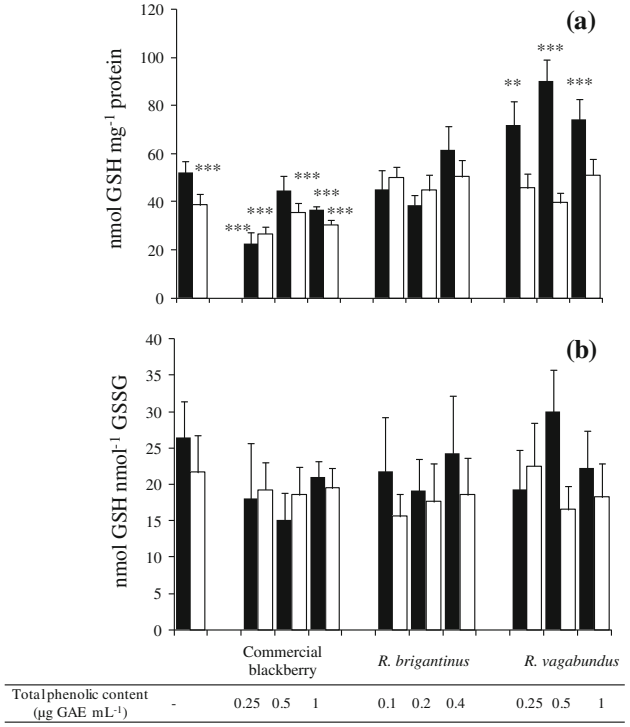


Fig. 5 Quantification of GSH and ratio GSH/GSSG. SK-N-MC neuroblastoma cells pretreated with digested blackberries extract for 24 h, in the absence of stress (*black bar*) and in cells subjected to stress (300 μM H_2O_2 for 24 h) (*open bar*) **a** GSH; **b** ratio GSH/GSSG. Cells were harvested and analyzed for their content in GSH and GSSG by HPLC. Statistical differences between treatments in relation to control were determined for $p < 0.01$ and are denoted as $^{**}p < 0.01$, $^{***}p < 0.001$. All values are mean ± SD, $n >= 3$

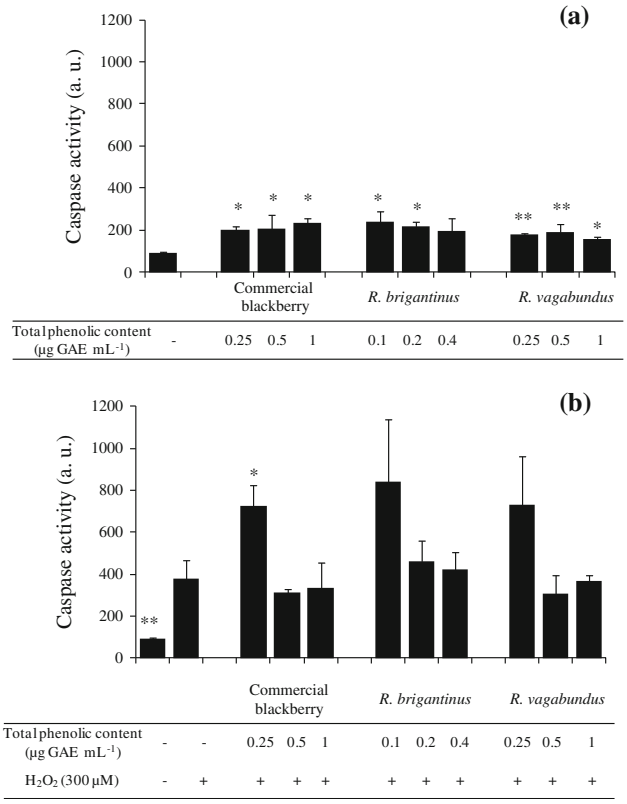


Fig. 6 Caspase 3/7 relative activity in SK-N-MC cells pretreated with digested fractions for 24 h. **a** In the absence of H_2O_2 injury. **b** In the presence of H_2O_2 (300 μM, 24 h) injury. Values of caspase 3/7 activity were normalized by cell viability determined by flow cytometry. Statistical differences are denote as $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. All values are mean ± SD, $n = 3$

(0.1–0.25 μg GAE mL^{-1}) strongly promote caspase activation.

Discussion

Wild species represent a rich source of phytochemicals that can be explored with nutritional, nutraceutical or pharmaceutical purposes. *R. brigantinus* and *R. vagabundus* are two *Rubus* sp. endemic from the North of Iberia Peninsula with a very restrict occurrence [18]. These two species have, until now, not been chemically characterized. The occurrence of different (quantities of) phenolic compounds could promote different bioactivities, thereby identifying this germplasm as sources of compounds to be further explored and exploited. Blackberries are berries with a recognized high phenolic content [27]. However, although the TPC of the three blackberry extracts was very similar, the wild blackberries exhibited higher ACs compared to the commercial variety, especially *R. brigantinus* (Table 1). LC–MS analysis of blackberry extracts before IVD showed some quantitative differences among species concerning anthocyanins, ellagitannins, flavonols and ellagic acid (Fig. 1a, Fig. S1 on Supplementary material). However, the physical–chemical alterations during gastrointestinal digestion have a major impact on the quantity and diversity of the phenolic compounds present in fruits [20]. After IVD, the TPC of the IN fractions was reduced to less than 10% of the original content (Table 1). These values are in accordance with recoveries obtained in previous work on red wine and red cabbage [28, 29]. The AC was also greatly reduced (Table 1). Nevertheless, the IN fractions presented relatively higher AC/TPC ratio than the undigested extracts (Table 1). IN fractions from *R. brigantinus* were the most potent followed by the commercial blackberry and then by *R. vagabundus*. These differences, reflecting the different chemistries, could be reflected in different biological potencies and/or effects. After IVD, the amounts of anthocyanins and ellagitannins detected in the IN fractions were considerably reduced (Fig. 1b). Total anthocyanin content was reduced to 12.8% in commercial blackberry and to ~5% in the endemic species; these recoveries are in agreement with other work on red cabbage, red wine and pomegranate juice [28–30]. Anthocyanins are stable in

acidic conditions of the stomach, but less stable at elevated pH of the small intestine [28, 30, 31]. Also ellagitannins, a characteristic group of metabolites in the Rosaceae, were extensively reduced in all species. A proportion of the "missing ellagitannins" was recovered in the OUT sample having been unable to pass through the dialysis tubing presumably as a result of their larger size or binding to proteins and/or bile acids [32]. The increased levels of ellagic acid in the IN fractions is indicative of ellagitannin breakdown. After IVD, quercetin derivatives were present in higher levels in extracts from the wild species than those from the commercial blackberry. These higher recoveries reflect the greater stability of flavonols to IVD [29] as other components were degraded during IVD but also reflect higher levels in the undigested extracts. The most obvious difference between the *R. brigantinus*, *R. vagabundus* and the blackberry IN samples was the presence of a component with properties indicating a methyl ellagic glucuronide structure. Methyl ellagic glycosides have been identified in blackberry and raspberry [33, 34], and methyl ellagic glucuronides have been identified in other plants [35]. However, further work is required to confirm this putative identification. Although this component appeared greatly enriched in the IN samples after digestion compared to the commercial blackberry IN sample, it still only comprised a small proportion of the total phenolic content. The IN samples contained many peaks, presumably breakdown products, that could not be identified, and these metabolites may also have important biological properties.

Based on the cytotoxicity profiles, ranges of non-toxic concentrations of IN fractions were selected as 0–1 μg mL^{-1} GAE for commercial blackberry and *R. vagabundus* and 0–0.4 μg mL^{-1} GAE for *R. brigantinus*. These ranges correspond to 0–6 μM GAE and 0–2.4 μM GAE, respectively. These levels are near to the physiological concentrations of certain phenolic components noted in human plasma (0–4 μM) [36]. Then, these non-toxic physiological concentrations were tested on a neurodegeneration cell model.

SK-N-MC neuroblastoma cells were pre-incubated for 24 h in the presence or absence of IN fractions and then challenged with H_2O_2. Neuroprotection was then evaluated by monitoring cells that maintained both high $\Delta\Psi m$ and cell membrane integrity (Q4 positive cells; Fig. 3b). Unlike the commercial blackberry variety, the wild species were able to recover both parameters, ensuring cell viability and functionality (high cell membrane integrity and high $\Delta\Psi m$). Therefore, *R. brigantinus* and *R. vagabundus,* but not the commercial blackberry, extracts contain phytochemicals with a potential to be explored in a neuroprotective perspective. However, the mechanism(s) by which those metabolites are exerting a beneficial effect are unknown. In order to unravel possible mechanisms behind the neuroprotection detected, some cellular redox/death mechanisms were tested.

Intracellular ROS levels were monitored (Fig. 4) at two different times of pre-incubation, 2 and 24 h, to cover different time events. Within 2 h, direct scavenging effects from metabolites can be determined, and at 24 h, indirect scavenging resulting from activation/modulation of other molecular mechanisms can be assessed. Diverse phytochemicals can act directly through scavenging properties or indirectly through modulation of thiol-containing molecules (such as glutathione), ROS-inactivating enzymes (e.g., superoxide dismutase, catalase) or phase II detoxifying enzymes (e. g. glutathione S-transferases, heme oxygenase-1) [37]. All blackberry extracts at 2 and/or 24 h pre-incubation (Fig. 4a) reduced basal ROS production. This effect suggests a possible preventive role in oxidative stress-associated diseases, such as neurodegeneration. These results agree with previous work on various phenolic components (e.g., naringin, nobiletin, luteolin, quercetin, gingerol) [38–40]. Following the application of a stress of 200 μM H_2O_2 for 1 h (Fig. 4b), *R. brigantinus* was the only species that maintained reduced ROS levels after 24 h pre-incubation. The different composition of *R. brigantinus* digested metabolites could activate indirect antioxidant systems more effectively than the other species. Digested metabolites from commercial blackberry, as already described [20], and from *R. vagabundus* lost part of their intracellular antioxidant capacity especially against H_2O_2-induced stress.

GSH and GSSG were determined after pre-incubation with blackberry fractions and after application of H_2O_2 stress (Fig. 5). Different IN fractions caused different responses. The commercial blackberry fraction caused a GSH depletion (Fig. 5a), while, conversely, *R. brigantinus* and *R. vagabundus* fractions augmented GSH levels with *R. vagabundus* being more significant (Fig. 5a). Intracellular GSH depletion is an early hallmark in the progression of cell death in response to different apoptotic stimuli [41]. H_2O_2 insult induced a GSH depletion (Fig. 5a). Pretreatment with commercial blackberry metabolites could not maintain GSH levels under H_2O_2 insult, which corresponds to the lack of neuroprotection by commercial blackberry digests (Fig. 3b). However, no significant changes were detected in GSH/GSSG ratio, induced by H_2O_2 stress or IN fraction pre-incubation (Fig. 5b). This redox homeostasis is due to changes in GSH that are accompanied by changes in GSSG. Pre-incubation with the IN fractions from the wild blackberries, although not changing GSH/GSSG ratio, increased GSH levels (Fig. 5a), which could occur via de novo synthesis of GSH by γ-glutamylcysteine ligase. This increase should contribute to the cellular protection, since after imposition of oxidative stress, levels of GSH were

maintained around control levels (Fig. 5b) and cell death was prevented (Fig. 3c). The decrease in GSH and GSSG levels after pre-incubation with wild blackberries fractions followed by the oxidative stress could be due to their mobilization by other molecules, such as proteins. This mechanism-denominated S-glutathionylation is an important post-translational modification that provides protection of proteins against irreversible modifications and protein damage in response to higher levels of oxidative stress [42].

Acute and chronic neurodegenerative conditions have been associated with both apoptotic and necrotic cell death, and caspases can play a role in both mechanisms [43]. A range of pure phenolics have been shown to cause caspase inhibition and reduce cell death [38, 39, 44]. However, pre-incubation with IN metabolites in the absence of H_2O_2 injury enhanced caspase activity up to twofold (Fig. 6a) without reducing cell viability (Fig. 3a). When cells were challenged with H_2O_2, caspase activation was augmented by comparison with the control (Fig. 6b). The same augment in caspase activity was verified when cells were pre-incubated with any of the blackberry IN samples and submitted to stress. However, for the fractions from wild blackberries, this caspase activation was not accompanied by a decrease in cell viability, in contrast to cells only challenged with H_2O_2 (Fig. 3c). This protective effect was not verified for the commercial blackberry fraction. Again, differences in polyphenol composition must be responsible for these observed differences. Curiously, although lower levels of endemic metabolites (0.1–0.25 μg GAE mL^{-1}) strongly promote caspase activation, they did not diminish cell death in stressed cells (Fig. 3c). Results suggest that the existing mechanism of death in the presence of metabolites is not caspase-dependent. Different levels of caspases-3 and caspases-7 activation by metabolites pre-incubation (Fig. 6a) did not lead to different levels of death (Fig. 3a). Also, similar caspases-3 and caspases-7 activation by metabolites (Fig. 6b) led to reduced levels of death after oxidative injury (Fig. 3c). Exposure to components in the digests from wild blackberries could be an initiating event that leads to protection against subsequent, potentially lethal stimuli. This mechanism, also known as preconditioning or hormetic effect, has been reported as an effective neuroprotective mechanism, where activation of caspase-3 could be involved [45–47]. In those cases where caspase-3 is activated, arrest of cell death can be triggered by downstream mechanisms like defense against apoptosome assembly and cleaved caspases. This protection can be mediated by factors such as calbindin, inhibitors of apoptosis (IAPs), Bcl-2 family and heat-shock proteins (HSPs), induced by sublethal insults [46, 47].

The evaluation of (in vitro) digested metabolites, as opposed to the common approach employing undigested/pure compounds, revealed a differential metabolic response indicating that considering dietary polyphenols metabolism is crucial to effectively assess and determine physiological bioefficacy [20]. In fact, the metabolism of these compounds is quite complex. Polyphenols can be absorbed in the stomach and small intestine, by diffusion or transport [36]. Once absorbed, they are biotransformed through phase II enzymatic conjugation in both the small intestine and liver. Flavonoids not absorbed in the upper gastrointestinal tract reach the colon and are subjected to colonic microflora action and their catabolites absorbed into the circulatory system.

Once we aim to study digestion-derived metabolites in neurodegenerative disorders, we should also take into account that circulating metabolites must cross and/or interact with blood–brain barrier. There are numerous studies reporting polyphenol-mediated neuroprotection. However, there is paucity of information regarding the interaction of polyphenols metabolites with the brain endothelial cells forming the blood–brain barrier, which has complicated identification of polyphenols compounds entering the central nervous system. Among polyphenols found in berries, there are some reports indicating the presence of anthocyanins and flavonols in brain tissues [48–52].

In summary *R. brigantinus* and *R. vagabundus* were revealed to be promising sources of metabolites with neuroprotection capabilities. Digested metabolites from these blackberries, at levels that could be found in human plasma, activated adaptative cellular stress response pathways such as caspase activation, GSH modulation and also ROS diminishment. These effects protected neuronal cells against oxidative injury, one of the most important features of neurodegeneration. This is the first report highlighting a neuroprotective effect by a digested food matrix involving activation of caspases, suggesting a preconditioning effect. It has been already described for some isolated phytochemicals that beneficial effects could be achieved through neurohormesis pathways, such as stimulating the vitagene system, the production of antioxidant enzymes, protein chaperons and other proteins that help cells to withstand stress [53]. Therefore, these molecular targets should be a goal for future studies into the neuroprotection mediated by digested metabolites from wild blackberries.

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