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Merlot grape pomace hydroalcoholic extract improves the oxidative and inflammatory states of rats with adjuvant-induced arthritis

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ABSTRACT

Grape pomace is an agro-industrial residue produced worldwide. The purpose of this study was to identify and quantify the main phenolics present in an hydroalcohlic extract of a Merlot grape pomace and to investigate its effect on the oxidative and inflammatory states of adjuvant-induced arthritic rats. Daily doses of 250 mg of the extract per kg body weight were administered during 23 days. Several oxidative stress indicators in arthritic rats were maintained at their normal or closely normal levels in the plasma, liver and brain by the treatment. Additionally, the grape pomace also showed significant anti-inflammatory effects. From the 25 phenolics identified in the grape pomace extract the most abundant ones were catechin and catechin derivatives, which are possibly the most important antioxidant agents. The results suggest a potential applicability of the Merlot grape pomace hydroalcoholic extract in the improvement of the oxidative and inflammatory states in arthritic patients.

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1. Introduction

Rheumatoid arthritis (RA) is an autoimmune disease characterized by chronic inflammation of the joints (the synovial membrane) and subsequent destruction of cartilage and bone. The disease involves well known immunological events, such as participation of T cells, B cells, macrophages and pro-inflammatory cytokines (Stolina et al., 2009). The progression of the disease is also accompanied by an increased oxidative stress in the lesion sites (Seven, Güzel, Aslan, & Hamuryudan, 2008). Furthermore, as a multisystem disease, rheumatoid arthritis also affects organs such as liver, heart, brain and vascular tissue. The liver of rats with adjuvant-induced arthritis, for example, presents increased rates of oxygen uptake, inhibition of gluconeogenesis and stimulation of glycolysis, alterations in the urea cycle and modifications in calcium homeostasis (Fedatto-Jr et al., 1999, 2000; Utsunomiya, Scaliante, Bracht, & Ishii-Iwamoto, 2013; Yassuda-Filho et al., 2003). Substantial oxidative stress and molecular damage has also been demonstrated to occur in the plasma, liver, brain and heart of both arthritic patients (Mateen, Moin, Khan, Zafar, & Fatima, 2016) and experimental animals (Bracht et al., 2016; Comar et al., 2013; Schubert et al., 2016; Wendt et al., 2015).

There has been a systematic search for natural products able to attenuate the deleterious effects of arthritis and other inflammatory diseases. With this purpose several preparations rich in antioxidant flavonoids or purified flavonoids have been investigated (Somasundaram & Oommen, 2013). *Clereodendrum inerme*, for example, exerts anti-inflammatory actions that have been attributed mainly to its flavonoid constituents (Khanam et al., 2014; Somasundaram & Oommen, 2013). *Tamarindus indica* seed extracts, rich in procyanidins, show protective efficacy against arthritis-associated oxidative damage (Sundaram et al., 2014). Another example is green tea, which has been shown to inhibit the inflammatory responses and the development of arthritis in animal model studies (Singh, Akhtar, & Haqqui, 2010) and also to improve in a significant way the oxidative state of arthritic rats

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(Gonçalves et al., 2015). These actions have been attributed to the catechins (polyphenolics), which are important constituents of green-tea.

Within the perspective of an implemented use of flavonoids in food supplements, it could be of interest to use abundant and relatively inexpensive sources of these compounds. One such source could be the most important by-product of the wine industry, namely, grape pomace (Fontana, Antoniolli, & Bottini, 2013; Hogan, Canning, Sun, Sun, & Zhou, 2010; Ky & Teissedre, 2015; Ribeiro et al., 2015; Souad, Faten, Emad, Emad, & Doha, 2014). The latter consists mainly of skins, but also of seeds, pedicels and peduncles and the pulp remaining after pressing the grapes. Grape pomace is an agro-industrial residue produced worldwide and already employed for animal feed or as a fertilizer. Grapes contain many different phenolic compounds distributed in pulp, seeds and skin. These compounds are the main determinants of colour, taste, mouth feel and oxidation (Shi, Yu, Pohorly, & Kakuda, 2003). During the winemaking process, a fraction of the phenolic compounds is transferred to the wine, but a significant proportion still remains in the residues, especially in the grape pomace (Iora et al., 2015; Ribeiro et al., 2015; Shi et al., 2003). It is, thus, of interest to characterize and quantify the phenolics and biological effects of grape pomaces within the perspective of their future use in formulations of medicinal foods.

The presence of polyphenolics strongly suggests that grape pomace could be effective against oxidative stress that accompanies arthritis in a way similar to that observed for green tea extracts (Gonçalves et al., 2015). It is also of interest to know if the preparation exerts anti-inflammatory effects. For this reason, we decided to investigate the action of a hydroalcoholic extract of a grape pomace of the Merlot variety on several parameters in rats with adjuvant-induced arthritis. The latter is an experimental immunopathology that presents similarities to human rheumatoid arthritis: synovial hyperplasia, systemic inflammation, cachexia, and high levels of plasma pro-inflammatory cytokines and lesion sites (Bendele et al., 1999; Szekanecz et al., 2000). In order to support our conclusions in molecular terms, an exhaustive screening of the Merlot grape pomace for its content in flavonoids and related compounds was also done. The results obtained in the latter procedures should be helpful in identifying the most probable active principles. At least to our knowledge, this is the first study analyzing in detail effects of a grape pomace extract under *in vivo* conditions.

2. Material and methods

2.1. Chemicals

Acetonitrile (99.9%) was of HPLC grade from Fisher Scientific (Lisbon, Portugal). Phenolic standards were from Extrasynthèse (Genay, France). Ethanol and all other chemicals were of analytical grade and purchased from common sources. Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA). Dinitrophenylhydrazine,2′7′-dichloro-fluores cein diacetate, oxidized dichloro-fluorescein, 1,1′,3,3′-tetrae thoxy-propane, horse-radish peroxidase, *o*-phthal-aldehyde, reduced glutathione (GSH), oxidized glutathione (GSSG), glutathione reductase, 2,2-azinobis 3-ethyl-benzothiazoline-6-sulfo nate (ABTS), Freund's adjuvant, 5,5-dithiobis 2-nitrobenzoic acid and formic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were of analytical grade.

2.2. Preparation of the Merlot grape pomace extract

Samples of grape pomace from *Vitis vinifera* of the Merlot variety, were obtained from companies located in the Paraná State, Brazil. The samples were by-products of the winemaking process, obtained after the grapes had been pressed. Once in the laboratory, the Merlot grape pomace sample was dried in a convection oven (Marconi MA 035, Brazil) at 80 °C for 36 h. The dried residues were milled, stored in polyethylene film bags packed under vacuum and stored at −20 °C until use (Ribeiro et al., 2015).

The Merlot grape pomace extracts were prepared using a mixture of 40% ethanol and 60% distilled water (Ribeiro et al., 2015). Extraction was done with a solute/solvent ratio of 1:50 (m/v). The suspensions were shaken in a rotary shaker for 24 h at 100 rpm and at a temperature of 25 °C. After this period of time the mixture was centrifuged at 3500*g* for 25 min. Ethanol was eliminated with a rotary evaporator and the aqueous solution was lyophilized and stored in a freezer until use (Haminiuk, Plata-Oviedo, Mattos, Carpes, & Branco, 2014; Ribeiro et al., 2015). One gram dried and milled grape pomace resulted in 130 mg lyophilized material (13%).

2.3. Animals and treatment

Male Holtzman rats were fed *ad libitum* with a standard laboratory diet (Nuvilab, Colombo, Brazil) and maintained on a regulated light dark cycle. For the induction of arthritis, animals weighing 180–210 g were injected in the left hind paw with 0.1 mL of Freund's complete adjuvant (heat-inactivated *Mycobacterium tuberculosis*, derived from the human strain H37Rv), suspended in mineral oil at a concentration of 0.5% (w/v) (Pearson & Wood, 1963). Rats of similar weights were injected with mineral oil and served as controls. All experiments of adjuvant arthritis induction were done in accordance with the world-wide accepted ethical guidelines for animal experimentation and previously approved by the Ethics Committee for Animal Experimentation of the University of Maringá (Protocol n° 8119260115-CEUA-UEM).

For evaluating the effects of the grape pomace extract on the oxidative status of arthritic rats, the animals were divided into 4 groups (6 rats each): (1) group I were healthy animals (controls); (2) group II were healthy animals treated with grape pomace extract (treated controls); (3) group III were the arthritic rats;(4) group IV comprised the arthritic rats treated with the grape pomace extract (treated arthritic rats). Treatment of the arthritic rats consisted in the administration of the grape pomace extract during 5 days prior to the induction of arthritis and during additional 18 days after initiating the induction. The daily doses were 250 mg extract per kg body weight. This dose was established based on previous experiments with green tea extracts (Gonçalves et al., 2015). The treated controls received the same doses during 23 days. Non treated animals, healthy or arthritic, received distilled water for 23 days. Fig. 1A in Appendix A offers a schematic view of the main experimental design with emphasis on the induction of arthritis, grape pomace treatment and evaluations. The numbers in Fig. 1A correspond to the four experimental groups mentioned above.

For evaluating the anti-inflammatory activity of the grape pomace extracts, rats were divided into 4 groups (6 animals each): (1) healthy animals (controls); (2) arthritic rats; (3) arthritic rats treated with ibuprofen; and (4) arthriitic rats treated with the grape pomace extract. Treatment of the arthritic rats consisted in the administration of ibuprofen or grape pomace extract during 5 days prior to the induction of arthritis and during additional 18 days after initiating the induction. The daily doses were 35 mg/kg for ibuprofen treatment and 250 mg extract/kg for the grape pomace treatment. Non-treated animals, healthy or arthritic, received distilled water for 23 days.

2.4. Preparation of liver and brain homogenates

After 18 h of fasting, the rats were decapitated, the brain and the liver were immediately removed, freeze-clamped, and stored in

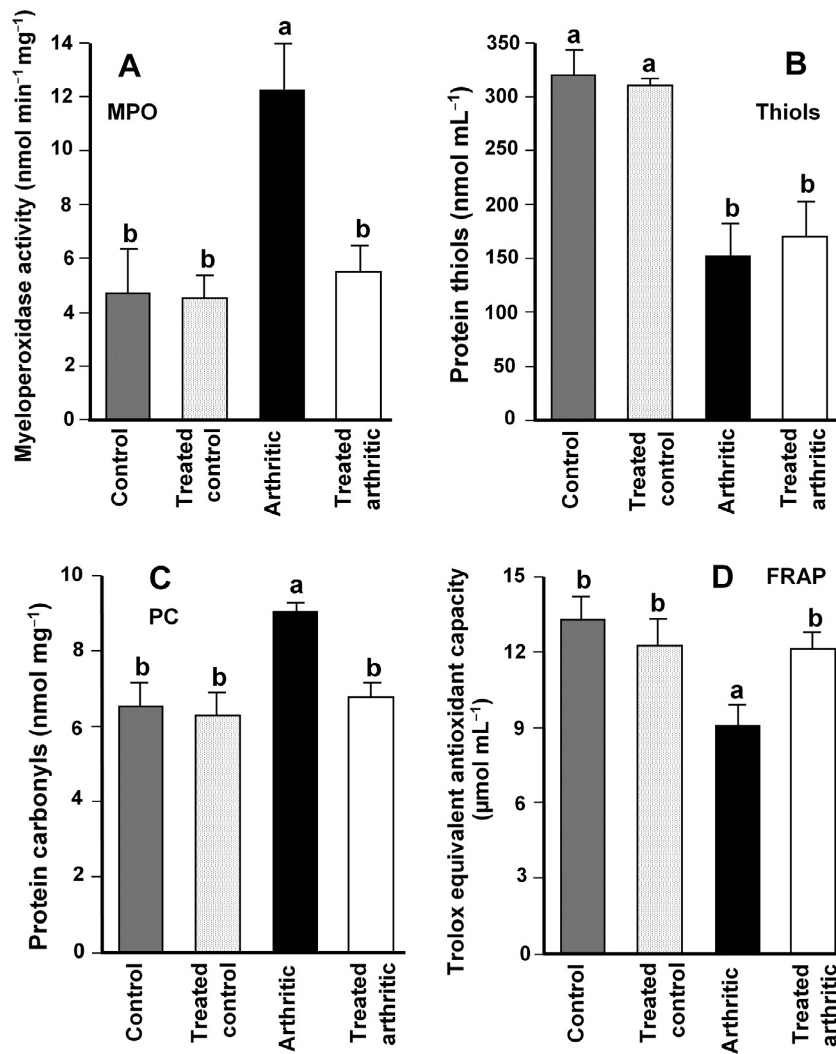

Fig. 1. Treatment of normal and arthritic rats with the grape pomace extract: effects on plasmatic oxidative stress indicators. The plasma samples were obtained and processed as described in Section 2. Values represent the mean ± standard error of the mean of 6 animals for each experimental condition. Columns labeled with the same symbol on each graph do not differ statistically from each other ($p \leq 0.05$) according to one-way ANOVA followed by Student-Newman-Keuls post hoc testing. Legends: MPO, myelo-peroxidase activity; PC, protein carbonyls; FRAP, ferric ion antioxidant reducing power.

liquid nitrogen. The tissues were then homogenized separately in a van Potter homogenizer with 10 volumes of ice-cold 0.1 M potassium phosphate buffer (pH 7.4) and aliquots were separated for use as total homogenates. The remaining homogenates were centrifuged at 11,000*g* during 15 min and the supernatants separated as the soluble fractions of the homogenates (Comar et al., 2013; Gonçalves et al., 2015; Wendt et al., 2015). Protein concentration was measured with the Folin-Ciocalteu reagent, using bovine serum albumin as a standard (Lowry, Rosebrough, Farr, & Randall, 1951).

2.5. Blood collection and plasma preparation

Rats fasted for 18 h were anesthetized with intraperitoneal injection of sodium pentobarbital (50 mg/kg) and the blood was collected by puncturing the inferior vena cava after laparotomy and transferring it into tubes containing anticoagulant. The samples were centrifuged at 1000*g* for 10 min to separate the plasma fraction (Bracht et al., 2016).

2.6. Reactive oxygen species and protein carbonyls

Reactive oxygen species were assessed in the homogenate supernatants. The total ROS content was quantified via the 2′,7′-dichlorofluorescein-diacetate (DCFH-DA) assay (Siqueira, Fochesatto, da Silva-Torres, Dalmaz, & Alexandre-Netto, 2005). The formation of DCF was measured immediately after stopping the reaction on ice with a spectrofluorimeter RF-5301 (Shimadzu) in which the excitation and emission wavelengths were set at 504 and 529 nm, respectively. A standard curve with oxidized dichlorofluorescein (DCF) was used to express the results as nmol per mg of protein.

The contents in protein carbonyl groups were measured spectrophotometrically in the liver and brain homogenates and in the plasma using 2,4-dinitrophenylhydrazine (DNPH) ($\varepsilon_{370} = 22 \times 10^3\ M^{-1}\ cm^{-1}$) and the values were expressed as nmol per mg protein (Levine et al., 1990).

2.7. Oxidative state indicators and antioxidant capacity

Reduced (GSH) and oxidized glutathione (GSSG) were measured in the total homogenate. The GSH and GSSG contents were measured spectrofluorimetrically (excitation 350 nm and emission 420 nm) by means of the *o*-phytalaldehyde (OPT) assay as described previously (Hissin & Hilf, 1976). The fluorescence was estimated as GSH. For the GSSG assay, the sample was previously

incubated with 10 mM N-ethylmaleimide (NEM) and subsequently with a mixture containing 1 M NaOH and 0.4 μM phthalaldehyde to detect the fluorescence. The results were calculated using a standard curve prepared with GSH or GSSG and the values were expressed as nmol per mg protein.

The reduced protein thiol groups in the plasma were determined using the reaction with 5,5′-dithiobis 2-nitrobenzoic acid (DTNB) (Sedlak & Lindsay, 1968). The reaction product was measured spectrophotometrically at 412 nm and the molar extinction coefficient of $1.36 \times 10^4\ M^{-1}\ cm^{-1}$ was used to express the results as nmol per mg protein.

The antioxidant capacity of the plasma was estimated as the ferric ion antioxidant reducing power (FRAP) (Benzie & Strain, 1996). The FRAP reagent contained (final concentrations): 0.83 mM 2,4,6[2-pyridyl]-s-triazine (TPTZ), 1.67 mM $FeCl_3{\cdot}6H_2O$ and 250 mM acetate buffer at pH 3.6. Plasma was added for a final dilution of 34-fold. The temperature of the assay was 37 °C and the reaction was monitored for up to 30 min at 595 nm. The standard curve was prepared with Trolox (6-hydroxy-2,5,7,8-tetramethyl chroman-2-carboxylic acid) and the activity was expressed as μmol Trolox equivalents per mL plasma.

2.8. Enzyme assays

Antioxidant enzymatic activities were assessed in the homogenate supernatant. The catalase (CAT) activity was estimated by measuring changes in absorbance at 240 nm using H_2O_2 as substrate and expressed as μmol min^{-1} (mg protein)$^{-1}$ (Aebi, 1974). The superoxide dismutase (SOD) activity was estimated by its capacity of inhibiting the pyrogallol autoxidation in alkaline medium. The latter was measured at 420 nm (Marklund & Marklund, 1974). One SOD unit was considered the quantity of enzyme that was able to promote 50% inhibition and the results were expressed as units per mg protein.

The myeloperoxidase activity (MPO) was determined by the *o*-dianisidine dihydrochloride-H_2O_2 method (Bradley, Priebat, Christensen, & Rothstein, 1982). The plasma samples (30 μL) were added to 900 μL of the reaction mixture containing 50 mM phosphate buffer pH 6.0, 0.167 mg/mL *o*-dianisidine-dihydrochloride and 0.0005% hydrogen peroxide. The change in absorbance at 460 nm was measured for 1 min. One unit of MPO activity was defined as the amount of enzyme degrading one micromole of peroxide per minute at 25 °C.

2.9. Determination of the number of leukocytes recruited in the femoro-tibial joint cavity

The determination of the number of leukocytes recruited in the femoro-tibial joint cavity of the legs (injected and not injected) of arthritic rats was done on day 18 after induction of arthritis. The rats were euthanized and the femoro-tibial joints were exposed by surgical sectioning of the patellar tendon. Next, the joint cavity was washed with 40 μL of a buffered saline solution containing EDTA. May-Grünwald-Giemsa staining was used to differentiate and count polymorpho-nuclear and mononuclear leukocytes.

2.10. Paw edema

The inflammatory response induced by Freund's complete adjuvant was evaluated in the hind paws of arthritic rats on days 0, 1, 3, 6, 9, 13, 15, 17 and 18. The volume of the injected and contralateral paw was assessed with a plethysmograph (Ugo Basile®), according to the technique described by Winder, Max, and Been (1957). The result was expressed in terms of the increase in paw volume relative to the initial volume.

2.11. Arthritic lesions score

The evaluation of the arthritic lesion scores (in the ears, front legs and tail) was started on the 10th day and continued until the end of the experiment on day 18. The lesions were evaluated through the numerical grading system proposed by Rosenthale (1970), in which points are attributed for each of the following events: (+1) for nodules on the tail; (+1 or +2) for nodules appearing in one or both ears and (+1 or +2) for swelling of one or both forelimbs. The severity of the injury was graded from zero to 5, zero indicating no injury.

2.12. Phenolic profile of the Merlot grape pomace extract

The phenolic profile was determined using the lyophilized extracts, re-dissolved in an ethanol:water (40:60, v/v) mixture, by LC-DAD-ESI/MSn (Dionex Ultimate 3000 UPLC, Hermo Scientific, San Jose, CA, USA).

2.12.1. Non-anthocyanin compounds

These compounds were separated and identified as previously described by Bessada, Barreira, Barros, Ferreira, and Oliveira (2016). Double online detection was performed using a DAD (280 and 370 nm used as preferred wavelengths) and a mass spectrometer (MS) connected to the HPLC system via the DAD cell outlet. The MS detection was performed in negative mode, using a Linear Ion Trap LTQ XL mass spectrometer (Thermo Finnigan, San Jose, CA, USA) equipped with an ESI source.

2.12.2. Anthocyanin compounds

Chromatographic separation was achieved with a Waters Spherisorb S3 ODS-2 C18 (3 μm, 4.6 mm × 150 mm, Waters, Milford, MA, USA) column working at 35 °C. The solvents used were: (A) 0.1% trifluoroacetic acid (TFA) in water, (B) acetonitrile. The gradient elution followed these parameters: 10% B for 3 min, from 10 to 15% B for 12 min, 15% B for 5 min, from 15 to 18% B for 5 min, from 18 to 30% B for 20 min, from 30 to 35% B for 5 min, and from 35 to 10% B for 10 min. The resulting total run time was 60 min, followed by column reconditioning of 10 min, using a flow rate of 0.5 mL/min. Double online detection was carried out in the DAD using 520 nm as the preferred wavelength and in a mass spectrometer (MS) connected to HPLC system via the DAD cell outlet. MS detection was performed in positive mode, using a Linear Ion Trap LTQ XL mass spectrometer (Thermo Finnigan, San Jose, CA, USA) equipped with an ESI source. Nitrogen served as the sheath gas (50 psi); the system was operated with a spray voltage of 4.8 kV, a source temperature of 320 °C, a capillary voltage of 14 V. The tube lens offset was kept at a voltage of 75 V. The full scan covered the mass range from m/z 100 to 1500. The collision energy used was 20 (arbitrary units). Data acquisition was carried out with Xcalibur® data system (Thermo Finnigan, San Jose, CA, USA).

2.12.3. Identification and quantification

The identification of the phenolic compounds (non-anthocyanin and anthocyanin compounds) was performed using standard compounds, when available, by comparing their retention times, UV–vis and mass spectra; and also, comparing the obtained information with available data reported in the literature giving a tentative identification. For quantitative analysis, a calibration curve for each available phenolic standard was constructed based on the UV signal. For the identified phenolic compounds for which a commercial standard was not available, the quantification was performed through the calibration curve of the most similar available standard. Compounds 1, 3 and 8 were quantified using gallic acid ($y = 208{,}604x + 173{,}056$; $R^2 = 0.999$), compounds 2, 4–7 and 9–12 were quantified using (+)-catechin ($y = 84{,}950x - 23{,}200$;

R^2 = 0.999), compound 13 with myricetin (y = 23,287x – 581,708; R^2 = 0.999), compounds 14–20 with quercetin-3-O-glucoside (y = 34,843x – 160,173; R^2 = 0.999) and compounds 21–25 with peonidin-3-O-glucoside (y = 122,417x – 447,974; R^2 = 0.999). The results were expressed as mg/g of extract.

2.13. Statistical analysis

The error parameters presented in graphs and tables are standard errors of the means. Statistical analysis was done by means of the GraphPad Prism Software (version 5.0). Evaluation of the statistical significance was done by means of the variance analysis (ANOVA) followed by post hoc Student-Newman-Keuls testing. The 5% level ($p < 0.05$) was adopted as the significance criterion.

3. Results

3.1. Effects on the oxidative state of arthritic rats

3.1.1. Plasma

Four parameters were measured in the plasma of control and arthritic rats: the myeloperoxidase activity (MPO), the protein thiol levels, the protein carbonyl levels (PC) and the antioxidant capacity (FRAP). The results are shown in Fig. 1. The myeloperoxidase activity (panel A), which can be regarded as an indicator for both the oxidative and inflammatory states (Kothari et al., 2011), was not modified when control (healthy) rats were treated with the grape pomace extract. Arthritis, on the other hand, more than doubled the myeloperoxidase activity (2.5-fold), but the grape pomace treatment totally prevented this increase. The protein thiol groups were 59% diminished by arthritis (panel B). In this case the grape pomace treatment was without significant effects on both healthy and arthritic rats. The protein carbonyls content (panel C), an indicative of protein damage, was increased by arthritis (41%). Treatment with the grape pomace did not affect this parameter in healthy rats, but it prevented in full the protein damage in arthritic rats. The evaluation of the antioxidant capacity in the plasma, estimated as the ferric ion antioxidant reducing power, is shown in panel D of Fig. 1. Grape pomace treatment did not increase the antioxidant capacity of healthy rats. Arthritis, on the other hand, clearly impaired the antioxidant capacity by 36%. This impairment, however, was prevented by the grape pomace treatment.

3.1.2. Liver and brain

Protein damage in both liver and brain were estimated as the tissue content of protein carbonyl groups. The results are shown in panels A and B of Fig. 2 (PC). Corroborating previous results, arthritis increased protein damage in both tissues, 55% in the liver

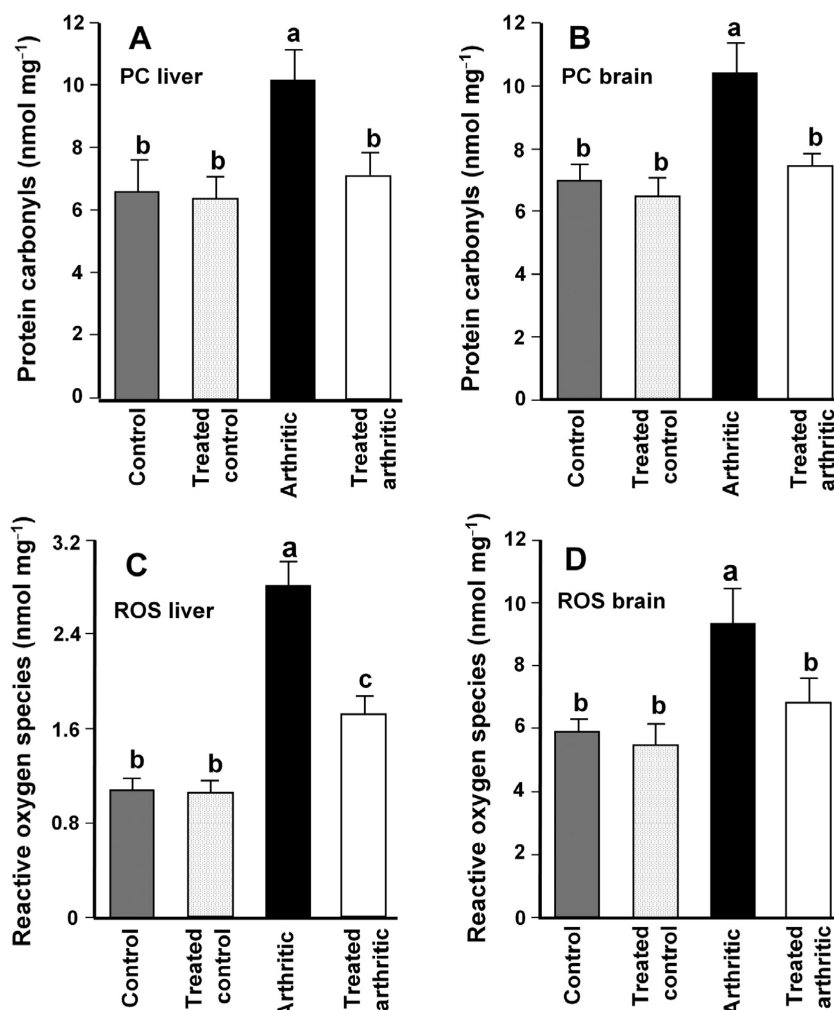

Fig. 2. Treatment of normal and arthritic rats with the grape pomace extract: effects on liver and brain oxidative stress indicators. Freeze-clamped tissues were homogenized for the assays, as described in Section 2. Values represent the mean ± standard error of the mean from 4 to 6 animals for each experimental condition. Columns labeled with the same symbol on each graph do not differ statistically from each other ($p < 0.05$) according to one-way ANOVA followed by Student-Newman-Keuls post hoc testing. Legends: PC, protein carbonyls; ROS, reactive oxygen species.

and 51% in the brain. Treatment with the grape pomace prevented protein damage caused by arthritis in both tissues, but did not modify the protein carbonyl levels in healthy rats.

The levels of reactive oxygen species (ROS), estimated by means of the traditional 2′,7′-dichlorofluorescein-diacetate assay, are shown in panels C and D of Fig. 2. No modifications were seen in healthy rats treated with the grape pomace extract. As expected from previous reports, arthritis caused a quite pronounced increase of 164% in the ROS levels of the liver and a less pronounced increase of 60% in the brain. Treatment of arthritic rats with the grape pomace extract partly prevented the rise in the ROS levels. In the liver, treated arthritic rats still presented increased ROS levels, but only 59.5% above the control levels, what represents a diminution of 64%. In the brain the ROS level of treated arthritic rats was not significantly different from that of the control rats.

Two antioxidant enzymes were assayed in both liver and brain, namely superoxide dismutase (SOD) and catalase (CAT). The results are shown in Fig. 3. No clear diminution of the superoxide dismutase activity (SOD) in the liver homogenate of arthritic rats was found (panel A), although a diminishing tendency was apparent, without statistical significance, however. Similarly, the effects of the grape pomace extract treatment did not present statistical significance. In the brain, however, arthritis clearly diminished the SOD activity by 61% (panel B). The grape pomace treatment prevented this diminution without affecting the activity in healthy rats. Arthritis diminished the CAT activity in the liver by 77% (panel C). This pronounced decrease was not prevented by the grape pomace treatment, although a tendency in this direction was apparent. The diminution of the CAT activity in the brain of arthritic rats was less pronounced than in the liver (35%) and the dispersion of the data do not allow to conclude in favour of an effective prevention of this diminution by the grape pomace extract (panel D).

The results of the GSH and GSSG measurements in liver and brain are shown in Table 1. The sum of the GSH + GSSG contents (expressed in GSH units) and the GSH/GSSG ratios were also calculated. In agreement with previous reports arthritis diminished the GSH levels in both liver (42%) and brain (36%) (Comar et al., 2013; Wendt et al., 2015). The grape pomace treatment attenuated these decreases in both tissues. The GSSG levels were somewhat increased by arthritis in the brain, a phenomenon prevented by the grape pomace treatment. Decrease in GSH and increase in GSSG also leads to decreased GSH/GSSG ratios, which can be regarded as redox indicators for the $NADP^+$/NADPH systems of each tissue. This phenomenon, more pronounced in the brain, was also prevented by the grape pomace treatment.

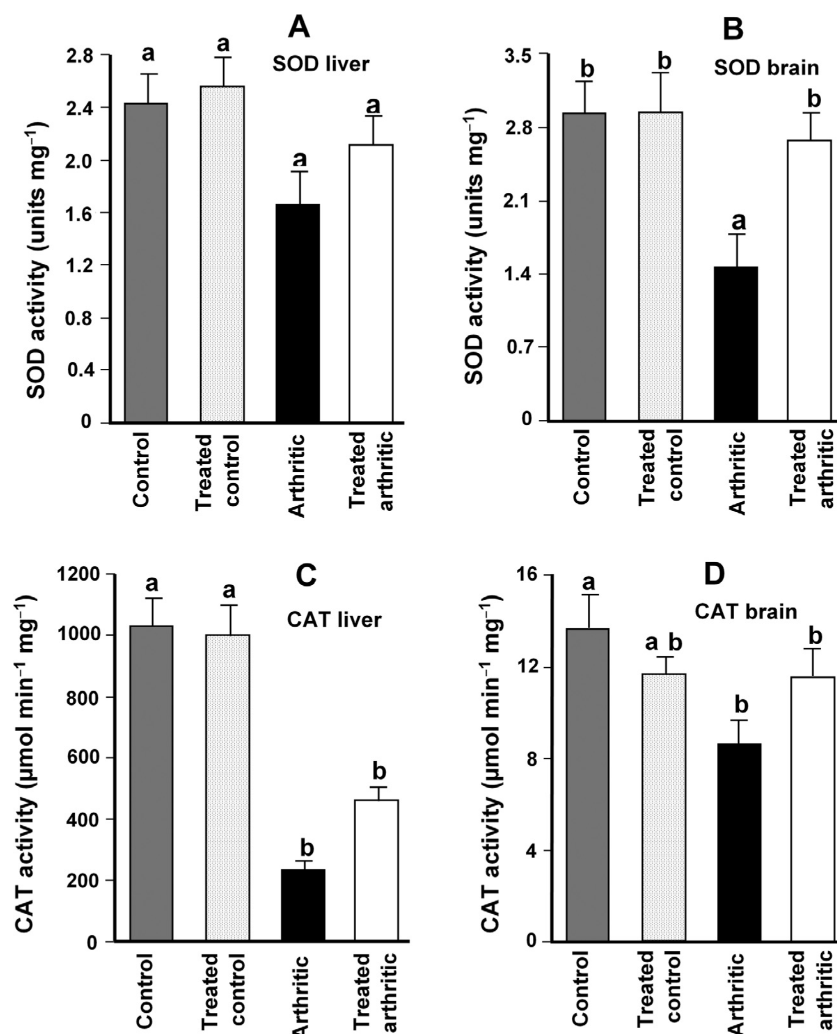

Fig. 3. Treatment of normal and arthritic rats with the grape pomace extract: effects on liver and brain antioxidant enzymes. Freeze-clamped tissues were homogenized for the assays, as described in Section 2. Values represent the mean ± standard error of the mean from 6 animals for each experimental condition. Columns labeled with the same symbol on each graph do not differ statistically from each other ($p < 0.05$) according to one-way ANOVA followed by Student-Newman-Keuls post hoc testing. Legends: SOD, superoxide dismutase; CAT, catalase.

Table 1
Effects of arthritis and grape pomace extract treatment on the contents of GSH and GSSG in liver and brain.

Parameters	Tissue	Control	Treated control	Arthritic	Treated arthritic
GSH (nmol/mg)	Liver	15.71 ± 1.74[a] (n = 4)	17.47 ± 0.74[a] (n = 4)	9.09 ± 1.48[b] (n = 4)	13.43 ± 0.55[a] (n = 4)
	Brain	7.91 ± 0.79[a,c] (n = 6)	9.90 ± 0.91[c] (n = 6)	5.04 ± 0.34[b] (n = 6)	7.30 ± 0.55[a] (n = 6)
GSSG (nmol/mg)	Liver	1.75 ± 0.03[a] (n = 4)	1.77 ± 0.09[a] (n = 4)	1.76 ± 0.04[a] (n = 4)	1.78 ± 0.05[a] (n = 4)
	Brain	0.64 ± 0.06[a] (n = 6)	0.68 ± 0.07[a] (n = 6)	0.96 ± 0.04[b] (n = 6)	0.73 ± 0.05[a] (n = 6)
GSH + 2 × GSSG (nmol GSH units/mg)	Liver	19.21 ± 1.76[a] (n = 4)	21.0 ± 0.91[a] (n = 4)	12.60 ± 1.43[b] (n = 4)	17.01 ± 0.65[a] (n = 4)
	Brain	9.42 ± 0.89[a,c] (n = 5)	11.80 ± 0.91[b] (n = 5)	6.95 ± 0.29[c] (n = 5)	8.40 ± 0.38[c] (n = 6)
GSH/GSSG	Liver	8.15 ± 0.57[a] (n = 4)	9.91 ± 0.20[c] (n = 4)	5.21 ± 0.89[b] (n = 4)	7.75 ± 0.24[a] (n = 4)
	Brain	15.36 ± 1.09[a] (n = 5)	14.00 ± 1.58[a,c] (n = 6)	5.60 ± 0.23[b] (n = 5)	11.21 ± 1.09[c] (n = 6)

Control and arthritic rats were treated with the aqueous extract of grape pomace (250 mg/kg) according to the experimental protocol described in Section 2. The data represent the mean ± standard error. Values with the same superscript in the same line do not differ statistically from each other ($p \leq 0.05$) according to one-way ANOVA followed by Student-Newman-Keuls post hoc testing.

3.2. Effects on inflammation markers

The development of arthritis was assessed by measuring the edema formation after adjuvant injection in the posterior paws. The results obtained with non-treated rats and with grape pomace-treated and ibuprofen-treated rats (positive controls) are shown in Fig. 4. The sequence of events in Fig. 4 can be best interpreted when analyzed together with the sequence of experimental procedures illustrated by Fig. 1A in Appendix A. An inflammatory reaction in the injected paw was observed on the first day post-adjuvant injection (panel A). After the initial increase in the first day the volume of the injected paw further increased progressively up to day 18. Ibruprofen reduced this increase by 37%. The grape pomace treatment also reduced the edema formation in the injected paw, but the effect was less pronounced and at day 18 the edema volume was 19% under the control. The inflammatory response in the non-injected paw started at day 10 and progressed further thereafter (panel B). Here the effect of ibruprofen was more pronounced, with 53% inhibition. The grape pomace extract, on the other hand, reduced the edema in the non-injected paw by 29%. The appearance of the secondary lesions, as shown in Fig. 4C, was considerably delayed by the grape pomace extract, but after day 14 they reached at once the levels found in the non-treated rats. A similar delay in the appearance and development of secondary lesions was observed in ibuprofen injected rats and the score still was under the arthritic control levels at day 18.

Leukocyte migration into the knee cartilages is a well known phenomenon that accompanies arthritis in both patients and adjuvant-induced arthritic rats (Wright, Moots, Bucknall, & Edwards, 2010). In the present study the influence of the grape pomace extract treatment on the infiltration of mono- and polymorphonucelar leukocytes in the right and left femoro-tibial joint cavities of the legs was evaluated at day 18 after arthritis induction. The results are summarized in Table 2. As expected, arthritis greatly increased the infiltration of leukocytes in both joint cavities. Treatment with ibuprofen or with the grape pomace extract

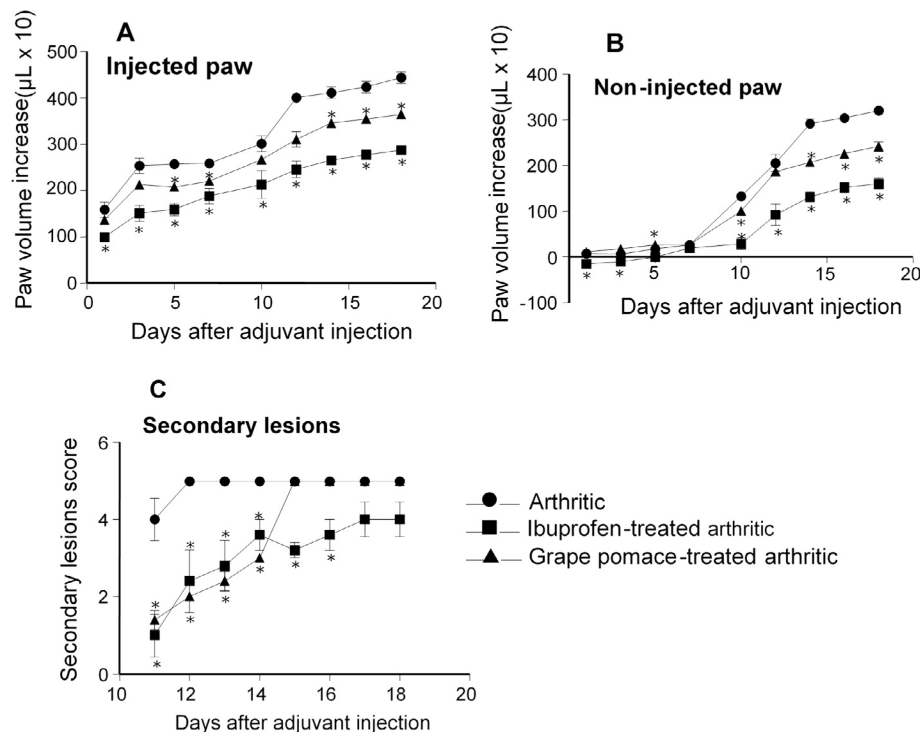

Fig. 4. Effects of the and grape pomace (250 mg/kg) extract and ibuprofen treatments (35 mg/kg) on the development of the inflammatory response to Freund's adjuvant injection. A: volume increase of the injected paw (left hind paw); B: volume increase of the non-injected paw (right hind paw); C: secondary lesions score (ears, front legs and tail). Details are given in Sections 2.11 and 2.12. Each point represents the mean ± SEM of 5 animals. Asterisks indicate statistical significance ($p < 0.05$) relative to the control arthritic animals.

Table 2
Effects of the grape pomace extract and ibuprofen treatments on the leukocyte counts of the right and left the femoro-tibial joint cavities of adjuvant-induced arthritic rats.

Groups	18 days after injection CFA (cells/mm³)			
	Right femoro-tibial joint cavity		Left femoro-tibial joint cavity	
	Mononuclear leukocytes	Polymorphonuclear leukocytes	Mononuclear leukocytes	Polymorphonuclear leukocytes
Control	1.85 ± 0.06	0.44 ± 0.02	0.88 ± 0.12	0.32 ± 0.01
Arthritic	84.93 ± 8.43[a]	274.11 ± 31.67	138.75 ± 21.26[b]	329.01 ± 59.13
Arthritic + ibuprofen	118.09 ± 15.10[a]	70.39 ± 14.09	170.30 ± 19.84[b]	114.50 ± 18.11[c]
Arthritic + grape pomace	105.79 ± 3.48[a]	158.73 ± 4.99	194.97 ± 5.59[b]	183.59 ± 9.75[c]

Arthritic rats were treated with ibuprofen (35 mg/kg) and with the aqueous extract of grape pomace (250 mg/kg) according to the experimental protocols described in Section 2. The data represent the mean ± standard errors of the means from 3 to 5 animals. Values labeled in the same column with the same letter do not differ statistically from each other ($p < 0.05$) according to one-way ANOVA followed by Student-Newman-Keuls post hoc testing.

did not diminish the infiltration of mononuclear leukocytes. On the contrary, there was even a tendency of increasing the infiltration of these cells, with no statistical significance, however. The infiltration of polymorphonuclear leukocytes, on the other hand, was diminished by both ibuprofen and grape pomace extract treatments. The diminution caused by the grape pomace extract treatment was similar in both right and left femoro-tibial joint cavities, more specifically 42 and 44%. For comparison, the diminutions caused by the ibuprofen treatment ranged 74 and 65% in the right and left femoro-tibial joint cavities, respectively.

3.3. Chemical composition of the grape pomace extract

The characterization of the non-anthocyanins and anthocyanin compounds present in the grape pomace was performed by HPLC-DAD-MS analysis, and data regarding the retention time, λ_{max}, pseudomolecular ion, main fragment ions in MS^2, tentative identification and quantification of phenolic compounds are presented in Table 3 (non-anthocyanin compounds) and Table 4 (anthocyanin compounds). The HPLC phenolic profiles of the grape pomace recorded at 280, 370 and 520 nm are shown in Fig. 2A in Appendix A, where the peaks were labeled with the same numbers as in Tables 3 and 4. Up to 25 phenolic compounds were identified, corresponding to nine flavan-3-ols derivatives, three galloyl derivatives, eight flavonols derivatives and five anthocyanins derivatives. The same family of compounds has been also identified by Rodriguez-Rodriguez et al. (2012).

Flavan-3-ols (i.e., catechins and proanthocyanidins) were the most relevant flavonoids found in the grape pomace extract (compounds 2, 4–7 and 9–12; Table 3). Compounds 5 and 9 were identified as (+)-catechin and (−)-epicatechin by comparison of their UV and MS spectra as also the retention time with commercial standards. Compounds 2, 4, 6 and 7 presented the same pseudo molecular ion $[M-H]^-$ at m/z 577 and MS^2 fragmentation patterns coherent with B-type (epi)catechin dimers (i.e., (epi)catechin units with C4–C8 or C4–C6 interflavan linkages). Characteristic product ions were observed at m/z 451 (−126 mu), 425 (−152 mu) and 407 (−152–18 mu), attributable to the HRF, RDA and further loss of water from an (epi)catechin unit, and at m/z 289 and 287, that could be associated to the fragments corresponding to the lower and upper (epi)catechin unit, respectively. These compounds, especially peak 2, were the most abundant compounds present in the samples. Other signals at m/z 865 (peaks 10 and 11) and 1153 (peak 12) were associated to B-type procyanidin trimers and tetramers, respectively.

Compounds 1, 3 and 8 were identified as galloyl derivatives, due to their MS fragmentation pattern and UV–vis spectra. Compound 1 presented the pseudo molecular ion $[M-H]^-$ at m/z 325,

Table 3
Retention time (R_t), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectrometric data, and tentative identification of non-anthocyanin phenolic compounds in the Merlot hydroalcoholic extract.

Non-anthocyanin compounds						
Compounds	R_t (min)	λ_{max} (nm)	$[M-H]^-$ (m/z)	MS^2 (m/z)	Tentative identification	Quantity (mg/g)
1	4.68	280	325	169(100), 125(8)	Galloylshikimic acid	3.37 ± 0.04
2	5.6	280	577	451(23), 425(100), 407(22), 289(12), 287(10)	B-type (epi)catechin dimer	10.2 ± 0.2
3	5.74	267	495	343(100), 191(8), 169(3)	Digalloylquinic acid	2.30 ± 0.01
4	6.3	280	577	451(23), 425(100), 407(22), 289(12), 287(10)	B-type (epi)catechin dimer	6.7 ± 0.2
5	7.16	280	289	245(100), 203(50), 187(10), 161(9), 137(3)	(+)-Catechin	7.27 ± 0.12
6	7.62	280	577	451(23), 425(100), 407(22), 289(12), 287(10)	B-type (epi)catechin dimer	3.2 ± 0.1
7	7.94	280	577	451(23), 425(100), 407(22), 289(12), 287(10)	B-type (epi)catechin dimer	5.33 ± 0.02
8	8.57	278	477	325(100), 169(3), 125(2)	Digalloylshikimic acid	1.88 ± 0.03
9	9.65	280	289	245(100), 203(35), 187(6), 161(8), 137(3)	(−)-Epicatechin	7.3 ± 0.3
10	11.06	279	865	739(78), 713(47), 695(100), 577(62), 575(42), 425(12), 407(9), 289(6), 287(11)	B-type (epi)catechin trimer	4.9 ± 0.1
11	11.1	279	865	739(78), 713(47), 695(100), 577(62), 575(42), 425(12), 407(9), 289(6), 287(11)	B-type (epi)catechin trimer	3.6 ± 0.1
12	12.3	280	1153	865(25), 739(78), 713(47), 695(100), 577(62), 575(42), 425(12), 407(9), 289(6), 287(11)	B-type (epi)catechin tretramer	6.2 ± 0.3
13	15.61	350	479	317(100)	Myricetin-*O*-hexoside	1.42 ± 0.01
14	18.4	350	477	301(100)	Quercetin-3-*O*-glucuronide	0.56 ± 0.02
15	19.3	350	463	301(100)	Quercetin-3-*O*-glucoside	0.52 ± 0.01
16	19.6	350	493	331(100)	Laricitrin-*O*-hexoside	0.36 ± 0.02
17	21.56	349	433	301(100)	Quercetin-*O*-pentoside	0.40 ± 0.01
18	22.52	348	447	301(100)	Quercetin-*O*-rhamnoside	0.38 ± 0.01
19	23.49	350	477	315(100)	Isorhamnetin-3-*O*-glucoside	0.51 ± 0.01
20	24.36	351	655	509(15), 501(49), 475(63), 347(20), 329(100), 314(13)	Methylisorhamnetin derivative	0.31 ± 0.01
				Total non-anthocyanin compounds		66.6 ± 0.7

Table 4
Retention time (R_t), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectrometric data, and tentative identification of anthocyanin phenolic compounds in the Merlot hydroalcoholic extract.

Anthocyanin compounds						
Compounds	R_t (min)	λ_{max} (nm)	$[M+H]^+$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Quantity (mg/g)
21	40.1	520	479	317(100)	Petunidin-3-*O*-glucoside	0.592 ± 0.001
22	43.2	520	463	301(100)	Peonidin-3-*O*-glucoside	1.555 ± 0.002
23	44.2	520	493	331(100)	Malvidin-3-*O*-glucoside	3.407 ± 0.001
24	53.3	520	505	301(100)	Peonidin-3-*O*-acetylglucoside	0.694 ± 0.001
25	53.8	520	535	331(100)	Malvidin-3-*O*-acetylglucoside	0.740 ± 0.001
					Total anthocyanin compounds	6.988 ± 0.003

releasing a fragment at *m/z* 169 [gallic acid–H]$^-$ (−156 mu, loss of a shikimic acid moiety), being tentatively identified as a galloylshikimic acid. Similarly, compound 8 ($[M-H]^-$ at *m/z* 477) was associated to a digalloylshikimic acid. Furthermore, compound 3 presented a pseudomolecular ion $[M-H]^-$ at *m/z* 495 showing three MS^2 fragments, one at *m/z* 343 ($[M-152-H]^-$, loss of a galloyl group), another at *m/z* 191, related to the elimination of the second galloyl moiety (−152 μ) and corresponding to quinic acid and finally the formation of the deprotonated gallic acid at *m/z* 169. This compound was identified as digalloylquinic acid.

Compounds 13–20 were identified as flavonols, especially quercetin, myricetin, laricitrin (3′-*O*-methylmyricetin) and isorhamnetin glycoside derivatives. Compounds 14, 15,17 and 18 presented pseudomolecular ions $[M-H]^-$ at *m/z* 477, 463, 433 and 447 releasing an MS^2 fragment at *m/z* 301 (−176 μ, −162 μ, −132 μ and −146 μ, corresponding to the losses of glucuronyl, glucosyl, pentosyl and rhamnosyl moieties, respectively); these compounds were identified as quercetin-3-*O*-glucuronide, quercetin-3-*O*-glucoside (by comparison with commercial standards), quercetin-*O*-pentoside and quercetin-*O*-rhamnoside, respectively. Compounds 13 ($[M-H]^-$ at *m/z* 479), 16 ($[M-H]^-$ at *m/z* 493) and 19 ($[M-H]^-$ at *m/z* 477) were assigned to a myricetin-*O*-hexoside, laricitrin-*O*-hexoside and isorhamnetin-3-*O*-glucoside according to its UV and mass fragmentation pattern and in the case of the last compounds with comparison with commercial standards. Finally, it was not possible to achieve an identification for compound 20 ($[M-H]^-$ at *m/z* 479) which was tentatively assigned as a methyl isorhamnetin derivative.

The anthocyanin compounds (peaks 21–25 in Table 4) were identified taking into account the identifications performed by Kammerer, Kljusuric, Carle, and Schieber (2005), being identified as petunidin glycoside derivative (peak 20), peonidin glycoside derivatives (peaks 22 and 24) and malvidin (peaks 23 and 25). The most abundant compound present in grape pomace extract was malvidin-3-*O*-glucoside, as also described by Kammerer et al. (2005).

4. Discussion

4.1. Antioxidant action

This study presents experimental evidence that components of an hydroalcoholic extract of the Merlot grape pomace are able to attenuate the modifications in the oxidative state caused by arthritis. Several oxidative stress indicators in arthritic rats were maintained at their normal or closely normal levels by the grape pomace treatment in the plasma, liver and brain. The list comprises protein damage in the plasma and the ROS levels and protein damage in both brain and liver. Furthermore, the grape pomace extract was also capable of maintaining the normal levels of the antioxidant capacity in the plasma as well as in the liver and brain. For the first compartment this is indicated by a direct measurement of the antioxidant capacity (FRAP) and for the liver and brain mainly by the GSH levels. The concomitant and coherent action in three compartments of the body suggests that a similar action can be expected to occur in all tissues and organs.

The same model of adjuvant induced arthritis in rats was used in previous studies to demonstrate the improvement of the oxidative state by a tamarind seed extract (Sundaram et al., 2014) and a green-tea extract (Gonçalves et al., 2015). Both extracts exert almost the same effects as the Merlot grape pomace extract used in the present study. The differences are mainly quantitative. With respect to the green tea extract, for example, it was more effective in preserving the reduced thiols levels in the plasma whereas a mere tendency in this direction was observed with the grape pomace (Fig. 1B). The same applies to the catalase activities in both liver and brain. It should be noted that the Merlot grape pomace did not modify the oxidative state of healthy rats. This is consistent with a study in which the action of Norton grape pomace treatment did not modify the oxidative state of diet induced obese mice in which the oxidative stress markers remained within the normal range (Hogan et al., 2010). Protection against CCl_4-induced hepatic injury was also demonstrated for a methanolic grape pomace extract, which was able to restore the activity of several enzymes and to prevent lipid peroxidation (Murthy, Singh, & Jayaprakasha, 2002).

4.2. Anti-inflammatory action

An anti-inflammatory action was also demonstrated for the grape pomace extract. This conclusion is allowed by the observations that the grape pomace treatment was able to delay and to diminish the development of the paw edema in adjuvant-injected rats. Delay of the appearance of secondary lesions is a further indicator of an anti-inflammatory action. Moreover, the grape pomace treatment was also able to diminish the infiltration of polymorphonuclear leukocytes (neutrophiles) in the femoro-tibial joint cavities of the legs, with an effectiveness that was not very far from that observed with ibuprofen at the administered doses (Dancevic, Daniel, & McCulloch, 2014). Consistently, the grape pomace treatment was equally able to diminish the myeloperoxidase activity in the plasma. The level of this enzyme is usually regarded as a marker of the neutrophil proliferation and severity of inflammation (Kothari et al., 2011). Inhibition of neutrophile infiltration can be important because these cells are responsible for the production of several agents able to cause tissue damage such as proteases and cytokines (Kothari et al., 2011).

The observed anti-inflammatory effects of the Merlot grape pomace extract is in accordance with a few similar observations. Norton grape pomace treatment, for example, attenuated inflammation in diet-induced obese mice, as indicated by a 15.5% decrease in the C-protein levels (Hogan et al., 2010). An anti-inflammatory action was also demonstrated for the Petit Verdot grape pomace and the red grape pomace (Denny et al., 2014; Nishiumi, Mukai, Ichiynagi, & Ashida, 2012). Treatment of mice

with a hydroalcoholic extract of Petit Verdot grape pomace was effective in diminishing the carrageenan induced paw edema and the inflammatory cytokine levels in the peritoneal cavity (Denny et al., 2014). The anti-inflammatory action of the red grape-pomace was indicated by the supression of the LPS/GalN-induced activation of the factor nuclear kappa B and expression of the inducible nitric oxide synthase and cyclooxygenase-2 proteins (Nishiumi et al., 2012).

4.3. Compounds involved

The grape pomace extract that was given daily to the rats during 23 days in the present study was extensively scrutinized for its content in phenolic compounds (Tables 3 and 4), which are probably involved in the antioxidant activity that was described. The most abundant polyphenolics in the extract are catechin, epicatechin and derivatives. Several derivatives of quercetin and gallic acid are also present. Quantification of the antioxidant capacity of each compound varies considerably because several methods have been used by several authors. However, by analyzing six different studies, catechin, gallic acid and quercetin are clearly among the most effective ones as free radical scavengers (Iacopini, Baldi, Storchi, & Sebastiani, 2008; Karamac, Kosińska, & Pegg, 2005; Khanduja & Bhardawy, 2003; Kwon, Kim, Lee, & Kim, 2010; Lu, Khoo, & Wiart, 2014; Weingerl, 2012). An important factor in addition to abundance and effectiveness as free-radical scavengers is bioavailability. Concerning this subject, a compilation of pharmacokinetic data from 97 bioavailability studies in humans concluded that gallic acid is better absorbed than the other polyphenols, followed by catechins and quercetin glucosides (Manach, Williamson, Morand, Scalbert, & Rémésy, 2005). This combination of abundance and good bioavailability, thus, reinforces again the importance of catechin and its derivatives in the grape pomace extract used in the present study. As a group, the anthocyanin compounds are relatively abundant in the grape pomace extract (Table 3). Although several of them may have a medium or even strong antioxidant activity (Kähkönen & Heinonen, 2003), their bioavailability is usually not very pronounced (Manach et al., 2005). In consequence their contribution to the overall antioxidant effects observed in the present work is possibly less important.

It is also likely that the catechin and epichatechin derivatives found in the Merlot hydroalcoholic extract may be involved in the anti-inflammatory activity. In this respect, there are reports of anti-inflammatory activity exerted by pure or nearly pure preparations of catechin and epichatechin derivatives. It was shown, for example, that (-)epigallocatechin-3-gallate suppresses osteoclast differentiation and ameliorates experimental arthritis in mice (Morinobu et al., 2012). Another example is the prevention of collagen-induced arthritis in mice, which was achieved by treatment of these animals with a polyphenolic fraction of green tea (Haqqi et al., 1999).

It must be remarked that attention of this work was focused on the phenolic compounds in the grape pomace extract because a possible effect on the oxidative stress that accompanies arthritis was also the main objective of the present work. One cannot exclude, however, that other compounds could be involved, especially in the anti-inflammatory effects that were also observed. This is an aspect that certainly deserves attention in the future.

4.4. Concluding remarks

At least to our knowledge, this is one of the first studies analyzing in detail the effects of a grape pomace extract in arthritic animals. The results suggest a potential applicability of the Merlot grape pomace hydroalcoholic extract in the improvement of the oxidative and inflammatory states in arthritic patients. For use in food supplements, the grape pomaces have the advantage of being abundant and relatively inexpensive (Fontana, Antoniolli, & Bottini, 2013). It should be stressed, however, that our results apply specifically to the Merlot grape pomace as the composition of the grape pomaces varies considerably among the various cultivars (Ribeiro et al., 2015). On the other hand, it would be of interest to investigate if the grape pomace treatment is equally able to prevent the metabolic modifications that are associated to arthritis which, especially in the liver, lead to modifications in important metabolic pathways such as glycolysis, gluconeogenesis and ureogenesis as well as in the calcium homeostasis (Fedatto-Jr et al., 1999, 2000; Utsunomiya et al., 2013; Yassuda-Filho et al., 2003).

Disclosure statement

The authors declare no conflict of interest.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jff.2017.04.009.

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