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Hydroethanolic Extract of Grape Peel from *Vitis labrusca* Winemaking Waste: Antinociceptive and Anti-Inflammatory Activities

Running head: Winemaking Residue and Its Biological Activities

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SUMMARY

Research background. Extracts from grape pomace, including the wine, showed many biological effects such as antioxidant and anti-inflammatory activities. Unfortunately winemakers discard the bagasse and the waste is less useful, however it contains bioactive compounds which result in antioxidant and anti-inflammatory properties. The work aimed to analyze the hydroethanolic extract of peels from agro-industrial waste from *Vitis labrusca* and to evaluate its antinociceptive and

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anti-inflammatory assays. This study is relevant for reusing a residue and adding value to the grape economic chain.

Experimental approach. A representative sample of pomace was obtained and the peels were applied to produce the extract. The phenolic compounds were determined by multiple reaction monitoring mode of mass spectrometry methods and Folin-Ciocalteu, using gallic acid as standard. The biological analyzes were carried out using mice orally treated with crude extract at doses (30, 100, and 300 mg/kg). We evaluated mechanical hyperalgesia by the von Frey method, thermal heat hyperalgesia using a hot plate at 55 °C, paw edema using a pachymeter, and neutrophil recruitment by measurement of myeloperoxidase enzyme activity. The nephrotoxicity and hepatotoxicity were evaluated by biochemical analyses using blood samples that were collected after the *Vitis labrusca* administration.

Results and conclusions. The peels correspond to 75 % of all wet winemaking residue and 59 % on a dry basis. We identified nine anthocyanins (3-O-glucoside: peonidin, delphinidin, petunidin, and malvidin; 3-p-coumaroyl-glucoside: cyanidin, peonidin, petunidin, and malvidin, and malvidin-3,5-diglucoside), five flavonoids (apigenin-7-glucoside, luteolin-7-glucoside, quercetin-3-galactoside, isorhamnetin-3-glucoside, and myricetin-3-rutinoside), and 26.62 mg GAE/g of phenolic compounds. *In vivo* assays, showed that *Vitis labrusca* extract at concentrations 100 and 300 mg/kg reduced carrageenan-induced mechanical and thermal hyperalgesia, 50 % of the paw edema, and neutrophil recruitment. In addition, there were no nephrotoxicity and hepatotoxicity. Our extract obtained from winemaking residue has analgesic and anti-inflammatory action, related at least in part to the presence of phenolic compounds, and it has no toxicity to renal and hepatic tissues.

Novelty and scientific contribution. We demonstrated that this waste can be used for the production of antioxidant and anti-inflammatory products (pharmaceutical and cosmetics) without toxicity, contributing to the environmental economy.

Key words: agro-industrial residue; analgesia; anthocyanins; phenolic compounds

INTRODUCTION

Plants are used by several communities as a remedy in the form of alternative treatments for many diseases, and as a common symptom, pain is the main reason for seeking specialized treatment. Thus, pain seeks a great demand for increasingly effective drugs (*1*). For this, medicines currently employed are non-steroidal anti-inflammatory drugs, widely used in different types of diseases and these anti-inflammatory drugs promote side effects such as gastritis, ulcers, blood and renal coagulation problems, hypertension, and heart failure (*2*). In this sense, in the constant search

for alternative treatments, natural products represent a valuable source for discovering molecules with promising anti-inflammatory effects (*3*).

Grape pomace is composed of seeds, stems, and peels, having a wide variety of bio-compounds, mainly phenolics. For this reason the empirical knowledge has stimulated the use of the extract obtained from grape pomace, becoming popular for presenting several biological activities such as antioxidant and antimicrobial activity, ability to inhibit nitrosation, and ability to modulate the activity of some enzymes (*4-6*). The high content of bioactive compounds is due to the maintenance of the primary and secondary metabolites present in the grape, and among their bioactive functions can relate to the anti-inflammatory and analgesic actions. Chung *et al*. (*7*) verified that the phenolic compounds present in the grape can act directly on the transcription factors involved in the inflammatory response, inhibiting the expression of cytokines. Ingestion of wine and grape juice is related to the reduction in levels of inflammatory markers such as cytokines and chemokines (*8*).

Wine has been used in diets that could confer benefits against chronic cardiovascular diseases, suggested by synergism between polyphenols and ethanol (*9*). Furthermore, red wine was considered a functional food which has healthy properties such as antimicrobial, anti-inflammatory, anti-carcinogenic, and potent antioxidants (*10*). In contrast, the wine industry is one of the largest producers of agro-industrial waste, and about 30 % of the total volume of grapes used in industrial production becomes waste or biomass. The reuse of this residue would reduce environmental impacts, such as surface and groundwater pollution, oxygen depletion, odor generation, and attraction of disease-transmitting insects (*11, 12*). Currently, in order to reduce these problems, the residue on a small scale and with criteria related to its use is used as a fertilizer, incorporated into animal feed, and the production of distillates (*13*). Besides being a bio-product with evidence of pharmacological potential, considerable values can be added as an economical alternative, which in the future may replace synthetic anti-inflammatories with the same pharmacological potential and lower side effects mainly for the pharmaceutical and cosmetic industries, making it a promising source for chemo-active substances (*14, 15*).

Considering the pharmacological properties of phenolic compounds (flavonoids and anthocyanins) and bioactive compounds of agro-industrial wine residues, the present study aimed to identify the contents of the chemical compounds of peel hydroethanolic extract (1:1 *V/V*) of *Vitis labrusca* agro-industrial waste. Specifically, we demonstrated *in vivo* analgesic and anti-inflammatory effects plus evaluating the nephrotoxicity and hepatotoxicity of this extract.

MATERIALS AND METHODS

Plant material and crude extract obtention

The agro-industrial residue of *Vitis labrusca* from the 2014/2015 crop was supplied by Cooperativa Agro-industrial dos Viticultores (COAVITI), located in Marialva - Paraná (23°28'20.1" S/51°48'57.9"W). The plant material consisted of peels, stems, and seeds kept frozen (-4 ºC) until the preparation of the extracts. This work is duly registered with the National System of Genetic Heritage Management and Associated Traditional Knowledge (SisGen Code: A14A92F).

The grape pomace was scattered over the surface of a workbench and divided in four parts, the percentage of the residue (peels, seeds, and stems) from the winemaking waste was then determined by a quarter of the representative sample, and recorded on a wet and dry basis. The quarter method was carried out in triplicate according to the Brazilian pharmacopeia (*16*).

The wet peels (50.0 g) were submitted to turboextraction (M Vithory, LQ 001, Catanduva, SP, Brazil) for 1 min with 250 mL of acidified hydroethanolic solution (1:1 *V/V*) (0.1 % HCl, Synth, Diadema, SP, Brazil) followed by ultrasonication (Ultronique, Q3.8/40, Indaiatuba, SP, Brazil) at controlled temperature (maximum 25 °C) and protected from light for 1 hour. The extract was then filtered and the extractive process was repeated. The solution was removed by rotary evaporator (Thermo Fisher Scientific, RC1022, Waltham, MA, USA) at 45 °C and the concentrated extract was maintained in the freezer (-4 °C), protected from light until use.

Chemical identification

The identification of the chemical compounds present in the extract of *Vitis labrusca* was carried out using Waters® (e2795, Milford, MA, USA) electrospray ionization (ESI) with a ThermoScientific HPLC pump (Waltham, MA, USA) with steady carrier solution flow in the triploquadrupole (QqQ) space spectrometer (Waters®, e2795, Milford, MA, USA). An aliquot of 4.5 mg of the *Vitis labrusca* dried extract was dissolved in 1 mL of methanol (UV/HPLC grade ≥99.9 % - Vetec, Burlington, MA, USA), sonicated for 10 minutes (Unique, Ultra Cleaner 1400, Indaiatuba, SP, Brazil), centrifuged (Hettich Zentrifugen, Universal 320 R, Tuttlingen Germany) at 4668 x *g* for 10 minutes, and filtered with a 0.22 µm microfilter (ThermoScientific, PTFE, Waltham, MA, USA). Subsequently, this extract was diluted according to the carrier solution, 0.1 % trifluoroacetic acid, and 0.1 % ammonium hydroxide solution (UV/HPLC grade ≥99.9 %, Sigma-Aldrich, Saint Louis, MO, USA), for the positive and negative modes respectively, in the ESI-MS/MS equipment.

The analysis parameters for multiple reaction monitoring (MRM) for positive mode ionization were: gas flow, Argon, 0.14 mL/min, desolvation temperature, 250 °C, capillary, 3500 V, cone, 30 V, collision energy, 30 V, syringe flow rate, 50 µL/min, cycle time, 2 s, number of channels, 31. For the

negative mode of ionization were: gas flow, Argon, 0.14 mL/min, desolvation temperature, 250 ºC; capillary, 2500 V, cone, 40 V, collision energy, 30 V, syringe flow rate, 50 µL/min, cycle time, 2.7 s, number of channels, 27.

For the positive mode fragmentation conditions, for delphinidin-3-glucoside, ESI-MS/MS were: gas flow, Argon, 0.14 mL/min, desolvation temperature, 250 °C, capillary, 3500 V, cone, 30 V, collision energy, 15 V, resolution, 12, syringe flow rate, 50 µL/min. For petunidin-3-glucoside and malvidin-3-glucoside were: gas flow, Argon, 0.14 mL/min, desolvation temperature, 300 ºC, capillary, 3500 V, cone, 30 V, collision energy, 30 V, resolution, 13, syringe flow rate, 50 µL/min.

Total phenolic compounds

The content of total polyphenols in the extract was determined by the colorimetric method using Folin-Ciocalteau (Sigma-Aldrich, Saint Louis, MO, USA) as a reagent and gallic acid (Vetec, Burlington, MA, USA) as standard (*17*). A solution of 2.42 mg/L was prepared, and 0.5 mL aliquots were subsequently mixed with 0.5 mL of the Folin-Ciocalteau reagent and 0.5 mL of Na_2CO_3 10 % (Anidrol, Diadema, SP, Brazil). The reaction was incubated for 1 hour at room temperature, and the absorbance was measured at 760 nm (Perkin Elmer Spectrometer Lambda 25, Waltham, MA, USA). A blank sample was run under the same conditions and the results of the total phenolic compounds were expressed in gallic acid equivalent (mg GAE/g). For this, we obtained a curve of the straight line constructed with different concentrations of gallic acid (4-32 µg/mL) (Equation 1).

$$y = 0.03419x - 0.03030 \quad /1/$$

where y is the absorbance, 0.03419 is the slope, x is the gallic acid concentration (µg/mL), and -0.03030 is the linear coefficient.

Biological experimental protocol

The experiments were carried out in male Swiss mice (20-25 g) that were kept in the Bioterium of the State University of Londrina, with free access to water and feed, for two days before the experiments, using light/dark cycle (12/12 h). The animals were divided into standard polypropylene cages (41 x 34 x 16 cm, Insight®) according to the experimental groups (maximum 12 animals per cage). They adapted to the experimental environments and conditions for at least 1 hour before the experiments. The procedures for the care and handling of animals were by the guidelines of the International Association for the Study of Pain (IASP), which was submitted to the Ethics Committee of the State University of Londrina for approval (protocol number 7534.2016.83).

The animals were treated orally by gavage with crude extract (peels grape *Vitis labrusca*, 20 % Tween 80 in saline) at doses of 30, 100, and 300 mg/kg 60 min before intraplantar stimulus with carrageenin (100 µg/animal, i.e. pl.).

Evaluation of edema

Measurement of the paw volume of the animals was evaluated using a pachymeter (Tramontina PRO 300 mm - 12", Canoas, RS, Brazil) before the inflammatory (basal) stimulus with carrageenan (100 µg/animal, i.pl.) and at the intervals of 1, 3, and 5 h after. The results were calculated by the difference between the mean of two measurements after the stimulus and the mean of two measurements before the stimulus (basal) (*18*).

Evaluation of mechanical and thermal hyperalgesia

Mechanical hyperalgesia was evaluated in mice by the von Frey method, as previously described by Cunha *et al*. (*19*). The mice were accommodated in acrylic boxes with a metal grid floor, in a quiet temperature-controlled room (23 ± 1 °C), 30 min before the test started. The animals were tested before (baseline) and at intervals of 1, 3, and 5 h after carrageenan stimulation (100 µg/animal, i.pl.). The results were calculated by the difference between the mean of 3 measurements after the stimulus (1-5 h) and before the stimulus (baseline).

Thermal heat hyperalgesia was performed using a hot plate (Lucadema, 43/03, São José do Rio Preto, SP, Brazil) at 55 °C ± 1 °C (*20*). The animals were tested at the same intervals of 1, 3, and 5 h after the carrageenan challenge, and the results were presented as residence values (in seconds) on the hot plate. The maximum time to remain on the hot plate was 20 s to avoid tissue damage.

Measurement of myeloperoxidase enzyme activity

Subcutaneous plantar tissue samples were collected 5 h after carrageenan (100 µg/animal, i.pl.) and stored in Potassium phosphate dibasic (K_2HPO_4) buffer (pH=6.0, Anidrol, Diadema, SP, Brazil) containing 0.5 % HTAB. The samples were homogenized with Polytron® (PT3100, Montreal, Canada) and centrifuged (16100 x *g*, 4 °C, 2 min) and the supernatant was collected. Aliquots of 10 µL of the supernatant sample were mixed with 200 µL of 50 mmol/L phosphate buffer solution at pH=6.0 containing 0.167 mg/mL O-dianisidine dihydrochloride and 0.015 % hydrogen peroxide. The absorbances of the samples were recorded in a microplate spectrophotometer (ThermoScientific, MultskanGO, Waltham, MA, USA) at a wavelength of 450 nm and myeloperoxidase enzyme activity (MPO) was compared to a standard neutrophil curve, and the result expressed as myeloperoxidase activity (number of neutrophils x 104/mg tissue) (*21*).

Renal function tests

Blood samples were collected after the *Vitis labrusca* administration by cardiac puncture and added into microtubes containing an anticoagulant (EDTA, 5,000 IU/mL, Sigma-Aldrich, Saint Louis, MO, USA). The plasma was separated by centrifugation (200 × *g*, 10 min, 4 °C) and processed according to the manufacturer's instructions (Labtest Diagnóstico S.A.) to evaluate urea and creatinine levels as indicators of nephrotoxicity. Results are presented as mg/dL of plasma urea or creatinine (adapted from Staurengo-Ferrari *et al*. (*22*)).

Enzymatic markers of liver injury

Blood samples were collected 5 h after *Vitis labrusca* administration by cardiac puncture and added into microtubes containing an anticoagulant (EDTA, 5,000 IU/mL, Sigma-Aldrich, Saint Louis, MO, USA). The plasma was separated by centrifugation (200 × *g*, 10 min, 4 °C) and processed according to the manufacturer's instructions (LabtestDiagnóstico S.A.) to evaluate ALT and AST levels as indicators of hepatotoxicity. Results are presented as U/L of plasma ALT or AST (adapted from Staurengo-Ferrari *et al*. (*22*)).

Statistical analysis

The results of the hyperalgesic parameters were presented as mean ± SEM (standard error of the mean) of measurements performed in six animals per group with two replicates and two-way repeated-measures ANOVA followed by the Tukey post-test were used to compare groups, and doses at all times (curves) were determined by RStudio software (*20*), version 3.4.1-2017. For the remaining trials, the results were presented as mean ± SEM (standard error of the mean) and one-way ANOVA was used followed by the Tukey post-test for specific time experiments. For both analyzes significant differences were considered for $p < 0.05$ (adapted from Staurengo-Ferrari *et al*. (*22*)).

RESULTS AND DISCUSSION

Chemical analysis and phenolic compounds

The wet winemaking residue was composed of 75, 23, and 2 %, and the dry mass was 59, 38, and 3 % of peels, seeds, and stems, respectively. The percentages on a dry basis, are variable according to the wine production and the type of grape pomace; in the literature we found values of 51, 47, and 2 % (peels, seeds, and stem respectively) (*23*), and 38-52 % for seed and 5-10 % for peels (*24*) for the waste of *Vitis spp*. These percentages show that our work reuses most of this waste, being able to increase the income of cooperatives and industries and reducing the environmental impact. In addition, even though the seeds are not evaluated in this study, they have important

biological activities such as antimicrobial and antioxidant (*25,26*). Furthermore, the main compounds found in the grape pomace residue were unsaturated fatty acids, simple phenolics, and polyphenols (*11*).

Grape pomace is rich in phenolic compounds such as anthocyanins, flavonols, flavan-3-ols, cinnamic acids, benzoic acids, ellagic acids, and stilbenes (*27,28*). Only the crude extract from the peels of *V. labrusca* obtained from the agro-industrial waste, showed the presence of 14 compounds: m/z 431 apigenin-7-glucoside, m/z 447 luteolin-7-glucoside, m/z 463 quercetin-3-galactoside, m/z 477 isorhamnetin-3-glucoside, and m/z 627 myricetin-3-rutinoside for negative mode; and m/z 463 peonidin-3-glucoside, m/z 465 delphinidin-3-glucoside, m/z 479 petunidin-3-glucoside, m/z 493 malvidin-3-glucoside, m/z 595 cyanidin-3-p-coumaroyl-glucoside, m/z 609 peonidin-3-p-coumaroyl-glucoside, m/z 625 petunidin-3-p-coumaroyl-glucoside, m/z 639 malvidin-3-p-coumaroyl-glucoside, and m/z 655 malvidin-3,5-diglucoside for positive mode by ESI-MS/MS using the MRM function.

The crude extract of *Vitis labrusca* peels presented a response in the *in vitro* assay for phenolic compounds content of 26.62 mg GAE/g. Melo *et al*. (*15*) studied the extraction of Isabel grape pomace with 80 % ethanol and found 16.57 mg GAE/g of phenolic compounds. Rockenbach *et al*. (*29*) quantified the content of phenolic compounds in grape pomace (*Vitis labrusca* and *Vitis vinifera*) and obtained values between 32.62 and 74.74 mg GAE/g. Furthermore, our study indicated that the phenolic compounds are maintained in the peels from winemaking waste. However, the types and quantity of these compounds depend on the climatic and processing conditions and the extraction method.

Potential anti-inflammatory and analgesic in vivo

The reduction of paw edema, analgesic, and anti-inflammatory effects were observed in our results, owing to the extraction of anthocyanins, flavonols, and phenolic compounds which worked in synergism, reducing the inflammation.

The extracts at the doses of 100 and 300 mg/kg reduced paw edema at all-time points when compared to the vehicle-treated group (Fig. 1a). These doses reduced 50 % of the paw edema when compared to the control group at 1 and 3 hours. The same trend of reduction of paw edema was presented by Figueira et al. (*30*) with blueberry extract and ear edema after topical application of malvidin-3-glucoside, malvidin-3,5-diglucoside, and quercetin (*31*). In contrast, the control group indicated paw edema of approximately 0.4 mm/paw and the 30 mg/kg dose did not show statistical significance for all times (1, 3, and 5 h) for the control group.

Mechanical and thermal hyperalgesia (Fig. 1b and Fig. 1c) using a dose of 30 mg/kg of bark extract when compared to the carrageenan control group was not statistically different. Despite this,

the doses of 100 and 300 mg/kg were statistically different. A sour cherries extract (400 mg/kg) containing anthocyanins showed the same effect on mechanical and thermal hyperalgesia as the indomethacin group (32). However, these effects were seen in our study at lower doses. We tested using the same model as Tall et al. (32), unfortunately their study did not characterize the extract, and biological results depend on the types and amount of compounds present in the extracts.

The inflammatory pain results from increased sensitization of peripheral nociceptors resulting from the stimulation of pro-inflammatory mediators by cytokines and chemokines. The regulation of the analgesic pathway it's very complex, the anthocyanins have anti-inflammatory and antioxidant properties, acting in many phases of the pathway (*33*). In addition, the anthocyanins might have these effects owing to the displacement of π (pi) electrons generated by the conjugation extension present in their structures. Moreover, the central ring or ring B acts as an electrophile species capable of undergoing nucleophilic addition (*34*), which reacts on the enzymatic pathway.

One of the inflammatory triggers might be evaluated by the quantification of MPO, which is linked to the injured and reactive species of the tissues. The carrageenan stimulation significantly increased MPO enzyme activity after 5 hours (Fig. 2), in contrast, 100 and 300 mg/kg of extract of peels from *V. labrusca* from winemakers' waste reduced statistically the MPO. Herein, the extract of *Malva sylvestris* which contains anthocyanins, and anthocyanins isolates and flavonol culminated in the same results (*34*).

Medications can be toxic, causing liver, kidney, and other tissue damage. Biochemical tests are commonly used to observe changes in liver cells, monitored by enzymes such as AST and ALT, as well as blood urea and creatinine concentrations that may or may not demonstrate possible renal changes. All biochemical parameters evaluated in this study remained similar regardless of treatment, with no significant difference (Fig. 3), as observed by Figueira et al. (30). Thus, the toxicity of the tested grape skin extract was not demonstrated, since anthocyanins, flavonoids, and phenolic compounds are natural metabolites suggestive of low or no toxicity in liver and kidney, when evaluated by blood biochemical parameters.

Winemaking residue can be utilized for combustion, pyrolysis (*23*), and in pharmaceutical and cosmetic industries as an alternative raw material (*35*), generating incomes for the wineries and minimizing environmental impacts. In addition, anthocyanins are healthy and safe compounds that have anti-inflammatory and antioxidant properties (33); grape pomace, in addition to anthocyanins, contains flavonoids and phenolic compounds with similar properties.

CONCLUSIONS

Our work reuses the grape peels present in the majority of winemaking residue and the crude extract demonstrated analgesic and anti-inflammatory action, related at least in part to the presence of phenolic compounds. We also showed that there was no toxicity to renal and hepatic tissues. Therefore, this discarded residual biomass can be considered a cheap and widely available source for the extraction of phenolic compounds, demonstrating a possible alternative to anti-inflammatory and analgesic activities, generating economic gains and minimizing environmental impacts. In addition, the remainder of the pomace might be evaluated in future studies.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

AUTHORS' CONTRIBUTION

CFGS performed molecular identification, *in vitro* and *in vivo* analyses, statistical evaluations, and writing. VF was involved in the *in vivo* analyses, statistical evaluations, and writing. CRT performed *in vitro* experiments and writing. MASR and ECM carried out molecular identification and writing. RLNM and JBC performed molecular identification, *in vitro* analyses, and statistical evaluations and writing. EYH, JAR, SRG, MMB, and WAVJ prepared of manuscript, performed critical revision and writing. NSA delineated of the study, writing, and critical revision. All authors revised the manuscript.

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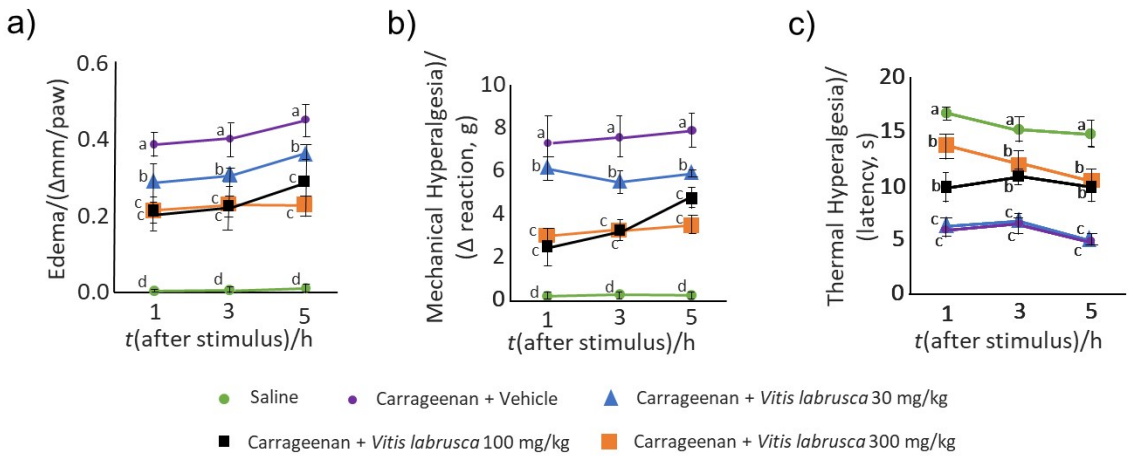

Fig. 1. Evaluation of (a) paw edema, (b) mechanical hyperalgesia, and (c) thermal hyperalgesia in three times (1, 3, and 5 hours) after intraplantar injection of carrageenan. The animals (n = 6 per experimental group) were pretreated (1 h) with saline (control group), vehicle control, and three doses (30, 100, and 300 mg/kg) of hydroethanolic extract from the peels of *Vitis labrusca* obtained from winemaker's waste. Results were expressed as mean ± standard error of the mean (different letters indicate statistical difference, $p < 0.05$)

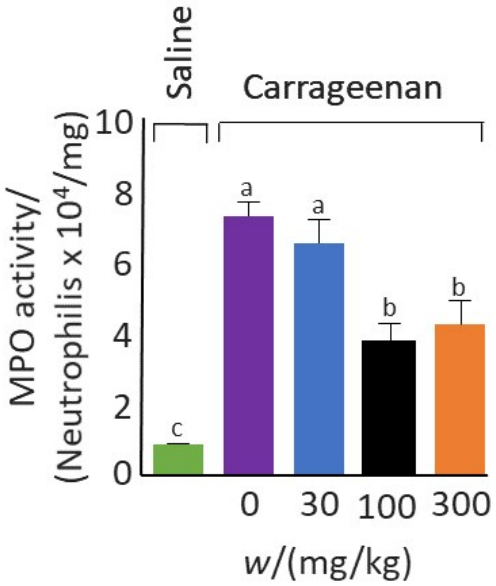

Fig. 2. Evaluation of MPO after 5 hours of intraplantar injection of carrageenan. The animals (n = 6 per experimental group) were treated with saline (control group), vehicle control, and three doses (30, 100, and 300 mg/kg) of hydroethanolic extract from the peels of *Vitis labrusca* obtained from winemaker's waste 1 h before the carrageenan induced inflammation. Results were expressed as mean ± standard error of the mean (different letters indicate statistical difference, $p < 0.05$)

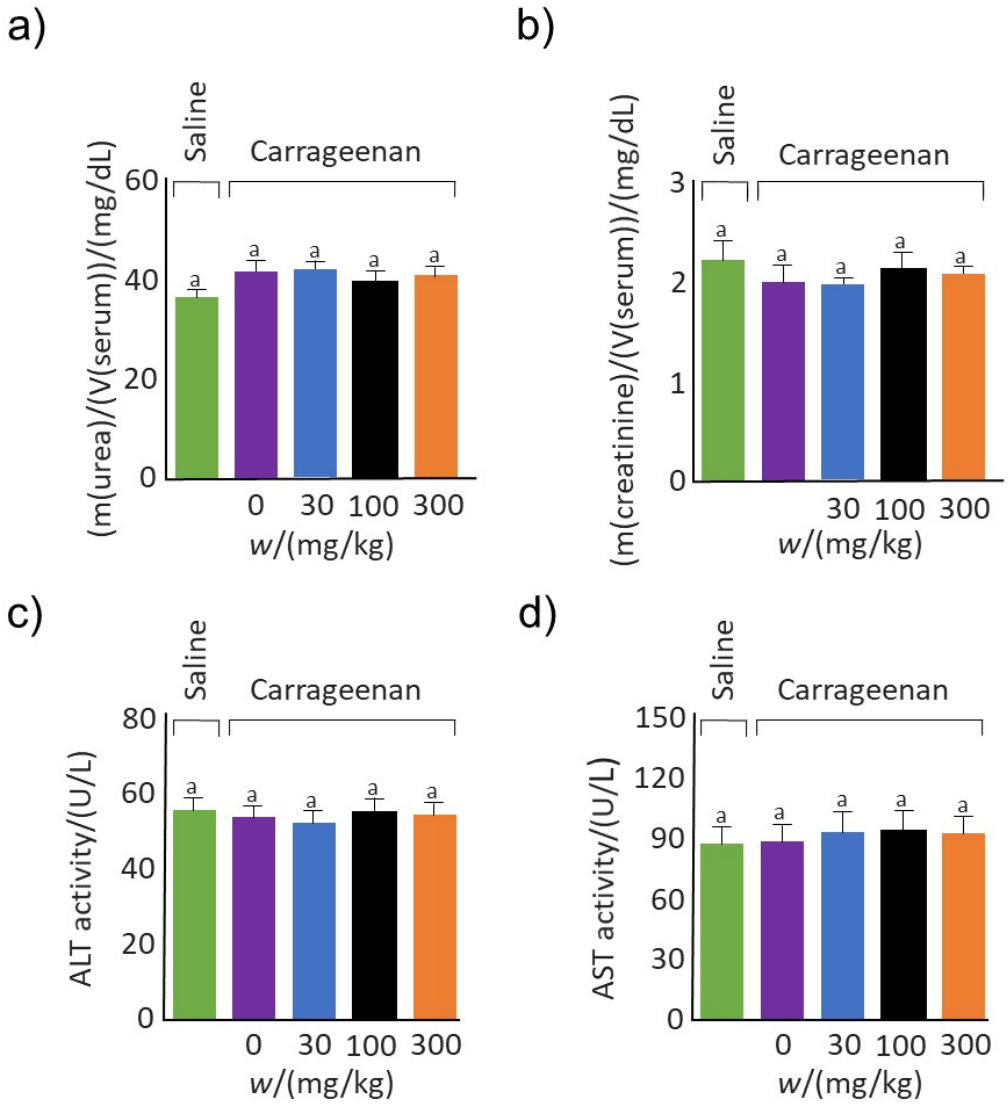

Fig. 3. Evaluation of biochemical parameters of serum: (a) Urea, (b) Creatinine, (c) ALT, and (d) AST after 5 hours of intraplantar injection of carrageenan. The animals (n = 6 per experimental group) were treated with saline (control group), vehicle control, and three doses (30, 100 and 300 mg/kg) of hydroethanolic extract from the peels of *Vitis labrusca* obtained from winemaker's waste 1 h before the carrageenan induced inflammation. Results were expressed as mean ± standard error of the mean (different letters indicate statistical difference, $p<0.05$)