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Natural deep eutectic solvent (NADES)-based blueberry extracts protect against ethanol-induced gastric ulcer in rats

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ABSTRACT

Blueberry is a polyphenol-rich fruit bearing great bioactive potential. Natural deep eutectic solvents (NADES) emerged as putatively biocompatible solvents that could substitute for toxic organic solvents in the extraction of fruit phenolic compounds for developing nutraceuticals or functional foods. Therefore, the aim of this study was to investigate the gastroprotective effects and the biocompatibility of a blueberry crude extract (CE) obtained using NADES and of the extract fractions (anthocyanin-rich fraction – ARF; non-anthocyanin phenolic fraction - NAPF) in a model of ethanol-induced gastric ulcer in rats. CE was the NADES-containing, ready-to-use extract that was obtained using choline chloride:glycerol:citric acid NADES (0.5:2:0.5 M ratio). ARF and NAPF were the NADES-free fractions obtained by solid phase purification of CE and were investigated to identify the bioactive fraction responsible for the effects of CE. Animals were treated for 14 days with water, NADES vehicle, CE, ARF, NAPF or lansoprazole (intragastric) and then received ethanol to induce gastric ulcer. CE decreased ulcer index and preserved the integrity of gastric mucosa. The pretreatment with CE or ARF reduced glutathione depletion and the inflammatory response. All treatments, including NADES vehicle reduced protein oxidation and nitric oxide overproduction in ethanol-treated rats. Additionally, ARF increased short-chain fatty acids in feces. These findings suggest that NADES can be used to obtain biocompatible extracts of blueberry that exhibit gastroprotective effects with no need of solvent removal. The gastroprotective effects were mainly associated to ARF but NAPF and even NADES vehicle also contributed to some protective effects.

1. Introduction

Affecting around 10% of population worldwide, the increase of gastric ulcer cases is associated with *Helicobacter pylori* infections, excessive ethanol consumption, stress, smoking, nutritional deficiencies and frequent ingestion of non-steroidal anti-inflammatory drugs

Abbreviations: ANOVA, Analysis of variance; AOPP, Advanced oxidation protein products; ARF, Anthocyanin-rich fraction; b.w., Body weight; CE, Crude extract; Cu/Zn SOD, Cu/Zn Superoxide dismutase; DTT, Dithiothreitol; EDTA, Ethylenediamine tetraacetic acid; ESI, Electrospray ionization; EtOH, Ethanol; GPx, Glutathione peroxidase; GR, Gutathione reductase; GSH, Reduced glutathione; GSSG, Oxidized glutathione; GST, Glutathione-S-transferase; HEPES, Hydroxyethyl piperazineethanesulfonic acid; HPLC, High-performance liquid chromatography; LAN, Lansoprazole; MPO, Myeloperoxidase; NADES, Natural deep eutectic solvent; NAPF, Non-anthocyanin phenolic fraction; NADPH, Reduced nicotinamide adenine dinucleotide phosphate; $^{\cdot}$NO, Nitric oxide; NOx, Nitrite and nitrate levels; Nrf2, Nuclear factor erythroid 2-related factor 2; NSAIDs, Non-steroidal anti-inflammatory drugs; ORAC, Oxygen radical absorbance capacity; PMSF, Phenylmethylsulfonyl fluoride; Q-TOF, Quadrupole time-of-flight; ROS, Reactive oxygen species; SCFA, Short-chain fatty acids; UV, Ultraviolet.

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(NSAIDs) (Lauret, Rodriguez-Peláez, Pérez, & Rodrigo, 2015). Gastric ulcer is characterized by an imbalance between protective (mucus, prostaglandins, bicarbonate and nitric oxide) and aggressive (pepsin and hydrochloric acid) agents in the gastric mucosa (Lauret et al., 2015).

The synthetic drugs for the clinical treatment of gastric ulcers pose serious complications after prolonged use (Freedberg, Kim, & Yang, 2017). Therefore, the discovery of natural compounds or nutritional strategies that can attenuate or prevent gastric ulcers are particularly relevant. Anthocyanins and other phenolic compounds have been shown to exhibit beneficial effects against gastric ulcer, by decreasing lipid peroxidation, increasing GSH levels and antioxidant enzymes, besides attenuating the inflammatory response (Kim et al., 2011; Li, Xu, Zhao, Chang, & Rhee, 2008). In this context, crude extracts of blueberry species as *Vaccinium leschenaultii* and *Myrtus communis* L., which were obtained using solvents as acetone, methanol and water, have demonstrated potential health benefits, including protection against gastric ulcers (Nagulsamy, Ponnusamy, & Thangaraj, 2015; Sumbul, Ahmad, Asif, Saud, & Akhtar, 2010).

The mechanisms responsible for the antioxidant action of polyphenols in the gastrointestinal tract are complex and generally attributed to their ability to directly eliminate free radicals and more recently to the upregulation of endogenous antioxidant defenses (Farzae, Abdollahi, & Rahimi, 2015). One of the studied alternatives is the modulation of nuclear factor erythroid 2-related factor 2 (Nrf2) that has been implicated in the protective effect of anthocyanins from black rice bran against naproxen-induced gastric ulcers (Kim, Kim, Shim, & Chang, 2014). The Nrf2 pathway is the main route involved in cell protection against oxidative stress because it upregulates the expression of antioxidant genes upon translocation into the nucleus (Jung et al., 2010). However, the involvement of the Nrf2 pathway in the protective effect of blueberry extracts against ethanol-induced gastric ulcers has not been investigated yet. In addition, polyphenol-rich extracts have been shown to modulate gut microbiota leading to increased production of short-chain fatty acids (SCFA) (Gowd, Karim, Shishir, Xie, & Chen, 2019). While SCFA have been shown to play a protective role in ulcerative colitis models by improving systemic and local immune response and gut intestinal barrier homeostasis (Maurer et al., 2019; Maurer, Cazarin, Quatrin, Minuzzi, et al., 2020), their involvement in the protective effects of polyphenols against gastric ulcer remains to be determined.

Although plant polyphenols have been demonstrated potential health benefits against gastric ulcers (Farzae et al., 2015), the extraction of these compounds is performed using organic solvents, which need to be removed before use and yields highly toxic wastes (Misan et al., 2019). Recently, a new green extraction technology, known as natural deep eutectic solvent (NADES), has emerged (Misan et al., 2019). NADES are based on mixtures of natural components: non-toxic quaternary ammonium salts (e.g. choline chloride) and uncharged hydrogen-bond donors (e.g. amines, sugars, alcohols and carboxylic acids) (Cvjetko, Vidović, Radojčić Redovniković, & Jokić, 2015). Recent *in vitro* studies on phenolic grape skin extracts obtained using NADES showed high antioxidant capacity and low cytotoxicity (Panić, Gunjević, Cravotto, & Redovniković, 2019; Radošević et al., 2016), suggesting that NADES could yield ready-to-use extracts for therapeutic and nutritional purposes. In addition, NADES-rutin solution has been demonstrated to yield greater rutin solubility and bioavailability in rats compared to an aqueous solution (Faggian et al., 2016; Murador, Mesquita, Vannuchi, Braga, & Rosso, 2019).

NADES have been recently demonstrated to be as efficient as organic solvents to extract blueberry anthocyanins and yielded an extract that has higher proportion of arabinoside anthocyanins than the organic solvent (Silva et al., 2020). Hence, NADES could be an alternative to obtain biocompatible, ready-to-use phenolic extracts bearing distinctive biological activity. Although NADES are biodegradable and putatively safe for biological use, the biological effects of NADES or NADES-based plant extracts has been scarcely investigated *in vivo* (Misan et al., 2019; Murador et al., 2019).

This study was aimed at evaluating whether a ready-to-use blueberry extract obtained using NADES has protective effects in a model of ethanol-induced gastric ulcer in rats. A secondary aim was to determine the bioactive fraction responsible for the effects and the role of NADES vehicle by comparing the protective effects of the NADES-containing crude extract to the effects of the NADES-free anthocyanin and non-anthocyanin phenolic fractions. The mechanisms involved in the gastroprotective effects were also investigated.

2. Materials and methods

2.1. Samples

Blueberry fruits of the O'Neal cultivar (*Vaccinium corymbosum L.* × *Vaccinium darrowii Camp.*) were collected in an orchard belonging to the Brazilian Agricultural Research Corporation (Embrapa Clima Temperado, Pelotas, Rio Grande do Sul) in November 2016. The fruits were frozen and lyophilized in a Terroni® model LS3000 (São Carlos, São Paulo, Brazil). Liophilized frozen samples were ground in a mill (Marconi, São Paulo, Brazil) in liquid nitrogen and stored at −20 °C in aluminum bags until the end of the experiments. This blueberry lyophilized powder had 11% moisture, 2.8% protein, 0.9% ash, 3.9% fat, 36.7% dietary fiber and 44.7% soluble sugars.

2.2. Preparation of NADES solvent and blueberry extract and fractions

NADES solvent was prepared by the mixture of choline chloride, glycerol, and citric acid at a molar ratio of 0.5:2:0.5 and was stirred in a flask at 80 °C for 30 min until forming a homogeneous liquid (Silva et al., 2020). Thereafter, NADES mixture was diluted with 25% water (v/v). Spectral and thermographic data of this solvent confirmed deep eutectic properties as recently described by our research group (Silva et al., 2020).

For obtaining the crude extract (CE), a total of 1.2 kg of blueberry powder were extracted with 3 L NADES solvent, as previously described by Silva et al. (2020) with the following modification (see Supplementary material). Extracts were submitted to an ultrasonic bath (40 kHz, 50 min, room temperature) and then centrifuged at 2100*g* for 15 min. The supernatants of all extractive batches were collected, polled and adjusted with NADES to a final volume of 3 L.

To obtain ARF and NAPF, the CE was subjected to a solid phase extraction (SPE) according to Rodriguez-Saona and Wrolstad (2001) modified by Bochi, Godoy, and Giusti (2015). The purified extracts were obtained with C-18 columns (max 10 g packed bed, 60 mL, Giga Tubes, Strata C18-E from Phenomenex, Torrance, USA) that were previously activated with 60 mL methanol and conditioned with three times of 60 mL acidified water (0.35%, v/v, formic acid). Samples were initially eluted with formic acid aqueous solution (0.35%, v/v) to remove NADES components and other polar interfering compounds. Then, samples were eluted with two volumes of ethyl acetate to release the less polar phenolic compounds (NAPF) and thereafter with methanol acidified with 0.1% (v/v) formic acid to release anthocyanins (ARF). Solvents were evaporated using a rotary evaporator (Bûchi-R3^{TM}, Flawil, Switzerland) at 38 ± 2 °C and samples were adjusted to a known volume (2.5 mL) using acidified water (0.35%, v/v, citric acid).

2.3. Chemical composition of blueberries extract and fractions

CE, ARF and NAPF from different extraction and purification batches were pooled to obtain a single sample for each extract type/fraction to be used in the animal assay. Thereafter, the composition of polyphenols of these samples were assessed by high-performance liquid chromatography (HPLC) coupled to photodiode array and mass spectrometer with quadrupole time-of-flight (Q-TOF) analyzer and electrospray ionization (ESI) source according to the methods reported earlier for phenolic compounds (Quatrin et al., 2019) and anthocyanins (Silva et al., 2020).

The tentative identification of anthocyanins and non-anthocyanin phenolics was based on the elution order in the C-18 reverse phase chromatogram, ultraviolet (UV) to visible spectral characteristics, and the fragmentation pattern in mass spectrometry analysis.

The blueberry CE had 4.57 mg of total polyphenols/mL of blueberry extract. The anthocyanin-rich fraction had 1.15 mg of cyanidin-3-glucoside (cy-3-glu) equivalents/mL of extract, whereas the non-anthocyanin polyphenols fraction had 1.11 mg of catechin equivalents (hydroxybenzoate derivatives)/mL and 2.30 mg of quercetin equivalents (flavonol derivatives)/mL of extract.

2.4. Animals and induction of gastric lesions by ethanol (EtOH)

Fifty-six adult male Wistar rats (150–200 g) were provided by the Central Animal House of the Federal University of Santa Maria (UFSM, RS). All procedures adopted were approved by the Committee for Care and Use of Experimental Animal Resources/UFSM (protocol number 3935111116/2017). Animals were housed in standard polypropylene cages (four rats/cage) and kept under controlled temperature (22 ± 2 °C) and humidity (55 ± 5%) at a 12/12 h light/dark cycle with free access to food and water. Animals were divided into seven experimental groups (n = 8/group) as follows (Fig. 1):

(I) Control group (Control): received distilled water by gavage for 14 days and was not submitted to gastric ulcer induction.
(II) Water-ethanol group (Water-EtOH): received distilled water by gavage for 14 days before induction of gastric ulcer.
(III) Vehicle-ethanol group (Vehicle-EtOH): received vehicle (NADES extraction solution) by gavage for 14 days before induction of gastric ulcer.
(IV) Crude extract of blueberry-ethanol group (CE-EtOH): received 10.0 mg/kg body weight (b.w.) of CE by gavage for 14 days before induction of gastric ulcer.
(V) Anthocyanin-rich fraction-ethanol group (ARF-EtOH): received 4.2 mg/kg b.w. of blueberry fraction rich in anthocyanins by gavage for 14 days before induction of gastric ulcer.
(VI) Non-anthocyanin phenolic fraction-ethanol group (NAPF- EtOH): received 5.8 mg/kg b.w. of blueberry fraction rich in non-anthocyanin phenolics by gavage for 14 days before induction of gastric ulcer.
(VII) Lansoprazole-ethanol group (LAN- EtOH): received 30 mg/kg b. w. of lansoprazole by gavage for 14 days before induction of gastric ulcer.

CE was the ready-to-use, NADES-containing blueberry extract, whereas ARF and NAPF were the NADES-free, isolated fractions obtained as described in Section 2.2. The doses of blueberry extracts were expressed as mg of total polyphenol compounds quantified by HPLC. The dose of 10 mg/kg was chosen on a preliminary study, where CE was administered at 5, 10 and 15 mg of total polyphenols/kg b.w. for 14 days and the best gastroprotective effects were observed at 10 mg/kg (Supplementary material, Fig. 1S and 2S). This preliminary study also revealed no hepatotoxicity of CE assessed by the activity of plasma transaminase activities (Fig. 3S). Polyphenols of CE were composed by 42% anthocyanins and 58% non-anthocyanin phenolic compounds (Supplementary material, Fig. 4S, Table 1S). The doses for the ARF and NAPF groups were equal to the amount of anthocyanins (4.2 mg/kg b. w.) or non-anthocyanin phenolic compounds (5.8 mg/kg b.w.) received by the CE group. LAN was used as a positive control as it is a standard drug used for the clinical treatment of gastric ulcers.

After 14 days of treatment, rats of all experimental groups were fasted for 12 h after the last gavage procedure to ensure an empty stomach and efficient induction of gastric lesions. Gastric lesions were induced by an oral administration of 75% EtOH (2 mL/kg b.w.) (Robert, 1979). EtOH stimulates the instability of gastric mucosa by increasing the mucosal permeability and release of vasoactive products, leading to oxidative stress, vascular damage and necrosis (Adinortey, Ansah, Galyuon, & Nyarko, 2013; Cadirci et al., 2007; Mota et al., 2011).

One hour after EtOH administration, animals were anesthetized with isofluorane and euthanized by cardiac puncture. The stomach of each rat was quickly removed and opened along the great curvature. The glandular gastric tissue was visually inspected for macroscopical analysis of gastric injury (expressed as ulcer index). Then, each stomach was dichotomized, one moiety was immersed in 10% formalin solution for histopathological analyses, whereas the other moiety was divided into 3 parts and stored at −80 °C for biochemicals analyses.

2.5. Gastric lesion index

After opening, the stomachs were fixed to assess the index of the gastric lesion. Flattened stomach samples were photographed and the ulcer area (mm^2) was measured using NIH Image J 1.36b imaging software (National Institutes of Health, Bethesda, MD, USA). The ulcer index was calculated as described by Ganguly (1969) using Eq. (1), where X = total mucosal area/total ulcerated area:

$$\text{Ulcer index (UI)} = 10/X \quad (1)$$

The inhibition of gastric lesions was calculated according to Eq. (2):

$$\text{Ulceration protection}(\%) = \left[\left(\text{UI}_{\text{Water group}} - \text{UI}_{\text{Pretreated group}}\right)/\text{UI}_{\text{Water group}}\right] \times 100 \quad (2)$$

2.6. Histological analysis

Samples of stomach tissue fixed in 10% buffered formalin solution were processed and paraffin embedded. Thereafter, 6 mm thick histological sections were cut and stained with hematoxilin and eosin. Two slides of each sample were prepared and each microscopic slide was evaluated in 10 histological fields by two independent operators in a double-blind method to detect alterations. Samples were evaluated for the integrity of gastric mucosa, hyperemia and neutrophil infiltration.

2.7. Biochemical analysis

Following the macroscopic analyses, stomach samples (0.2 g each), were homogeneized in 0.6 mL of 0.01 M phosphate buffered saline with 0.136 M NaCl (pH 7.4), on ice using an ultra-Turrax homogenizer and centrifuged at 4 °C (5000*g*, 10 min). The supernatants were collected and used for biochemical assays.

2.7.1. Markers of oxidative stress

Advanced oxidation protein products (AOPP) were determined at 340 nm after solubilization of lipids with citric acid (Hanasand et al., 2012). The levels of reduced glutathione (GSH) and oxidized glutathione (GSSG) were measured by the fluorimetric method as previously described by (Hissin & Hilf, 1976). Nitrite and nitrate levels (NO_x) were assessed using vanadium (III) combined with detection by Griess reaction, according to Miranda, Espey, and Wink (2001). Nitrite/nitrate was used as an estimate of nitric oxide ($^{\bullet}NO$) level as they are produced during the oxidative metabolism of $^{\bullet}NO$ (Radi, 2004).

2.7.2. Inflammatory marker

Myeloperoxidase (MPO) activity was determined in the stomach supernatant as previously described (Grisham, Hernandez, & Granger, 1986). Samples of stomach supernatant were mixed with potassium phosphate buffer (50 mM, pH 6.0) and hexadecyltrimethylammonium bromide (0.5%). The kinetic analysis of MPO activity was started by adding hydrogen peroxide (0.005%) and assessed at 450 nm at 25 °C.

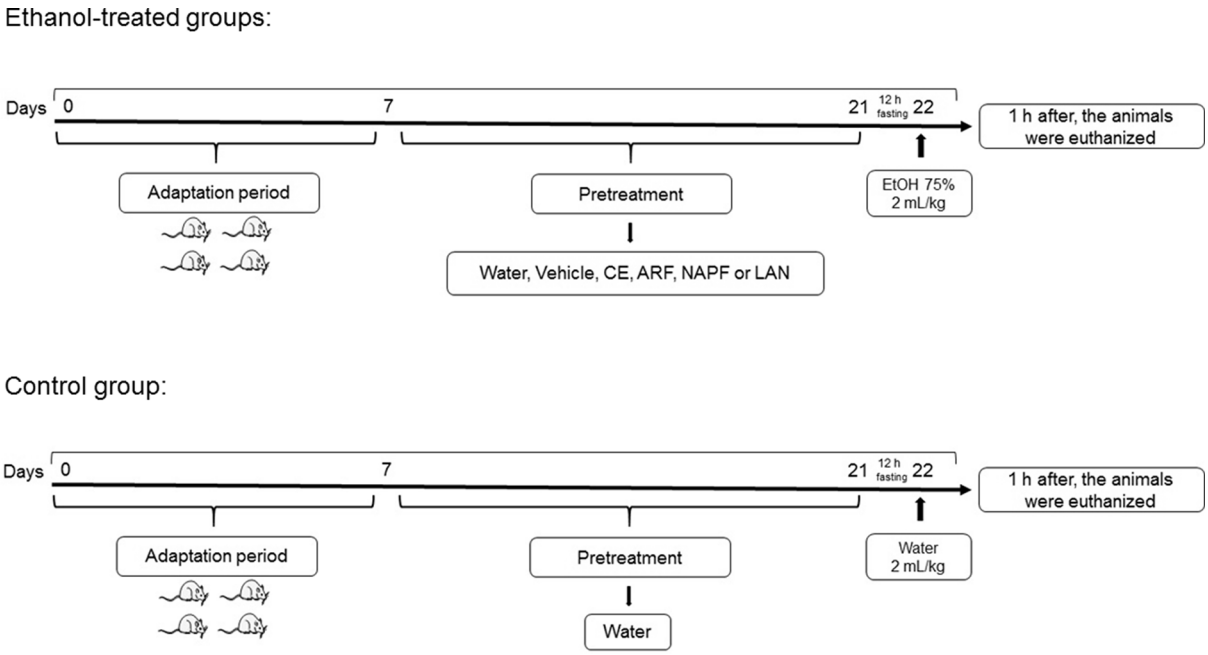

Fig. 1. Experimental design. CE = crude extract of blueberry; ARF = anthocyanins-rich fraction; NAPF = non-anthocyanin phenolics fraction; LAN = lansoprazole; EtOH = ethanol.

2.7.3. Antioxidant defense system

Cu/Zn superoxide dismutase (Cu/Zn SOD) activity was determined by inhibition of nitroblue tetrazolium (NBT) reduction at 560 nm (Spitz & Oberley, 1989). Glutathione reductase (GR) activity was determined using oxidized glutathione and reduced nicotinamide adenine dinucleotide phosphate (NADPH) at 340 nm (Carlberg & Manner, 1979). Glutathione-S-transferase (GST) activity was measured at 340 nm, using 1-chloro −2,4 – dinitrobenzene as the substrate (Habig, Pabst, & Jakoby, 1974). Glutathione peroxidase (GPx) activity was determined using reduced glutathione, GR and NADPH. The method is based on the oxidation of NADPH, which is indicated by a decrease in absorbance at 340 nm (Paglia & Valentine, 1967).

2.8. Nrf2 protein expression

Gastric tissue was homogenized in cold buffer A (pH 7.9), consisting of 50 mM hydroxyethyl piperazineethanesulfonic acid (HEPES), 1 M KCl, 1 M $MgCl_2$, 100 mM ethylenediamine tetraacetic acid (EDTA), 200 mM phenylmethylsulfonyl fluoride (PMSF), 100 μL Triton X-100, 100 mM Na_3VO_4, 1 M dithiothreitol (DTT) and left on ice for 15 min. Then, tissue lysates were centrifuged at 15,000*g* at 4 °C for 30 min and supernatants were collected (cytoplasmic fraction). The sediment was homogenized in cold buffer B (pH 7.9) containing 50 mM HEPES, 1 M KCl, 1 M $MgCl_2$, 5 M NaCl, 100 mM EDTA, 200 mM PMSF, 2.5% glycerol, 100 μL Triton X-100, 100 mM Na_3VO_4, 1 M DTT. Then, samples were sonicated, centrifuged at 15,000*g* at 4 °C for 30 min and the supernatant was collected (nuclear fraction). The protein content was determined according to Lowry (Lowry, Rosebrough, Farr, & Randall, 1951). Protein lysates (50 μg) were resolved on a 12.5% (for cytosolic Nrf2 and β-actin) or 7.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (for nuclear Nrf2 and lamin-B1) and transferred to nitrocellulose membranes. After membrane blocking (5% low-fat dried milk in Tris buffered saline for 1 h) membranes were incubated overnight at 4 °C with primary antibodies for Nrf2 (1:1000; ab31163, Abcam, Cambridge, MA, USA), lamin-B1 (1:4000; 33–2000, ThermoFisher Scientific, MA, USA) or β-actin (1:10000; ACTN05 (C4), ThermoFisher Scientific, MA, USA). Membranes were thereafter probed with HRP-conjugated secondary antibodies and revealed using the Enhanced Chemiluminescence system (Bio-Rad, Hercules, CA, USA). Membranes were scanned using ChemiDoc MP (Bio-Rad, Hercules, CA, USA). Band intensity was quantified with Quantity One software (Bio-Rad, Hercules, CA, USA) and normalized to β-actin (cytoplasmic fraction) or lamin-B1 (nuclear fraction) content.

2.9. Short-chain fatty acids (SCFA) in cecal feces

The SCFAs of fecal samples were extracted as described by Zhao, Nyman, and Jönsson (2006) with some modifications. About 0.5 g of frozen cecal feces were homogenized with 2 mL of ultrapure water, the pH was adjusted to 2–3 using an aqueous solution of 2 N HCl. Samples were centrifuged at 1700*g* for 20 min and the supernatant was collected. The internal standard, 2-ethyl-butyric acid (54.75 mM in 12% formic acid), was added to reach a final concentration of 1 mM. Samples were injected in an Agilent Technologies gas chromatograph (HP 6890 N) equipped with a capillary column Nukol TM (30 m × 0.25 mm; 0.25 μm) and flame ionization detector (FID). Chromatographic conditions were: temperature of injection port was maintained at 200 °C; carrier gas was nitrogen (1 mL/min); injected volume 1 μL with split ratio (10:1). The oven temperature was programmed as follows: kept at 100 °C for 0.5 min, then it was increased to 180 °C at 8 °C/min and maintained at this temperature for 1 min and then the temperature was increased to 200 °C at 20 °C/min and maintained at 200 °C for 10 min. A standard mix of volatile free fatty acids (46975-U, Sigma-Aldrich, St. Louis, MO, USA) were diluted in formic acid solution (12%) and used as external standard.

2.10. Statistical analysis

Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Duncan's *post hoc* test when appropriate. Data that did not meet the ANOVA assumptions were analyzed by Kruskal Wallis analysis, followed by multiple comparison tests. Results were expressed as the mean ± SEM and differences were considered statistically significant when $p \leq 0.05$. Data were analyzed using the

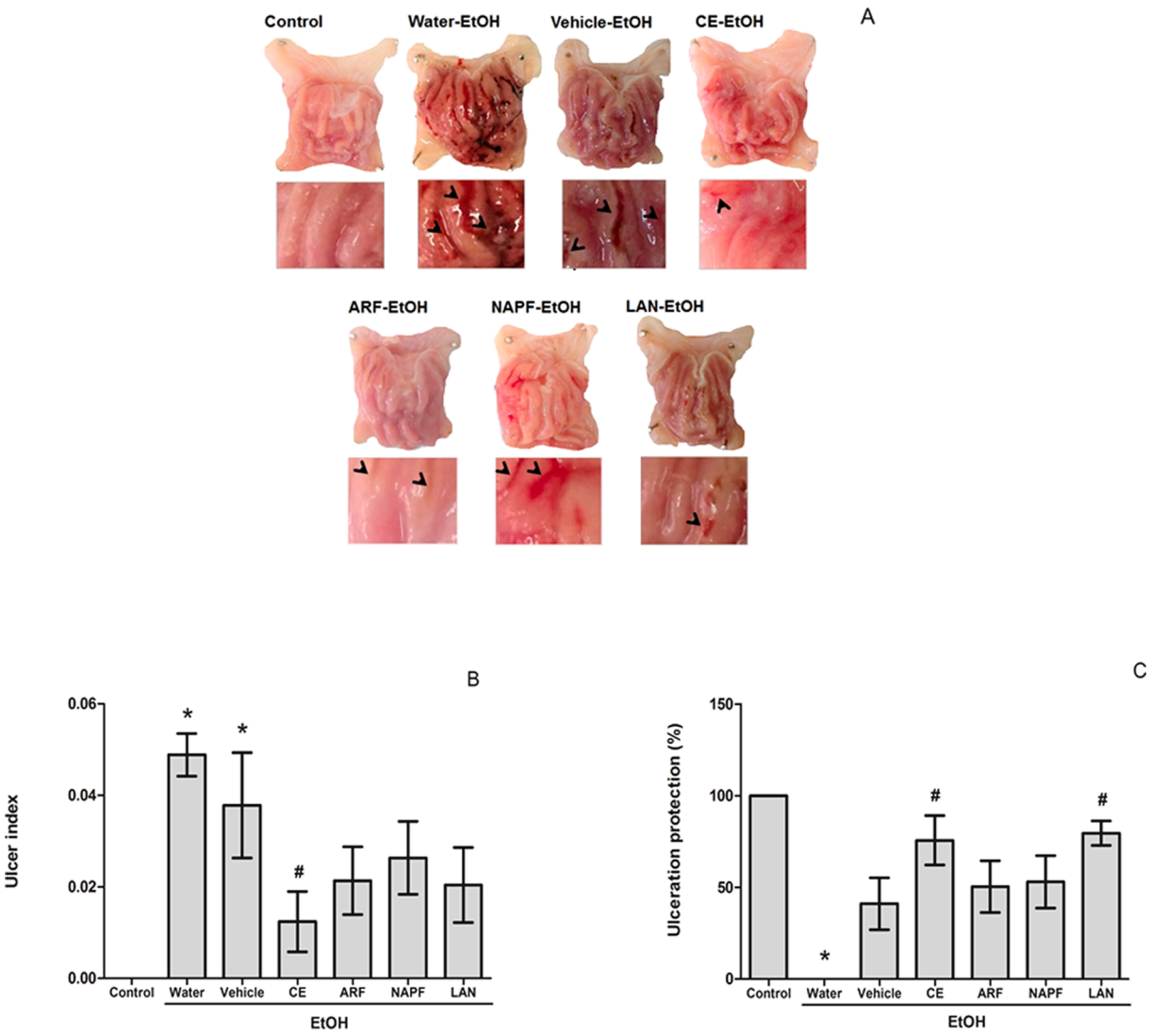

Fig. 2. Effect of blueberry CE and its anthocyanin and non-anthocyanin polyphenol fractions in the macroscopic appearance of stomach (A), ulcer index (B) and ulceration protection (C) (means ± SEM, n = 7). Note ulcerations with hemorrhagic craters (indicated by arrowheads in panel A). CE = crude extract of blueberry; ARF = anthocyanin-rich fraction; NAPF = non-anthocyanin phenolics fraction; LAN = lansoprazole; EtOH = ethanol. *Different from the control group ($p < 0.05$), #Different from the water group ($p < 0.05$).

Statistica® V.7 software system (Statsoft Inc., 2004).

3. Results and discussion

3.1. Composition of blueberry extracts

Blueberry polyphenols were composed by 42% anthocyanins and 58% non-anthocyanin phenolic compounds. Ten compounds were identified in the anthocyanin fraction of the blueberry extract (% of total anthocyanins): delphinidin 3-hexoside (23.78%), delphinidin 3-pentoside 4.49%), cyanidin 3-galactoside (11.90%), petunidin-3-hexoside (12.11%), petunidin 3-pentoside (5.37%), malvidin 3-hexoside (19.80%), peonidin 3-pentoside (3.24%), malvidin 3-pentoside (10.07%), petunidin 3-acetylhexoside (1.44%) and malvidin 3-acetylhexoside (3.72%). The non-anthocyanin polyphenol fraction of blueberry extracts contained (% of total non-anthocyanin polyphenols): catechin (1.41%), chlorogenic acid (69.96%), myricetin 3-hexoside (3.90%), quercetin-3-galactoside (16.52%), quercetin-3-glucoside (4.45%), laricitin 3-hexoside (1.36%) and quercetin-3-pentoside (2.40%) (Supplementary material, Table 1S and Fig. 4S).

3.2. Effect of NADES-based blueberry extracts on EtOH-induced gastric damage

NADES have been recently considered the solvents of the future due to their eco-friendly properties and putatively low toxicity (Misan et al., 2019). However, the feasibility of NADES for yielding ready-to-use plant extracts remains to be supported by appropriate *in vivo* data. The present study provided evidence on the gastroprotective effects and biocompatible properties of NADES-containing blueberry extract in a rat model of gastric ulcers induced by EtOH.

The intragastric administration of EtOH induced macroscopic morphological changes (Fig. 2A). The water-EtOH group had severe mucosal injury characterized by hemorrhagic ulceration and increased ulcer index when compared to the control group (Fig. 2A and B; $p \leq 0.05$), which is in accordance with previous reports on this model (Li et al., 2014). In addition to the macroscopic damage, EtOH also caused histological gastric damage evidenced by disseminated hemorrhage, inflammatory neutrophil infiltration and loss of glandular structure (water-EtOH group, Fig. 3B). Similar histological damage was observed in the vehicle-EtOH group (data not shown). EtOH increases vascular

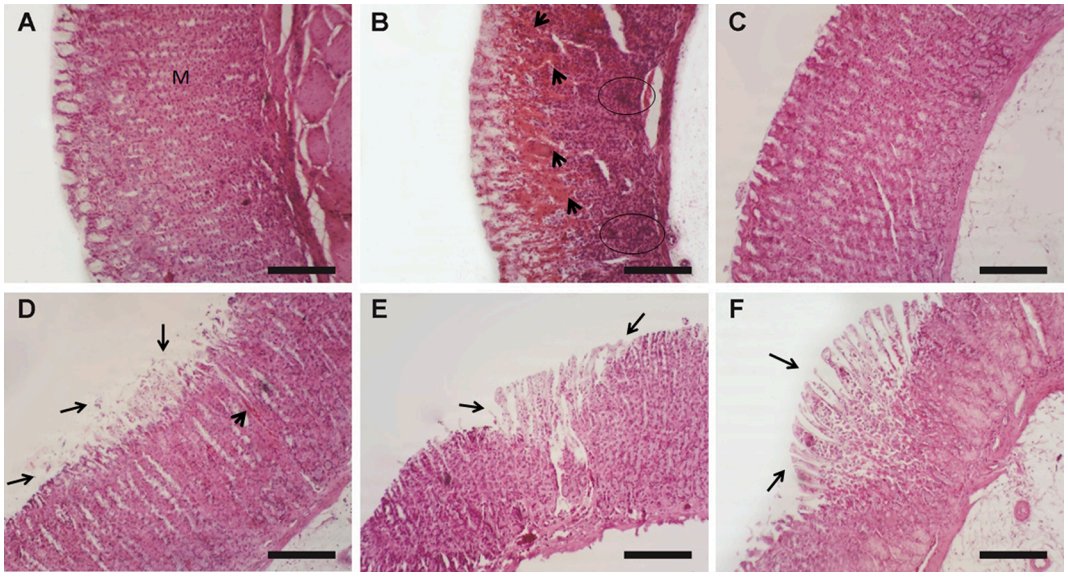

Fig. 3. Representative histology of the stomach tissue from: Control (A); Water-EtOH (B); CE-EtOH (C); ARF-EtOH (D); NAPF-EtOH (E); LAN-EtOH (F) groups. Note inflammatory neutrophil infiltration (circles), structural loss of tissue (arrows) and hyperemia (arrowheads). M = Mucosa; Stomach sections were stained with hematoxylin and eosin. (Scale bar 150x). CE = crude extract of blueberry; ARF = anthocyanin-rich fraction; NAPF = non-anthocyanin phenolics fraction; LAN = lansoprazole.

permeability and exposes gastric mucosa to the proteolytic and hydrolytic actions of pepsin and HCl, besides causing blood flow stasis and microvascular disruption (Mota et al., 2011).

The pretreatment with NADES-based blueberry CE showed notable protection against macroscopic and histopathological gastric lesions induced by EtOH administration ($p < 0.05$), which were clearly observed in the water- and vehicle-EtOH groups (Fig. 2; Fig. 3C vs. Fig. 3B). The ARF and NAPF groups yielded partial protection against EtOH-induced ulcer (Fig. 2), being characterized by superficial hemorrhagic infiltration, light dilation and loss of glandular structure, which was more severe in the NAPF group (Fig. 3). The pretreatment with the vehicle used to obtain the CE extract (NADES alone) did not attenuate EtOH-induced damage (Fig. 2B; $p > 0.05$). The gastric mucosa of animals that received LAN showed little preservation of the histological aspects and were only slightly more preserved than the water-EtOH group (Fig. 3F). As summarized in Table 1, both the anthocyanin and non-anthocyanin polyphenols of blueberry extract appear to have contributed to the protection against gastric tissue damage as rats pretreated with the isolated ARF and NAPF (at equivalent amounts to those found in the CE) had partial protective effect. Blueberry polyphenols appear to have high stability in the stomach as >90% of polyphenols were recovered during an *in vitro* gastric digestion assay (Correa-Betanzo et al., 2014). It is noteworthy that ARF and NAPF were as effective as LAN to prevent EtOH-induced damage, whereas CE had greater effect than LAN (Figs. 2 and 3). LAN is a proton pump inhibitor that has been widely used in the treatment and prevention of gastrointestinal disorders as gastric ulcers (Schubert, 2017) and was used as a positive control in this study. These data indicate that NADES blueberry CE has promising gastroprotective properties.

European blueberry extract (*Vaccinium leschenaultii,* bilberries) obtained using acetone and methanol solvents has been previously demonstrated to protect against EtOH-induced gastric ulcers in rats (Sumbul et al., 2010). However, NADES-based blueberry extract has great advantage over this previously studied extract as the present study demonstrates that NADES is biocompatible and therefore do not require solvent removal before use. This is the first study on the bioactive effect of blueberry NADES extracts and one of the few studies investigating the biological effects of phenolic compounds solubilized in a NADES vehicle in animal models (Murador et al., 2019).

3.3. *Effect of NADES-based blueberry extracts on EtOH-induced changes in gastric oxidative status and antioxidant enzymes*

In addition to mucosal damage, EtOH administration promotes ROS overproduction, which plays a crucial role in the pathogenesis of gastric ulcer (Cadirci et al., 2007; Mota et al., 2011). In the present study, the intragastric administration of EtOH (water-EtOH) caused oxidative stress characterized by increased protein oxidation (AOPP levels) and $^{\bullet}NO$ (assessed as NO_x levels) along with decreased GSH/GSSG ratio in comparison to the control group (Fig. 4; $p \leq 0.05$). GSH is a tripeptide that acts in the primary antioxidant defense system. The decrease of GSH/GSSG ratio has been used as a marker of oxidative stress (Zitka

Table 1
Summary of the gastroprotective effects of NADES-based blueberry extracts expressed as changes relative to the water-EtOH group in the gastric ulcer model.

Treatment	Gastric tissue damage	Oxidative stress	Antioxidant enzymes	Inflammatory response	SCFA production
NADES-vehicle	–	↓ AOPP; ↓ NO_x;⇑ GSH/GSSG	↓ GPx; ↓ GST	–	↓ PROP; ↓ BUT
CE	↓ UI; ↑ UP; ↓ HE	↓ AOPP; ↓ NO_x; ↑ GSH/GSSG	↓ GPx	↓ MPO	–
ARF	⇓ UI; ⇑ UP; ⇓ HE	⇓ AOPP; ↓ NO_x; ↑ GSH/GSSG	↑ Cu/ZnSOD; ↑ GR; ↓↓ GPx; ⇓ GST	↓ MPO	↑ ACET
NAPF	⇓ UI; ⇑ UP; ⇓ HE	↓ AOPP; ↓ NO_x	↓↓ GPx; ↓ GST	⇓ MPO	–
LAN	⇓ UI; ↑ UP; ⇓ HE	⇓ AOP; ↓ NO_x; ↑ GSH/GSSG	↓↓ GR; ↓↓ GPx	↓ MPO	–

CE, crude extract of blueberry; ARF, anthocyanin-rich fraction; NAPF, non-anthocyanin phenolics fraction; LAN, lansoprazole; EtOH, ethanol; UI, ulcer index; UP, ulcer protection; HE, hematoxylin-eosin histological damage; AOPP, advanced oxidation protein products; NO_x, nitrite and nitrate levels; GSH, reduced glutathione; GSSG, oxidized glutathione; Cu/ZuSOD, cupper, zinc-superoxide dismutase; GR, glutathione reductase; GPx, glutathione peroxidase; GST, glutathione-s-tranferase; MPO, myeloperoxidase activity; PROP, propionate; ACET, acetate; BUT, butyrate; SCFA, short-chain fatty acids; –, no change relative to the water-EtOH group ; ↑ or ↓, increase or decrease change relative to the water-EtOH group; ⇑ or ⇓, partial increase or partial decrease effect relative to the water-EtOH group.

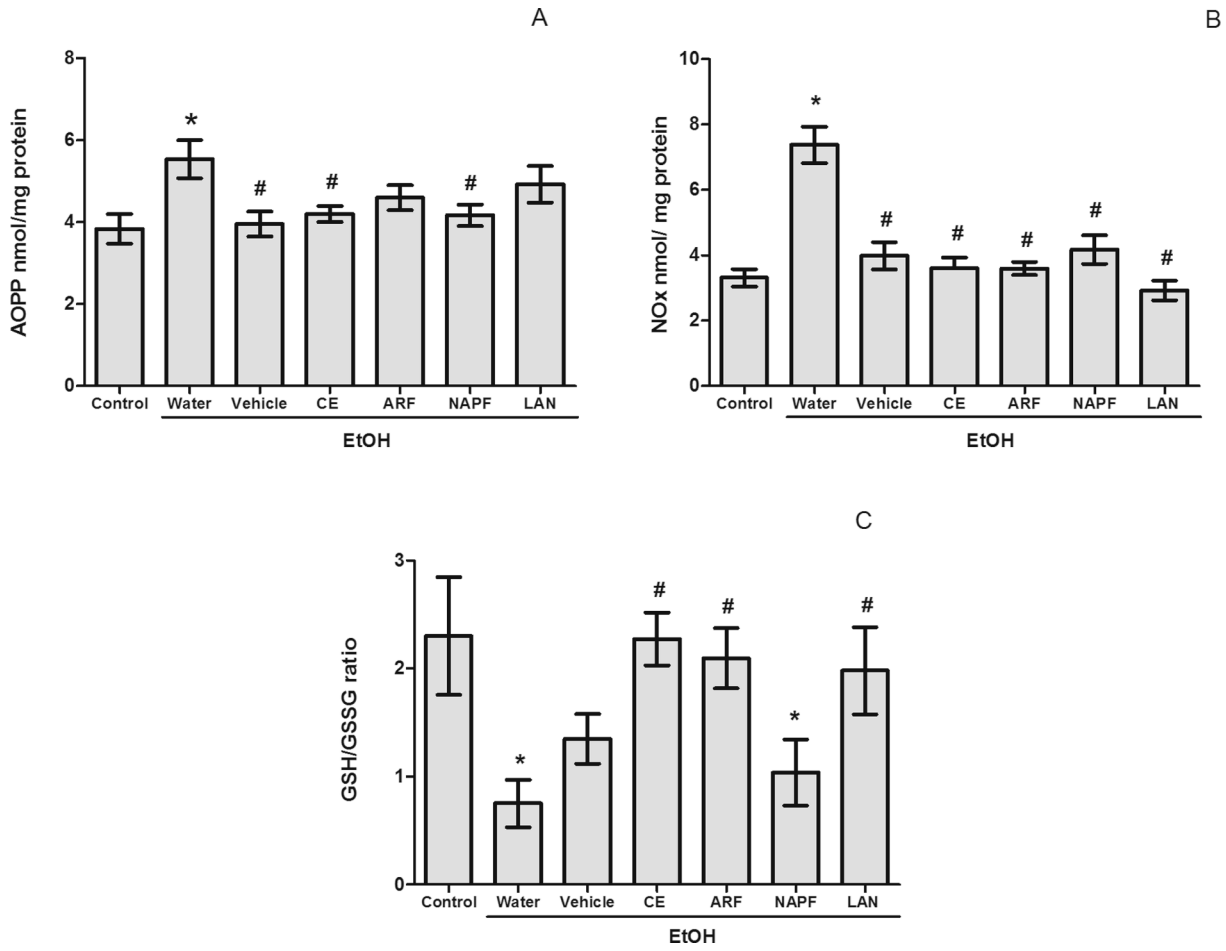

Fig. 4. Effect of blueberry CE and its anthocyanin and non-anthocyanin polyphenol fractions on AOPP (A) and NO_x levels (B) (means ± SEM, n = 8), and GSH/GSSG ratio (C) (means ± SEM, n = 4). AOPP = advanced oxidation protein products; NO_x = nitrite and nitrate levels. GSH = reduced glutathione, GSSG = oxidized glutathione; CE = crude extract of blueberry; ARF = anthocyanin-rich fraction; NAPF = non-anthocyanin phenolics fraction; LAN = lansoprazole; EtOH = ethanol. *Different from the control group ($p < 0.05$), #Different from the water group ($p < 0.05$).

et al., 2012). EtOH also induced remarkable changes in the activity of antioxidant enzymes (Fig. 5). Compared to the control group, GPx and GST activities were increased, whereas SOD and GR activities were decreased in the water-EtOH group (Fig. 5; $p \leq 0.05$). The concomitant increase of GPx and GST likely contributed to decrease the levels of GSH, which is a co-factor for these enzymes (Habig et al., 1974; Paglia & Valentine, 1967). Moreover, the activity of GR, which is responsible for recycling oxidized glutathione, was decreased by>50% in the water- and vehicle-EtOH groups. These results show that EtOH administration promotes oxidative stress along with remarkable changes in enzymatic and non-enzymatic antioxidant defense systems, collaborating to the damage of gastric mucosa.

The pretreatment with NADES-vehicle, CE and NAPF completely prevented AOPP changes, whereas ARF and LAN partially prevented this effect (Fig. 4A; $p \leq 0.05$). Additionally, pretreatment with all blueberry extracts, NADES-vehicle or LAN were able to completely prevent the increase in NO_x levels (Fig. 4B; $p \leq 0.05$). The decrease in GSH/GSSG ratio was completely prevented by CE, ARF and LAN treatment but partially prevented by NADES-vehicle (Fig. 4C; $p \leq 0.05$). The gastroprotective effects of blueberry CE and its fractions (ARFs and NAPFs) were associated to a decrease in the oxidative stress in gastric mucosa, namely, decreased AOPP and NO_x levels and increased GSH/GSSG ratio as summarized in Table 1. The protective effect against the EtOH-induced increase in protein oxidation and NO_x levels was associated to the anthocyanin and non-anthocyanin fractions of blueberry extract (Fig. 4; Table 1). In addition, the NADES vehicle (vehicle-EtOH group) also appears to contribute to the protective effect of crude blueberry extracts against increased protein oxidation and NO_x levels (Fig. 4. Table 1). This result reveals that NADES are not only biocompatible but may also have interesting protective properties *per se*. To our knowledge there are no previous data on the protective effects of NADES mixtures *in vivo*. Although the effect of NADES in a gastric ulcer model is being explored for the first time, one of the natural components of the NADES mixture, citric acid, has been demonstrated to contribute to the gastroprotective effect of black tea against gastric ulcers induced by ethanol (Raychaudhuri, Ghosh, Roy, & Snehasikta Swarnakar, 2019).

$^{\bullet}NO$ is a signaling molecule that maintains gastric mucosal integrity, however, there is huge evidence that $^{\bullet}NO$ also contributes to gastric injury during overactivation of inflammatory response (Al-Quraishy, Othman, Dkhil, & Abdel Moneim, 2017). Macrophage migration in the gastrointestinal tract promotes the generation of ROS, which activate proinflammatory cytokines and result in the overexpression of iNOs (inducible nitric oxide synthase) and increased $^{\bullet}NO$ levels. $^{\bullet}NO$ reacts with superoxide anion ($O_2^{\bullet-}$) and produces the potent prooxidant peroxynitrite (ONOO–), a biologically relevant oxidizing and nitrating agent (Radi, 2004). Accordingly, was observed that increased $^{\bullet}NO$ levels were accompanied by increased protein oxidation (AOPP levels) in the water-EtOH group (Fig. 4A and B). Thus, the protective effect of NADES blueberry extracts against EtOH-induced oxidative stress in the stomach may be secondary to an anti-inflammatory effect. Alternatively, this gastroprotective effect may be also related to the direct free radical scavenging capacity of flavonoid polyphenols, which has been mainly

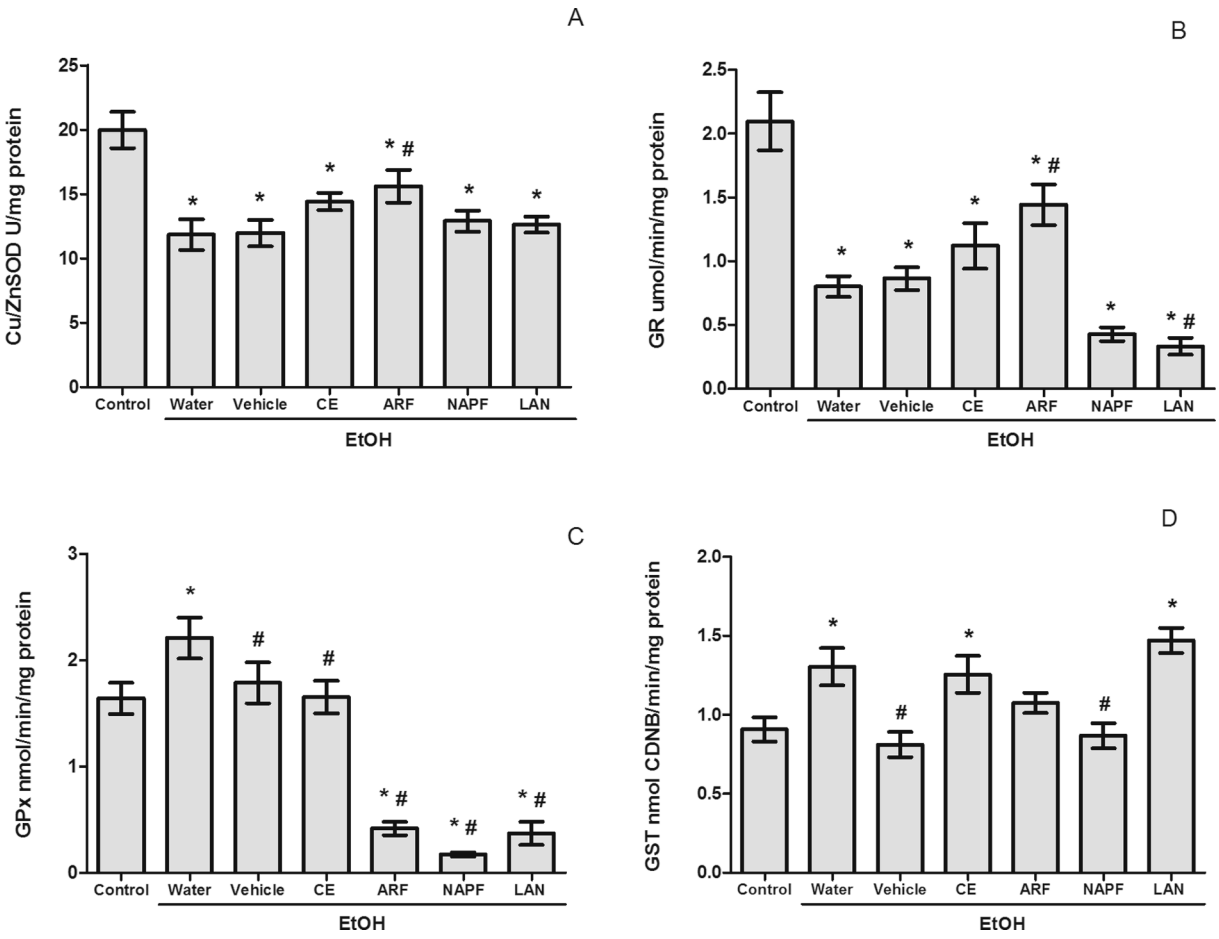

Fig. 5. Effect of blueberry CE and its anthocyanin and non-anthocyanin polyphenol fractions on CuZnSOD (A), GR (B), GPx (C) and GST (D) activities (means ± SEM, n = 8). CuZnSOD = cupper, zinc-superoxide dismutase; GR = glutathione reductase; GST = glutathione-s-tranferase; GPx = glutathione peroxidase; CE = crude extract of blueberry; ARF = anthocyanin-rich fraction; NAPF = non-anthocyanin phenolics fraction; LAN = lansoprazole; EtOH = ethanol. *Different from the control group ($p < 0.05$), #Different from the water group ($p < 0.05$).

attributed to the presence of an *ortho*-catechol group in B-ring (Silva et al., 2009). In fact, a direct radical scavenging assay revealed that blueberry ARF extracted using NADES solvent has greater potency to remove oxygen radicals (ORAC assay) than ARF obtained using conventional organic solvents (Supplementary material, Fig. 5S). This greater potency may be due to the fact that NADES improves the solubility and bioavailability of flavonoids (Faggian et al., 2016) and yields extracts bearing greater amount of arabinoside anthocyanins compared to the organic solvents (Silva et al., 2020).

Additionally, the present study highlights the protective effect of the anthocyanin fraction of blueberry extract, which was as effective as LAN to prevent GSH depletion and appears to be responsible for the protective effect of CE against GSH depletion (Fig. 4C; $p \leq 0.05$; Table 1). Moreover, ARF was the sole treatment that prevented the decrease of SOD and GR activities induced by EtOH (Fig. 5A and B; $p \leq 0.05$), whereas LAN pretreatment caused an additional decrease in GR activity (Table 1). Blueberry ARF is mainly composed by delphinidin and malvidin derivatives, followed by petunidin derivatives. Although there are no studies on these compounds in models of gastric ulcer, the anthocyanin cyanidin-3-glucoside, whose aglycone was also found in ARF, has been demonstrated to exhibit gastroprotective properties (Li et al., 2008). Moreover, blueberry ARF contained acylated anthocyanins as petunidin 3-acetylhexoside and malvidin 3-acetylhexoside, which have been demonstrated to be more stable than the non-acylated forms in the stomach and are able to remove superoxide radicals (Correa-Betanzo et al., 2014). The pretreatments with CE or NADES vehicle prevented the increase in GPx activity caused by EtOH, whereas the pretreatment with ARF, NAPF or LAN caused a remarkable decrease in the activity of this enzyme compared to the control group (Fig. 5C; $p \leq 0.05$). Similar to ARF, anthocyanins from *Rubus coreanus* exhibited antiulcer activity in a rat model of acute ulcer by preventing changes in the activities of SOD and GPx (Kim et al., 2011). In addition, an anthocyanin-rich extract from grape peel has been demonstrated to recover GSH levels by promoting the recycling of oxidized glutathione in a colitis model (Maurer, Cazarin, Quatrin, Nichelle, et al., 2020).

GSTs are phase II detoxifying enzymes that remove ROS and xenobiotics by catalyzing their conjugation to GSH (Habig et al., 1974). As expected, EtOH administration increased GST activity and contributed to decrease GSH/GSSG ratio (Fig. 4C and 5D; $p \leq 0.05$). The pretreatment with LAN did not reduce GST activity, most likely because this enzyme is involved in the metabolization of LAN (Agnihotri, Kaur, Kaur, & Sarotra, 2007). The pretreatment with blueberry fractions (ARFs and NAPFs) or NADES-vehicle partially or completely prevented EtOH-induced changes in GST, but surprisingly the pretreatment with CE did not prevent the increase in GST activity (Fig. 5D; $p \leq 0.05$). This finding suggests an interaction between polyphenols and NADES that abolished the decrease in GST activity. Thus, the recovery of GSH/GSSG ratio promoted by CE appears to be related to a direct sparing of GSH content rather than to increased GSH recycling (GR) or decreased consumption by GST (Table 1).

The induction of enzymatic antioxidant defense system is crucial for the homeostatic response against intracellular oxidative stress

(Carrasco-Pozo et al., 2016). The beneficial effects of flavonoid compounds in NSAIDs-induced gastric ulcer models has been associated to their ability to activate the Nrf2 pathway (Carrasco-Pozo et al., 2016; Jung et al., 2010). The gastroprotective effect of olive (*Olea europaea*) leaf methanolic extract against gastric lesions induced by HCl/EtOH was mediated by cytoprotective and antioxidant mechanisms attributed to the transcriptional activation of various genes implicated in the cell redox balance (Al-Quraishy et al., 2017). Nrf2 is a transcription factor that is sequestered in the cytosol by a Keap1 homodimer. Following the exposure to oxidative stimulus or activation of the MAPK/ERK (p38) pathway, Nrf2 is translocated to the nucleus, where it binds to the antioxidant response element (ARE) and promotes the transcription of antioxidant enzymes (Jung et al., 2010). EtOH-treated rats had no significant activation of Nrf2 pathway assessed by its translocation to the nucleus (Fig. 6; $p > 0.05$). Although ethanol is a potent inducer of oxidative stress, the short time between ethanol administration and rat euthanasia (1 h) was likely insufficient to promote detectable Nrf2 translocation to the nucleus. In fact, a significant reduction of the Nrf2 cytosolic fraction has been detected only when animals were euthanized 3 h after the administration of HCl in an acute model of gastric lesion (Ueda et al., 2008). The pretreatment with blueberry NADES extracts or LAN did not change Nrf2 translocation to the nucleus compared to the control or EtOH-treated rats (Fig. 6; $p > 0.05$). However, the possibility that polyphenol treatment might have induced translocation of Nrf2 at some earlier time during the treatment cannot be ruled out.

3.4. Effect of NADES-based blueberry extracts on EtOH-induced gastric inflammation

The activation and infiltration of neutrophils is involved in the start of gastric ulcer lesions due to an excessive inflammatory response (Araújo et al., 2018) that can be assessed by the increased activity of MPO in the tissue (Arab, Salama, Omar, Arafa, & Maghrabi, 2015). Accordingly, gastric mucosal lesions induced by EtOH were characterized by neutrophil infiltration (Fig. 2) and were accompanied by the acute rise of MPO activity (Fig. 7; $p \leq 0.05$). The pretreatment with CE, ARF or LAN protected the histological structure of the gastric mucosa by preventing the infiltration of inflammatory cells (neutrophils) and the increase in MPO activity (Fig. 7; $p \leq 0.05$), whereas NAPF had partial preventive effect. This result provides evidence that blueberry polyphenols have anti-inflammatory effects, with a greater effect for the anthocyanin-rich fraction compared to the non-anthocyanin fraction. These results are in line with previous studies where anthocyanin-rich extracts of *Aronia melanocarpa* (Black Chokeberry) reduced the levels of pro-inflammatory mediators like MPO in EtOH-induced gastric ulcers

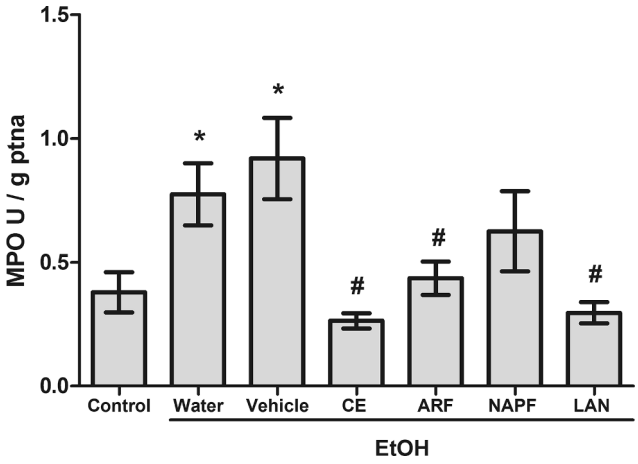

Fig. 7. Effect of blueberry CE and its anthocyanin and non-anthocyanin polyphenol fractions in MPO activity (means ± SEM, n = 4). MPO = myeloperoxidase activity; ARF = anthocyanin-rich fraction; NAPF = non-anthocyanin phenolics fraction; LAN = lansoprazole; EtOH = ethanol. *Different from the control group ($p < 0.05$), #Different from the water group ($p < 0.05$).

(Paulrayer et al., 2017). Interestingly, the anti-inflammatory effects of NADES-extracted blueberry polyphenols were comparable to the anti-ulcer reference drug, LAN.

3.5. Effect of NADES-based blueberry extracts on the fecal content of short chain fatty acids (SCFA) after EtOH administration

The fermentation of non-digestible carbohydrates by the intestinal microflora yields SCFAs, which have numerous beneficial effects in the intestinal mucosa (Den Besten et al., 2013). In addition to local effects on the gut, SCFAs can influence systemic molecular and cellular processes (Levy, Thaiss, & Elinav, 2016). Polyphenol-rich extracts have been demonstrated to modulate gut microbiota and the production of SCFAs (Fotschki, Juśkiewicz, Sójka, Jurgoński, & Zduńczyk, 2015; Gowd et al., 2019; Maurer et al., 2019). In the present study, the fecal concentrations of SCFA (acetic, propionic, and butyric acids) were not affected by the administration of ethanol when compared to the control group (Fig. 8). Only the pretreatment with ARF increased fecal concentration of acetic acid (Fig. 8A; $p \leq 0.05$), whereas NADES vehicle decreased propionic and butyric acid concentrations (Fig. 8B and C; $p \leq 0.05$). Interestingly, oral administration of acetate has been recently demonstrated to attenuate the gastric mucosal damage induced by

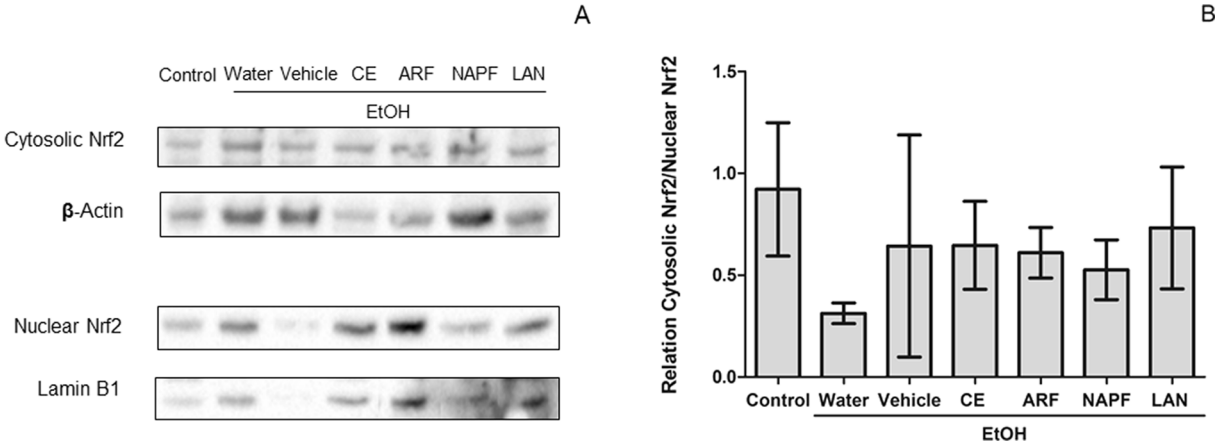

Fig. 6. Effect of blueberry CE and its anthocyanin and non-anthocyanin polyphenol fractions on Nrf2 translocation in gastric mucosa. (A) Representative blots of cytosolic and nuclear Nrf2, β-actin and lamin-B1 expression and (B) Cytosolic/Nuclear Nrf2 content ratio; ARF = anthocyanin-rich fraction; NAPF = non-anthocyanin phenolics fraction; LAN = lansoprazole; EtOH = ethanol. No significant differences were observed among groups ($p > 0.05$).

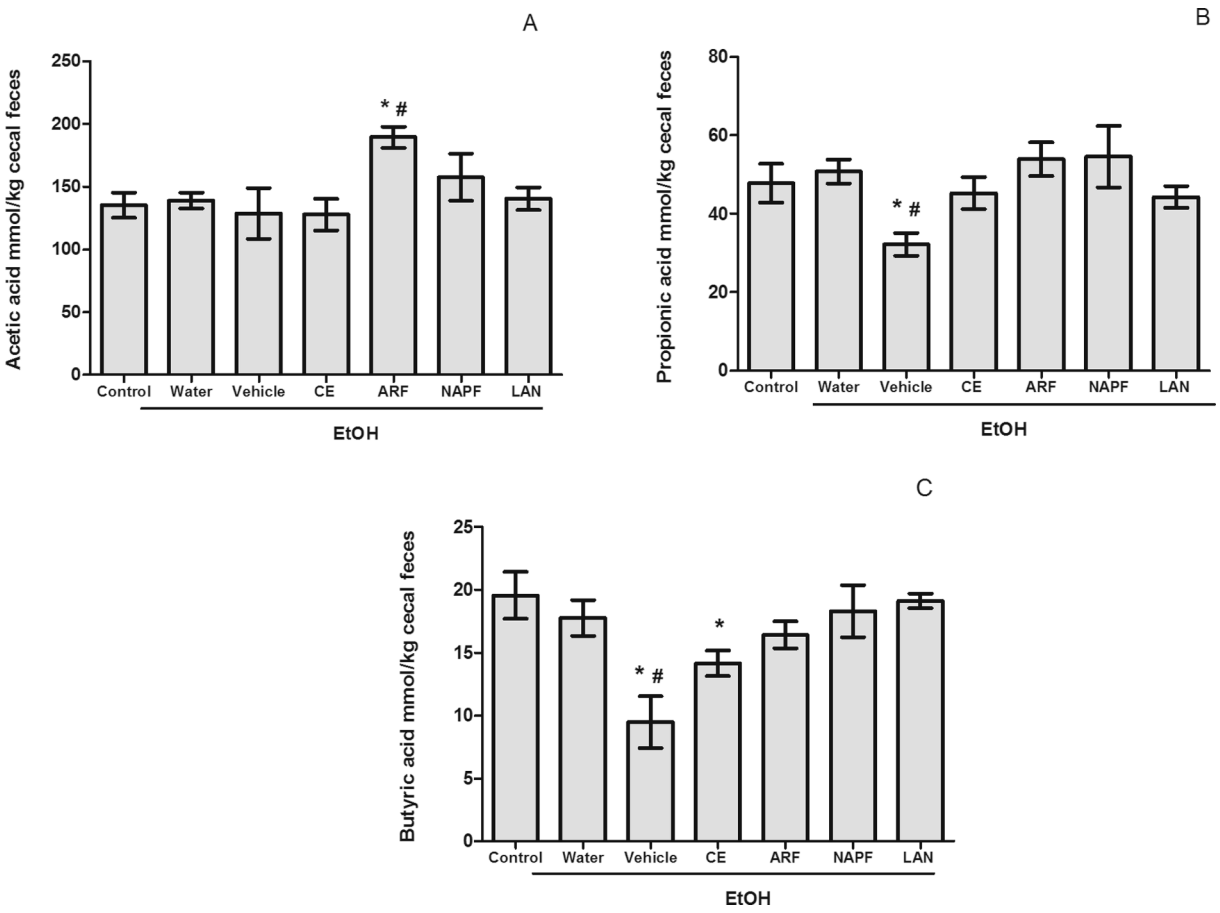

Fig. 8. Effect of blueberry CE and its anthocyanin and non-anthocyanin polyphenol fractions on short-chain fatty acid content of cecal feces. ARF = anthocyanin-rich fraction; NAPF = non-anthocyanin phenolics fraction; LAN = lansoprazole; EtOH = ethanol. *Different from the control group ($p < 0.05$), #Different from the water group ($p < 0.05$).

ethanol (Liu et al., 2017). Therefore, the increase in acetate levels is likely responsible for the protective effect of anthocyanins against EtOH-induced gastric ulcers.

4. Conclusion

The ready-to-use NADES-based crude extract of blueberry attenuated EtOH-induced gastric lesions corroborating the biocompatibility and revealing the gastroprotective properties of this extract. These gastroprotective properties were associated to the mitigation of oxidative stress and neutrophil infiltration. The anthocyanin fraction played a major role in the gastroprotective effects but there was also a contribution from the non-anthocyanin polyphenol fraction and from NADES vehicle that mitigated gastric oxidative stress. The anthocyanin fraction, which increased fecal SCFA levels, appears to be the sole responsible for preventing GSH depletion and had greater anti-inflammatory effect than the non-anthocyanin fraction. Thus, choline chloride:glycerol:citric acid NADES yields ready-to-use, biocompatible crude blueberry extracts that can be further exploited in human studies as a nutritional adjuvant strategy in the prevention of gastric ulcers.

CRediT authorship contribution statement

Dariane Trivisiol da Silva: Conceptualization, Investigation, Methodology, Data curation, Writing - original draft, Writing - review & editing, Visualization. **Renata Fritzsche Rodrigues:** Investigation, Methodology, Data curation, Visualization. **Natália Minuzzi Machado:** Investigation, Methodology, Data curation. **Luana Haselein Maurer:** Methodology, Formal analysis. **Lauren Fresinghelli Ferreira:** Investigation, Methodology. **Sabrina Somacal:** Methodology, Data curation, Formal analysis. **Marcelo Leite da Veiga:** Investigation, Resources. **Maria Izabel De Ugalde Marques Da Rocha:** Investigation, Resources. **Marcia Vizzotto:** Resources, Writing - original draft. **Eliseu Rodrigues:** Methodology, Resources. **Milene Teixeira Barcia:** Conceptualization, Supervision, Writing - original draft. **Tatiana Emanuelli:** Conceptualization, Resources, Supervision, Writing - original draft, Writing - review & editing, Funding acquisition, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2020.109718.

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