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Anti-inflammatory effect of the medicinal herbal mixture infusion, Horchata, from southern Ecuador against LPS-induced cytotoxic damage in RAW 264.7 macrophages

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ABSTRACT

The phytochemical composition and the antioxidant and anti-inflammatory activities of a mixture of 23 plants, named Horchata, traditionally consumed in Ecuador, have been evaluated. The study was carried out using the hydroalcoholic extract (HHext) and infusion (IHext) of the horchata plant mixture. It was verified that thermal treatment affected the contents of vitamin C and carotenoids, but hardly those of polyphenols, which would be the main bioactive compounds in the infusion, the common form of preparation of horchata for consumption. Among phenolic compounds, caffeoylquinic acids, flavones and flavonols (mostly quercetin glycosides) were prominent. Both HHext and IHext extracts managed to protect RAW 264.7 macrophages against LPS-induced cytotoxic damage, increasing the levels of endogenous antioxidant enzymes and modulating the production of pro-inflammatory and anti-inflammatory cytokines. Greater protective effects were obtained for HHext compared to IHext, which was in agreement with its higher content of phenolic compounds favoured by a more efficient extraction in the hydroalcoholic medium. Nonetheless, the infusion still maintained a significant antioxidant and anti-inflammatory activity, which would support the protective effects on health traditionally attributed to its consumption by the population.

1. Introduction

The horchata drink, also known as “aguas frescas” or “agua de frescos” is a popular herbal tea infusion, which has been consumed in the province of Loja, located in the southern inter-Andean region of Ecuador, since colonial times. The drink consists of a mixture of medicinal plants, normally ranging from 16 to 32 locally-produced plants, to which one adds sugar, honey or raw cane sugar and drops of lemon (Villamagua-Vergara, 2014). The infusion is particularly traditional among the indigenous people of the region who took the customs brought by the colonisers and adapted them to their culture, ancestral knowledge and the region's characteristics. So, the indigenous people combined plants locally known as “calientes” (warm) and “frías” (cold) in order to enhance the therapeutic effect of the drink (Villamagua-Vergara, 2014).

The tradition of consuming the horchata drink persists nowadays and is supported by the beneficial effects attributed to it, such as its capacity to promote healthy digestion and improve memory, as well as its hepatic anti-inflammatory capacity and diuretic action (Rios et al., 2017). In fact, these traditional beliefs have been supported by several

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studies that have confirmed that some culturally important medicinal plants are effective against digestive, circulatory, nervous and respiratory disease. Rios et al. (2017) found that 66% of the medicinal plants commonly used to prepare "horchata" were traditionally associated with anti-inflammatory capacities, while 51% were used by their supposed analgesic properties. Despite the potential that these plants have in the search for new bioactive compounds for use in health, the studies on drinks prepared with medicinal plants in Ecuador are still scarce, considering the ancestral knowledge of the indigenous populations of the region, as well as the floral richness that the Andean region offers (Bailon-Moscoso et al., 2017; Rios et al., 2017). On the other hand, the effect of cooking on the availability of the phytochemicals present in the plants used to make the horchata infusion is not known either. It is well known that cooking can affect the stability of plant and food constituents, including bioactive compounds, so that it could be expected that the thermal processing used for horchata preparation might change the profile of compounds present in the infusion compared to the original phytochemical composition of the different plants, thus affecting their biological properties. In this backdrop, the aim of this study was to determine the phytochemical composition and anti-inflammatory and antioxidant capacities of horchata obtained from a mixture of 23 plants medicinal plants commonly used to prepare this herbal drink. The contents of total phenolics, flavonoids, anthocyanins, free amino-acids, carotenoids and vitamin C, and the antioxidant capacity were determined in the infusion, the traditional form of consumption by the population, and in the hydroalcoholic extract of the plant mixture as a control to compare the efficiency of the infusion process and its influence on the phytochemical profile. The same variables were also analysed in each individual plant, in order to evaluate their contribution to the horchata bioactive profile.

2. Material and methods

2.1. Plant materials and preparation

Plant materials (0.5 kg per specimen) were collected on two different occasions during 2018 from the most important popular open market in Loja city, capital of the province of Loja, located in the southern inter-Andean region of Ecuador at an elevation of 2.060 m above sea level. The plants are usually sold in a group locally named as "atado", "manojo", "ramillete" or "tongo" that is equivalent to the term "portfolio" (Bussmann et al., 2007). A total of 23 plants from the list of the most frequent plant species used to prepare horchata (Rios et al., 2017) were obtained. Table 1 shows the common name, scientific name and voucher number of each of the specimens. The specimens were identified by specialists at the 'Jardín Botánico de Quito', Quito, Ecuador, using the reference samples found in the herbarium of this centre.

Plant samples were ground to a fine powder in liquid nitrogen using an analytical mill (IKA A11 basic) and stored at −20 °C until analysis. A mixture containing the 23 plants was used to prepare the horchata mix. For this, equal amounts of the fine powder of each plant were weighed independently and then combined to form a unique mixture of the plants. This mixture was used to obtain the horchata hydroalcoholic extract and the infusion. In addition, hydroalcoholic extracts and infusions were also prepared from each single plant.

The hydroalcoholic extracts were obtained as described by Wojdyło et al. (2007). Fine powder (1 g) from the plants or the horchata mix was extracted in 20 mL of 80% aqueous methanol solution, stirred and left for 24 h in the dark at room temperature. The mixture was centrifuged in two sequential times (10 min, 1500 *g*) and the supernatant was filtered through a 0.45 μm Minisart filter (RephiLe Bioscience Ltd). The infusions were prepared in the same way as for traditional horchata. Firstly, water was heated to boiling point and then removed from the fire, afterwards 1 g of fine powder from the horchata mix or a single plant was added to 20 mL of hot water and allowed to cool at room temperature. After that, the infusions were filtered through Whatman® cellulose filter paper (25 μm). The preparations of the horchata mixture were concentrated under vacuum to obtain dried hydroalcoholic (HHext) and infusion extracts (IHext), while the hydroalcoholic extracts from the different plants were stored at −20 °C until analysis and their infusions analysed in the same day of preparation.

Table 1
Medicinal plants used to prepare the "Horchata" infusion.

Common name	Scientific name	Voucher number
Ataco	*Amaranthus hybridus*	26921
Albahaca	*Ocimum basilicum* L.	26909
Borraja	*Borago officinalis* L.	26965
Cedrón	*Aloysia triphylla* (L'Hér.) Britton	26964
Culantrillo	*Adiantum concinnum* Humb. & Bonpl. ex Wild.	26967
Cola de caballo	*Equisetum bogotense* Kunth	26935
Congona	*Peperomia inaequalifolia* Ruiz & Pav.	26950
Canela	*Cinnamomum* sp.	26939
Escancel	*Aerva sanguinolenta* L. (Blume).	26923
Stevia	*Stevia rebaudiana* (Bertoni) Bertoni	26943
Hierba luisa	*Cymbopogon citratus* (DC.) Stapf	26917
Hoja de naranja	*Citrus* x *aurantium* L.	26920
Llantén	*Plantago major* L.	26966
Malva esencia	*Malva* sp.	26912
Malva olorosa	*Pelargonium odoratissimum* (L.) L'Hér.	26915
Menta	*Mentha* x *piperita* L.	26927
Manzanilla	*Matricaria chamomilla* L.	26974
Malva blanca	*Althaea officinalis* L.	26961
Orégano dulce	*Origanum vulgare* L.	26916
Pena – pena	*Fuchsia loxensis* Kunth	26970
Pimpinela	*Pimpinella aromatica* M. Bieb.	26910
Toronjil	*Melissa officinalis* L	26956
Violeta	*Viola odorata* L.	26973

2.2. Total phenolic, flavonoid, anthocyanins and free amino acids contents

Total phenolic, flavonoid, anthocyanins and free amino acids contents were determined in both the hydroalcoholic extracts and the infusions. The total phenolic content (TPC) was determined using the Folin-Ciocalteu method (Singleton et al., 1998) and the results were expressed as milligrams of gallic acid equivalents (GAE) (0.2–1.0 mM, $y = 1.5986x - 0.0565$, $R^2 = 0.995$) per 100 g of fresh weight (FW) of plant (mg GAE/100 g FW). Total flavonoid content (TFC) was determined spectrophotometrically (Dewanto et al., 2002) and the results expressed as milligrams of catechin equivalents (Cateq) (0.02–0.155 μg/mL, $y = 2.1942x - 0.026$, $R^2 = 0.9963$) per gram of fresh weight of plant (mg Cateq/g FW). Total anthocyanin (ACY) content was determined using a modified pH differential method (Giusti and Wrolstad, 2005), with minor modifications, and the results were expressed as milligrams of Pg-3-gluc equivalents (PgEq) (5–150 mg/mL, $y = 0.0064x - 0.0335$, $R^2 = 0.9881$) per gram of FW of plant (mg PgEq/g FW). Free amino acids were quantified by the Cd-ninhydrin method (Doi et al., 1981) and the results expressed as mg of leucine equivalents (LEeq) (2.4–60 mg/mL, $y = 0.0064x - 0.0027$, $R^2 = 0.9987$) per 100 g of fresh weight and mg of proline equivalents (PROLeq) (0.01–0.3 mg/mL, $y = 10.218x + 0.0014$, $R^2 = 0.9977$) per gram of fresh weight of plant (mg LEeq/100g FW; mg PROLeq/g FW).

2.3. Determination of total antioxidant capacity

The total antioxidant capacity (TAC) of the hydroalcoholic extracts and the infusions was determined using the Ferric Reducing Antioxidant Power (FRAP) assay (Benzie and Strain, 1996) and the 2,2-diphenyl-1-picrylhydrazyl free radical method (DPPH) (Prymont-Przyminska et al., 2014) and the results were expressed as μmol of Trolox equivalents (TEq) (5–500 μM, $y = 0.1181 + 0.6477$, $R^2 = 0.9922$) per 100 g of fresh weight of plant (μmol TEq per g FW) for

all the assays.

2.4. HPLC-UV determination of vitamin C content

Vitamin C content was determined directly in the fine powders and also in the infusions (Gasparrini et al., 2017). For the fine powder, 0.5 g of the sample was extracted in 10 mL of the extraction solution [metaphosphoric acid and acetic acid (73/84 p/v)], sonicated for 20 min in ice in the dark, filtered through a syringe filter (0.45 μm, RephiLe Bioscience Ltd) and 20 μL of this solution was immediately injected into the HPLC system, while for infusions, samples were filtered through a syringe filter (0.45 μm, RephiLe Bioscience Ltd) and then 20 μL was immediately analysed by HPLC.

The HPLC system (Agilent Technologies Series 1260, Santa Clara, California, United States) consisted of a Quaternary Pump (Agilent Technologies 1260 Infinity G1312B) and a Diode Array Detector (PDA) (Agilent Technologies 1260 Infinity G1315C DAD) set at an absorbance of 245 nm. The Eclipse Plus C18 column (5 μm, 4.6 × 150 mm) was used as the stationary phase and elution was performed with 50 mM KH_2PO_4 (pH 2.5) in an isocratic gradient at a flow rate of 1 mL/min for 20 min. Ascorbic acid standard (5 mg/L - 50 mg/L, $y = 235.54x + 53.13$, $R^2 = 0.9975$, LOQ: 0.598 mg/L and LOD: 0.179 mg/L) was used for the calibration curve and total vitamin C content was expressed as mg of ascorbic acid per 100 g of fresh weight of plant or plant mixture (mg Vit C/100 g FW) or mg of ascorbic acid per mL of infusion (mg Vit C/mL).

2.5. HPLC-UV determination of carotenoid content

Carotenoid content was determined in both the fine powders and the infusions. 100 mL of chloroform was added to 5 g of the plants powder, stirred for 24 h in the dark at room temperature and filtered it through a 0.45 μm Minisart filter (RephiLe Bioscience Ltd). The carotenoid extract was dried at vacuum and the resulting dry residue was saponified by the reflux method using 100 mL of a KOH (5%) methanolic solution for 4 h at 50 °C in the dark. The saponified sample was combined with 100 mL of petroleum ether and 100 mL of distilled water. The organic layer was collected and then dried in a vacuum in a rotary evaporator. The dried residue was dissolved in methanol-isopropanol (35:65, v/v), filtered through a 0.45 μm syringe filter and analysed by HPLC (Alvarez-Suarez et al., 2017).

For the analysis of carotenoids in the infusions, the extraction procedure was conducted following the method previously reported (Loranty et al., 2010) with some modifications. Infusion samples (50 mL) were frozen at −20 °C for 24 h and then freeze-dried using a Lyovapor™ L-200 freeze-drier (BÜCHI Labortechnik, AG, Germany). The freeze-dried residues (0.5 g) were saponified by adding 20 mL of KOH (5%) methanolic solution for 4 h at 50 °C in the dark. The saponified sample was combined with 10 mL of ether and 10 mL of distilled water, and the organic phase was collected and dried at vacuum in a rotary evaporator. The dried residue was dissolved in methanol-isopropanol (35:65, v/v), filtered through a syringe filter (0.45 μm, RephiLe Bioscience Ltd) and analysed by HPLC.

The HPLC system (Agilent Technologies Series 1260, Santa Clara, California, United States) consisted of a Quaternary Pump Agilent Technologies 1260 Infinity G1312B and a Diode Array Detector (PDA) Agilent Technologies 1260 Infinity G1315C DAD set at an absorbance of 450 nm and equipped with an Eclipse Plus C18 column (5 μm, 4.6 × 250 mm). Elution was performed with methanol-isopropanol (35:65, v/v) in an isocratic gradient at a flow rate of 1 mL/min for 15 min. β-Carotene (0.1 mg/mL – 10 mg/mL, $y = 1428.2x - 113.88$, $R^2 = 0.9978$, LOQ: 0.037 mg/L and LOD: 0.011 mg/L) and lutein (0.1 mg/mL – 50 mg/mL, $y = 248.51x + 32.49$, $R^2 = 0.9966$, LOQ: 1.393 mg/L and LOD: 0.418 mg/L) were used as external standards for the calibration curve and the results were expressed as μg per 100 g of fresh weight (FW) of plants or μg per mL of infusion.

2.6. HPLC-DAD/ESI-MS^n characterization of phenolic acids, flavonoid and anthocyanins

Dry hydroalcoholic horchata (HHext) and infusion horchata extracts (IHext) were dissolved in 0.5 mL of 0.1% formic acid: acetonitrile (70:30, v/v) and filtered through a 0.22 μm disposable LC filter disk for analysis by HPLC using double online detection by diode array spectrophotometry and mass spectrometry (MS). A Hewlett-Packard 1200 chromatograph (Hewlett-Packard 1200, Agilent Technologies, Santa Clara, CA, USA) provided with a binary pump and a diode array detector (DAD) coupled with an HP ChemStation (rev. A.05.04) data-processing station was used. The system was connected via the cell outlet to an MS detector API 3200 Qtrap (Applied Biosystems, Darmstadt, Germany) that was controlled by the Analyst 5.1 software. The separation was achieved on an Agilent Poroshell 120 EC-C18, 2.7 μm (4.6 × 150 mm) column thermostated at 35 °C. The solvents were (A) 0.1% formic acid, and (B) acetonitrile. The elution gradient established was isocratic 15% B for 5 min, 15–20% B over 5 min, 20–35% B over 10 min, 35–50% B over 10 min, 50–60% B over 5 min, isocratic 60% B for 5 min and re-equilibration of the column to the initial solvent conditions. The flow rate was 0.5 mL/min. Double online detection was carried out in the DAD at 280, 330 and 370 nm as preferred wavelengths and in the MS operated in the negative ion mode. Spectra were recorded between m/z 100 and m/z 1000. Zero grade air served as the nebulizer gas (30 psi) and as turbo gas (400 °C) for solvent drying (40 psi). Nitrogen served as the curtain (20 psi) and collision gas (medium). Both quadrupols were set at unit resolution and EMS and EPI analyses were also performed. The EMS parameters were: ion spray voltage −4500 V, DP -50 V, EP -6 V, CE -10 V and cell exit potential (CXP) −3 V, whereas EPI settings were: DP -50 V, EP -6 V, CE -30 V and CES 10 V. The individual phenolic compounds were tentatively identified from their UV and mass spectra, and comparison with data reported in the literature, as well as with standards when available.

2.7. Evaluation of anti-inflammatory properties

2.7.1. Cell culture and treatment

RAW 264.7 murine macrophage cell line was purchased from the American Type Culture Collection (ATCC-TIB71). RAW macrophage were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/mL of penicillin and 100 μg/mL of streptomycin, in a humidified atmosphere at 37 °C with 5% CO_2. The different tests and pellet preparations were conducted on cells between the 4th and the 6th passage. The HHext and IHext were resuspended in RPMI 1640 medium to achieve the final concentration of 80 μg/mL. RAW macrophage was treated with: (i) RPMI 1640 medium only (Ctrl group), (ii) dried extract of HHext or IHext for 24 h (HHext group and IHext group), (iii) LPS (*Escherichia coli* serotype 055:B5) at 1 mg/mL for 24 h (LPS group), or (iv) dried extract of HHext or IHext for 24 h and then with LPS at 1 mg/mL for 24 h (HHext + LPS or IHext + LPS group). The combination of dose/time of HHext, IHext and LPS treatments was established in previous MTT viability assays for cytotoxicity studies (data not shown).

2.7.2. Measurement of intracellular ROS level and cell adenosine triphosphate (ATP) content

Intracellular ROS levels in each of the groups (Ctrl group, HHext or IHext group, LPS group, HHext + LPS or IHext + LPS group) were determined using the 2′-7′-dichlorofluorescin diacetate (DCFH) method as previously reported (Wang and Joseph, 1999). After the treatments, RAW cells were incubated with 5 μmol/L of DCFH at 37 °C for 30 min under light protection. Fluorescence intensity was read using the microplate reader (Thermo Scientific Microplate Reader, Multiskan® EX, USA) at an excitation wavelength of 485 nm and an emission wavelength of 530 nm. The Bradford method (Bradford, 1976) was used to measure total proteins and data were expressed as arbitrary units of

fluorescent intensity/μg cell proteins.

ATP content was determined from cells in culture using the ATP Cell Titer-Glo® assay (Promega, Madison, WI, U.S.A.) according to the manufacturer's instructions, and results were expressed as fluorescence units × 1000 cells.

2.7.3. Antioxidant enzyme activities and biomarkers of oxidative damage of lipid and proteins

RAW cells were incubated with RIPA buffer on ice for 5 min and the obtained lysate was stored at −80 °C until analysis. The antioxidant enzyme activities (superoxide dismutase (SOD), catalase (Cat), glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione transferase (GST)) were determined spectrophotometrically from the cellular lysates by following the specific procedure previously described (Gasparrini et al., 2017). SOD and catalase activity were expressed as U/mg prot/min, where one unit of SOD was defined as the amount of enzyme that gave 50% inhibition of nitroblue tetrazolium reduction and one unit of catalase was defined as the amount of enzyme that decomposed 1 mmol of hydrogen peroxide (H_2O_2). The results of GPx and GR were expressed as nmole NADPH oxidized/mg protein/min, while GST activity was expressed as nmol 1-chloro-2,4-dinitro benzene (CDNB)-reduced glutathione (GSH) conjugate/mg protein/min.

Protein carbonyl content and thiobarbituric acid-reactive substance (TBARS) content were studied as biomarkers of protein and lipid oxidative damage, respectively, according to the previously methods reported (Alvarez-Suarez et al., 2016) and the results were expressed as nmol/mg prot for total protein carbonyl content, and nmol/100 mg prot for TBARS levels.

2.7.4. Measurement of nitrite levels and the productions of the cytokine TNFα, IL6 and IL10

After the treatments (Ctrl group, HHext or IHext group, LPS group, HHext + LPS or IHext + LPS group), the culture supernatant was collected and the production of NO was determined using the Griess reagent, while TNFα, IL6 and IL10 contents were analysed using their respectively enzyme-linked immunosorbent assay (ELISA) kits (Invitrogen | Thermo Fisher Scientific) following the manufacturer's protocol. NO content was expressed as μM of $NaNO_2/10^5$ cells, TNFα as ng/ml, while IL6 and IL10 were expressed as pg/mL of culture supernatant medium.

2.8. Statistical analyses

Statistical analyses were performed using STATISTICA software (Statsoft Inc., Tulsa, OK, USA). Data were subjected to a one-way ANOVA (analysis of variance) for mean comparison and significant differences among different treatments were calculated according to HSD Tukey's multiple range test. Data are reported as mean ± SD. Differences at $P < 0.05$ were considered statistically significant.

3. Results and discussion

3.1. Chemical composition of the horchata infusion

In this study, a total of 23 medicinal plants and a mixture of them, named horchata, were analysed for their phytochemical composition, i.e., total phenolics (TPC), total flavonoids (TFC), anthocyanins (ACY), free amino-acids, carotenoids and vitamin C, and total antioxidant capacity (TAC). The analyses were performed in both the infusion and a hydroalcoholic extract of the 'horchata', as well as in each of the individual plants used to prepare this plant mixture. In the particular cases of carotenoids and vitamin C, for which hydroalcoholic extraction was not efficient or not advised, the analyses were performed directly from the plant powders.

The phytochemical composition and TAC of the horchata hydroalcoholic extract and infusion are shown in Fig. 1. As expected, the hydroalcoholic extraction was more efficient than the infusion to extract the different analysed compounds, offering values significant higher ($P \geq 0.05$ for TPC, TFC, ACY and leucine content, and $P \geq 0.01$ for proline content) (Fig. 1A). These results are in agreement with those reported by several authors, demonstrating the greater effectiveness of the hydroalcoholic extraction compared to aqueous extraction (Do et al., 2014). Indeed, the combined use of water and alcohol would facilitate the extraction of phenolic compounds that are either soluble in water or organic solvent. Moreover, the extraction of other components besides phenolics, such as proteins and carbohydrates, could also have been enhanced with the combination of both solvents (Zieliński and Kozłowska, 2000).

Regarding the contribution of each plant to the horchata bioactives composition and TAC, cucharillo and canela were the major contributors in both hydroalcoholic extract and infusion. Fig. 2A and B shows the results of the factorial analysis and cluster obtained considering the variables FRAP and DPPH using varimax rotation. For the hydroalcoholic extract (Fig. 2A) one factor was extracted (PC1_E) that explained 71.76% of the variance. The Person's correlation coefficient between the two considered variables and the extracted factor were higher than 0.84 ($P < 0.01$) and positive in all cases. The analysis of the same components in the infusion (Fig. 2B) also reduced to one component (PC1_I) explaining 75.16% of the total variance. Similarly, the Person's correlation coefficient between FRAP, DPPH and the extracted factor were higher than 0.86 ($P < 0.01$) and positive in all cases. These factors (PC1_E, PC1_I) represented the combined antioxidant properties of the hydroalcoholic extract and infusion of the plants. Similar behaviour was observed in the dendrograms obtained considering the phytochemical composition in both hydroalcoholic extract and infusion (Fig. 2C and D, respectively), and the antioxidant capacity (Fig. 2E and F). In each case, the dendrograms created using Ward's method and the rescaled squared Euclidian distance shown similar results regarding plant grouping.

Vitamin C and carotenoid contents were also determined, in this case use the plant powder of the plants besides infusion. Vitamin C was only found in the plant mixture (7.13 mg/100 g FW), while not detected in the infusion. Vitamin C contents were also determined in the 23 analysed plants, with values ranging between 1.05 and 27.92 mg/100 g FW (data not shown). The absence of vitamin C in the infusion despite the high levels present in the plants should be explained by its sensitivity to thermal treatments. Indeed, it is well known that vitamin C is very sensitive to degradation under adverse handling conditions, such as extended storage, high temperatures, low relative humidity, physical damage and chilling injury (Lee and Kader, 2000). For the preparation of horchata, water is heated to boiling point, plants are added after removing from the fire and allowed cooling at room temperature, then filtered to be consumed throughout time, either cold or warm.

Significant differences ($P \geq 0.01$) were found in the carotenoids content between the plant mixture powder and the infusion. Both β-carotene and lutein levels were significantly higher ($P \geq 0.01$) in the powder than in the horchata infusion (Fig. 1C), which may be explained by their poor solubility in water. Despite carotenoids are fairly stable during cooking in water (Bunea et al., 2008; Kao et al., 2014, 2012; Murador et al., 2014), no lutein was detected in the water after boiling vegetables (Liu et al., 2007) or in the infusions of 25 different herbal teas (Liu et al., 2007; Loranty et al., 2010).

The profiles of polyphenols were analysed in both the hydroalcoholic extract and infusion of the plant mixture by HPLC-DAD-ESI/MS. Representative HPLC chromatograms are shown in Fig. 3A (flavonoids and phenolic acids) and 3B (anthocyanins), while absorption and mass spectral data are indicated in Table 2, together with the tentative identity of the peaks assigned based on their UV–visible characteristics, (pseudo)molecular ions and MS^2 fragmentation patterns, as well as comparison with our own chromatographic peak library. Up to 29 of the detected peaks could be tentatively assigned, corresponding to flavonols (16), flavones (4), flavanones (1), lignans (1),

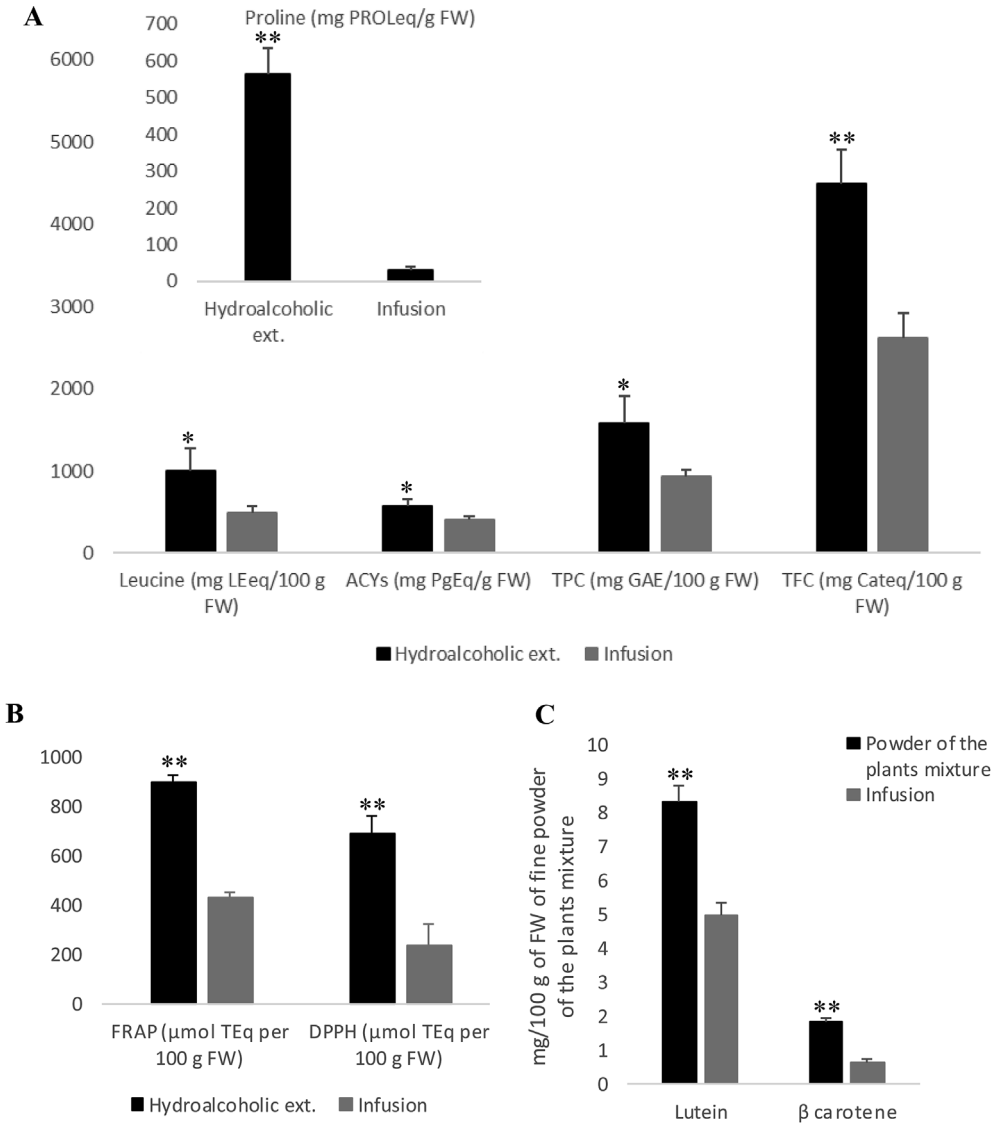

Fig. 1. Contents of different bioactive compounds (free amino-acids, total polyphenols, total flavonoids, total anthocyanins and carotenoids) and total antioxidant capacity of the hydroalcoholic extract and infusion obtained from *horchata*, consisting of the mixture of 23 medicinal plants. Results are reported as mean ± SD of three experiments. $*P < 0.05$, $**P < 0.01$, significant differences compared to infusion.

hydroxycinnamoyl derivatives (5) and anthocyanins (2). Similar phenolic profiles were obtained for the hydroalcoholic extract and the infusion, although with some differences in peak distribution. In general, the polyphenol extraction was favoured in the hydroalcoholic solvent compared to the infusion, especially in the case of the hydroxycinnamoyl derivatives (e.g., peaks 4, 20 and 23). As for anthocyanins, the two peaks observed in the hydroalcoholic extract (Fig. 3B) were hardly detected in the infusion, probably due to their poor stability to heating.

The detected phenolic acid derivatives corresponded to chlorogenic acids, namely caffeoylquinic and dicaffeoylquinic acids, whereas majority flavonoid peaks corresponded to quercetin glycosides, similar to what was previously reported for different medicinal herbs (Surveswaran et al., 2007; Wojdyło et al., 2007). Caffeoylquinic acids have been associated to antioxidant, antibacterial, anticancer and antihistamic effects, and an enhancement of ATP production (Miyamae et al., 2011), which might be associated, in a certain way, with the invigorating effects attributed to the horchata. Quercetin is the most abundant flavonol in nature and the human diet, and one of the most studied flavonoids regarding biological activity, being associated to significant antioxidant, anti-inflammatory and antitumor effects (Del Rio et al., 2013; Wang et al., 2014). An apigenin glycoside (peak 14) was the second predominant flavonoid after quercetin's in both hydroalcoholic extract and infusion. The antioxidant and anti-inflammatory properties of apigenin have been largely reported in animal models, and partly attributed to its ability to decrease the production of pro-inflammatory cytokines by inhibition of COX-2 and NF-kB activation (Wang et al., 2014). Minority levels were detected of syringaresinol (peak 3), a lignan for which anti-inflammatory activities have been described (Bajpai et al., 2018), as well as a capacity to inhibit Helicobacter pylori motility in vitro (Miyazawa et al., 2006). Anthocyanins have been shown to possess outstanding antioxidant, anti-inflammatory (Gasparrini et al., 2017) and antitumor properties (Amatori et al., 2016) and to induce beneficial effects in the cardiovascular system (Santhakumar et al., 2018), so they might also have some contribution to the attributed beneficial effects of horchata despite their low concentration in the infusion.

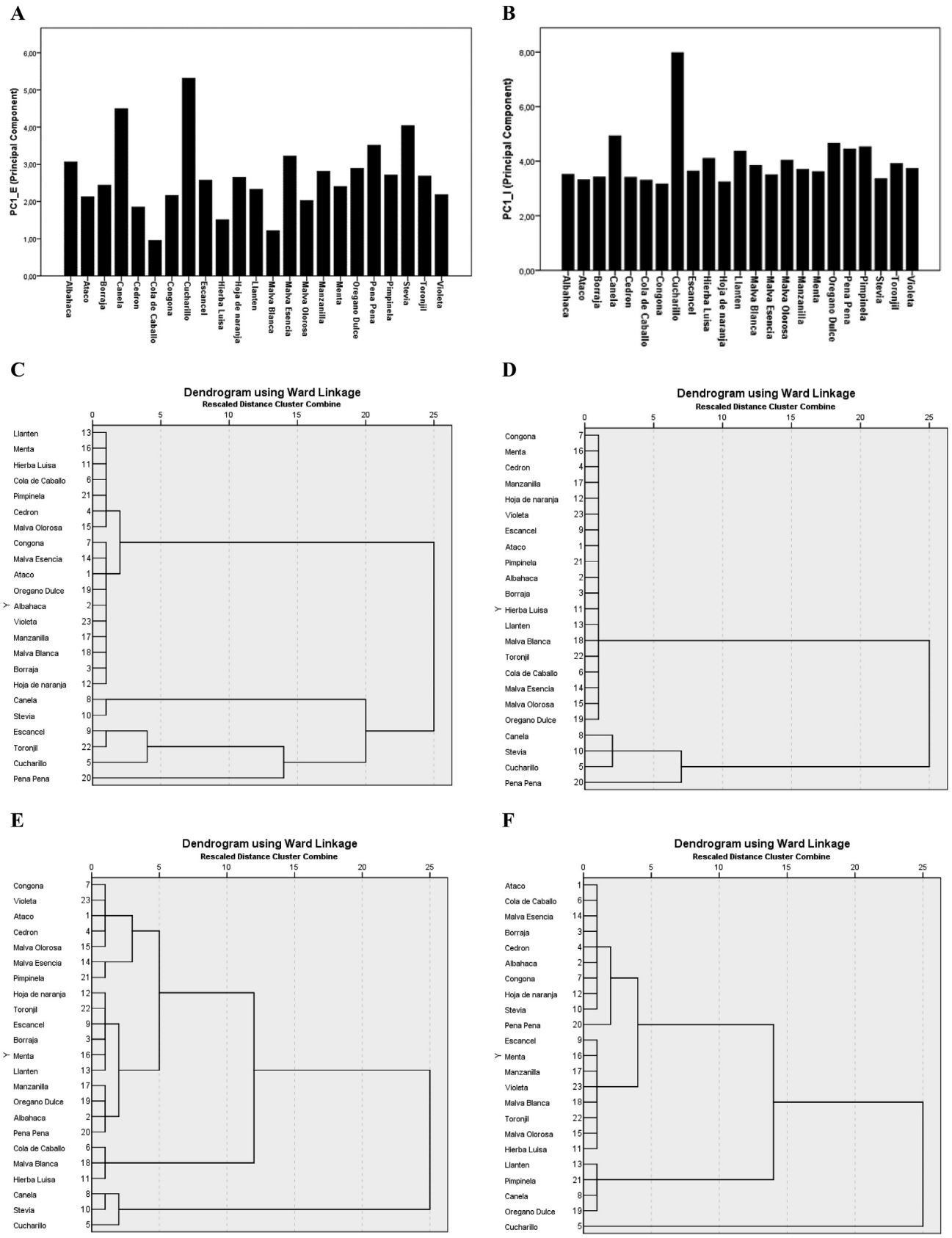

Fig. 2. Histograms obtained from the factorial analysis considering the variables FRAP and DPPH using varimax rotation for the hydroalcoholic extract **(A)** and infusion **(B)** of each plant conform the horchata drink. Dendrograms created using Ward's method considering the phytochemical composition for the hydroalcoholic extract **(C)** and infusion **(D)**, and the antioxidant capacity (DPPH and FRAP) for the hydroalcoholic extract **(E)** and infusion **(F)**.

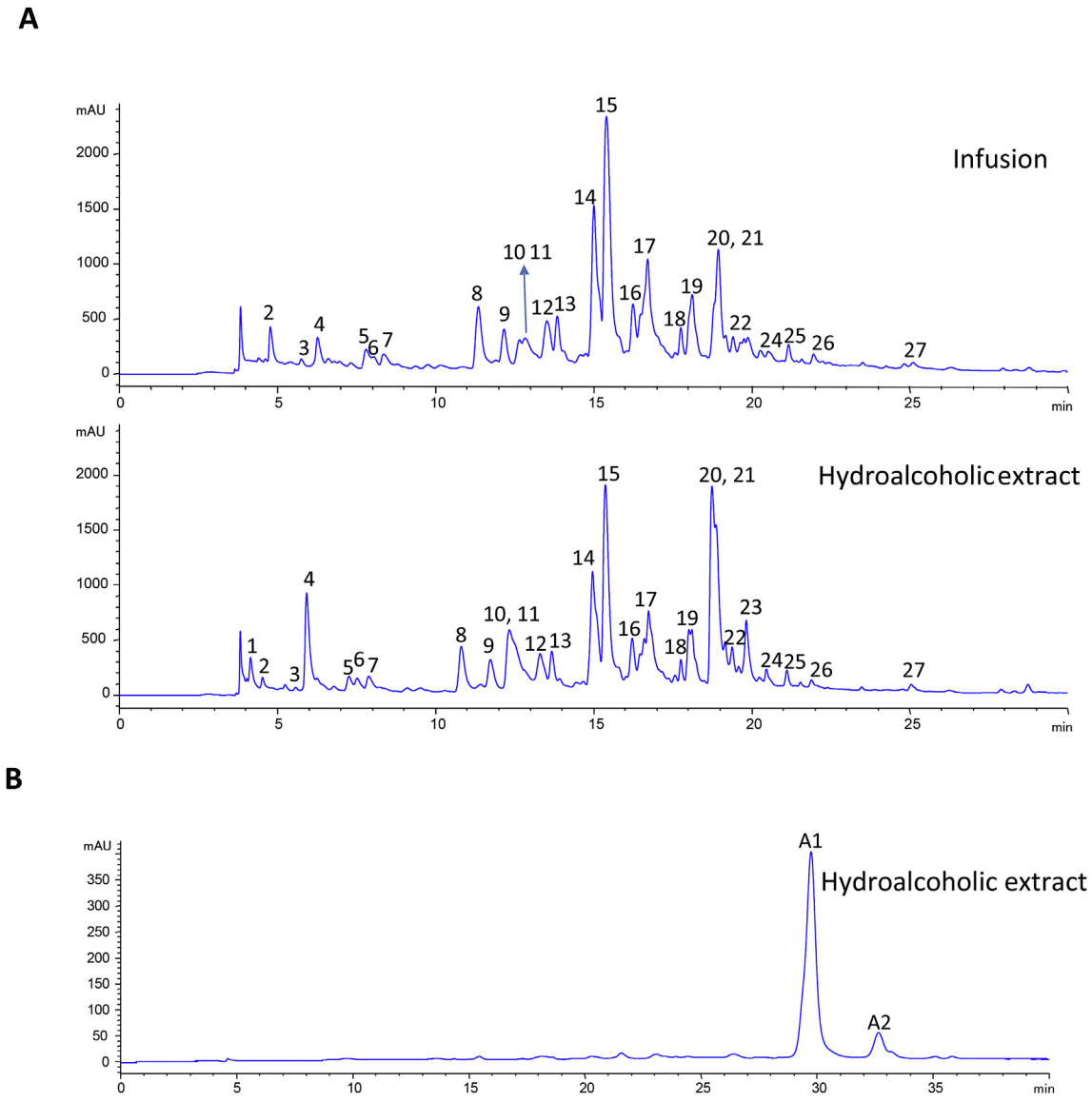

Fig. 3. HPLC chromatograms of (**A**) phenolic acid derivatives and flavonoids (recorded at 360 nm), and (**B**) anthocyanins (recorded at 520 nm) in the hydroalcoholic extract and infusion of the *horchata* plant mixture.

3.2. Horchata treatment stimulated the endogenous antioxidant defense system and reduced biomarkers of oxidative stress

Since the anti-inflammatory capacity of several medicinal herbs used to obtained the horchata infusion has been documented (Bailon-Moscoso et al., 2017; Rios et al., 2017), in this study the anti-inflammatory and the protective effects against cytotoxic damage mediated by the inflammation were evaluated for both the dried horchata infusion (IHext) and hydroalcoholic extract (HHext) using an in vitro model of RAW 264.7 murine macrophage cell line. Preliminary studies showed no cytotoxic effect in RAW 264.7 cells after incubation with HHext or IHext at the concentration and time assayed (data not shown). A concentration of 80 μg/mL was for both extracts, HHext and IHext, the lowest one able to protect RAW264.7 cells against the oxidative damage mediated by inflammation. After treatment with LPS, a burst in the intracellular ROS levels was produced in RAW 264.7 cells compared to the control ($P < 0.01$), which was significantly reduced when the cells were pre-incubated with HHext or IHext (Fig. 4A). Greater decrease in the intracellular ROS levels was observed in cells treated with HHext ($P < 0.01$) than in cells treated with IHext ($P < 0.05$), compared with the LPS-treated group.

An increase in the production of intracellular ATP was also observed LPS-treated cells ($P < 0.01$) in relation to control (Fig. 4B). The increase in the ATP values in LPS-treated cells was maintained when cells were pre-treated with HHext and IHext ($P < 0.05$) compared to the control groups, in this case with no differences between the effect of each extract (Fig. 4B). The increase in ATP levels would be in agreement with the observations of Gasparrini et al. (2017) who reported an increase in oxygen consumption in RAW 264.7 macrophages stimulated with LPS compared to untreated cells. This could represent a ROS-dependent oxidative stress response that leads cells to change their transcriptional status after activation, inducing expression of several molecules, such as cytokine receptors, cytokines or MHC II molecules for the presentation of antigens and co-receptors (Cruz et al., 2007), which could enhance the glycolytic flux and favour ATP generation, helping to develop and maintain the long term defensive and reparative functions in macrophages (Ruiz-García et al., 2011). It has been also reported that activated macrophages utilise glycolytic ATP to maintain mitochondrial membrane potential and prevent apoptotic cell death (Garedew et al., 2010).

Plant bioactive compounds have also been associated with a capacity to modulate the activity of diverse antioxidant enzymes reducing

Table 2
Absorption and mass spectral data, and tentative identification of the peaks detected in the *horchata* plant mixture.

Peak	UV–vis (λ_{max}, nm)	Pseudomolecular ion (m/z)	Main MS2 product ions (m/z)	Tentative identification
Phenolic acids and flavonoids				
1	256, 356	771	609, 463, 301,	Quercetin-O-deoxyhexosyl-hexoside-O-hexoside
2	309sh, 326	353	191, 179, 135	3-O-Caffeoylquinic acid
3	272	417	207, 152	Syringaresinol
4	309sh, 326	353	191	5-O-Caffeoylquinic acid
5	270, 336	593	473, 383, 353	Apigenin di-C-hexoside
6	270, 350	771	477, 315	Methylquercetin-O-pentosyl-hexoside-O-hexoside
7	257, 352	507	331, 193, 165	Methylmyricetin-O-glucuronide
8	256, 354	755	301	Quercetin-O-deoxyhexosyl-deoxyhexosyl-hexoside
9	266, 346	593	489, 369, 327	Luteolin-C-deoxyhexoside-C-hexoside
10	266, 352	785	477, 625, 315	Methylquercetin-O-deoxyhexosyl-hexoside-O-hexoside
11	260, 368	493	317, 179	Myricetin glucuronide
12	257, 350	595	301	Quercetin-O-pentosyl-hexoside
13	264, 352	771	609, 301	Quercetin-O-deoxyhexosyl-hexosyl-hexoside
14	272, 338	577	487, 473, 457, 383, 365, 353	Apigenin-C-deoxyhexoside-C-hexoside
15	256, 356	609	301	Quercetin-O-rutinoside
16	256, 352	463	301	Quercetin-O-galactoside
17	256, 352	463	301	Quercetin-O-glucoside
18	266, 348	593	285	Kaempferol-O-rutinoside
19	256, 354	433	300, 271	Quercetin-O-pentoside
20	332	515	353, 191, 179, 173	Dicaffeoylquinic acid
21	–	447	301	Quercetin-O-deoxyhexoside
22	326	515	353, 191, 179, 173	Dicaffeoylquinic acid
23	326	515	353, 191, 179, 173, 135	Dicaffeoylquinic acid
24	329	771	609, 301	Hesperetin-O-caffeoyl-deoxyhexosyl-hexoside
25	264, 344	431	285, 255	Luteolin-O-deoxyhexoside
26	301, 334sh	433	271, 163, 155, 151, 125	Naringenin hexoside
27	256, 374	301	179	Quercetin
Anthocyanins				
A1	277, 312, 531	773	611, 303	Delphinidin-O-coumaroylhexosyl-O-hexoside
A2	260, 314, 523	757	595, 287	Cyanidin-O-coumaroylhexosyl-O-hexoside

the damage induced by LPS and other stressors and restoring conditions similar to control levels in cellular models (Chichiriccò et al., 2019; Park et al., 2011; Zengin et al., 2019). The treatment with both extracts did not affect the activity of these enzymes in RAW 264.7 cells, while LPS stimulation significantly decreased their activity compared to the control ($P < 0.01$), which was partly prevented by cell pre-treatment with HHext and IHext (Fig. 4C and D). HHext showed greater capacity to protect the activity of the antioxidant enzymes than IHext in the LPS-stressed cells ($P < 0.05$). The stimulation of RAW 264.7 macrophages with LPS significantly increased ($P < 0.01$) the TBARS level and protein carbonyl content, as markers of lipid and protein oxidative damage, respectively (Fig. 4E). This increase was also significantly reduced ($P < 0.05$) by pre-incubation of the cells with HHext and IHext (Fig. 4E), thus reducing the level of damage in both types of macromolecules (Fig. 4E).

The damage produced by LPS stimulation in the enzymes activity, proteins and lipids might be due to the observed increase of the intracellular ROS levels and the consequent deleterious effect of free radicals on these molecules (Matés et al., 1999). The ability of HHext and IHext to counteract LPS-induced lipid and protein oxidative damage might be due to the capacity of certain bioactive compounds, such as phenolic compounds present in the extracts, to reduce the intracellular ROS levels and/or activate endogenous antioxidant enzymes. Actually, it has been proposed that polyphenols could exercise their protective action against oxidative damage through the activation of molecular pathways related to the antioxidant response. The activation of the AMPK/p-AMPK/Nrf2/ARE (Alvarez-Suarez et al., 2016; Gasparrini et al., 2017) and the insulin/IGF-1 signaling pathways (Ayuda-Durán et al., 2019) have been put forward as possible molecular mechanisms by which polyphenols protect cells against oxidative damage. The greater protective effect observed for HHext compared to IHext could be due to its higher content of phenolic compounds favoured by a more efficient extraction in the hydroalcoholic medium.

3.3. Horchata treatment positively affected nitrite production and inflammatory cytokines secretion in RAW 264.7 macrophages

The anti-inflammatory effect of the horchata extracts was evaluated by measurement of nitrite production, the secretion of the pro-inflammatory cytokines TNFα and IL-6, and the anti-inflammatory cytokine IL-10. Nitrite is a product from nitric oxide (NO) oxidation that acts as regulator of inflammatory responses and has traditionally been viewed as an acute biomarker of NO flux and formation in biological systems. Nitrite over-production reacts with superoxide causing cytotoxicity and tissue damage (Kevil and Lefer, 2010). The levels of nitrite significantly increased ($P < 0.01$) after the LPS stimulation of RAW 246.7 cells in comparison to the control, while the pre-incubation with HHext and IHext extracts significantly decreased ($P < 0.01$) these levels in the LPS-stressed macrophages (Fig. 5A).

Similar observations were made for the pro-inflammatory cytokines TNFα and IL-6, whose levels dramatically increased ($P < 0.01$) following LPS treatment, an effect that was partially prevented by pre-incubation with HHext and IHext, which significantly decreased the production of both cytokines ($P < 0.01$) in the LPS-stimulated group (Fig. 5B and C). As for the anti-inflammatory cytokine IL-10, a smaller increase in its levels was observed in LPS-treated cells ($P < 0.05$) compared to the control group, whereas a significant enhancement was produced in cells pre-treated with the horchata extracts ($P < 0.01$ for HHext and $P < 0.05$ for IHext) (Fig. 5D). The increase in pro-inflammatory cytokines could be explained by the activation of the signaling cascade initiated by the binding of the exotoxin LPS to its TLR4 receptor, while the increase in the anti-inflammatory cytokine IL-10 could be a consequence of the self-regulatory mechanism of the inflammatory response. It is known that high levels of inflammatory response can produce tissue damage once the pathogen has been eliminated, so self-regulatory mechanisms, such as induction of IL-10, play a key role in the prevention of such adverse effects (Chen et al., 2018).

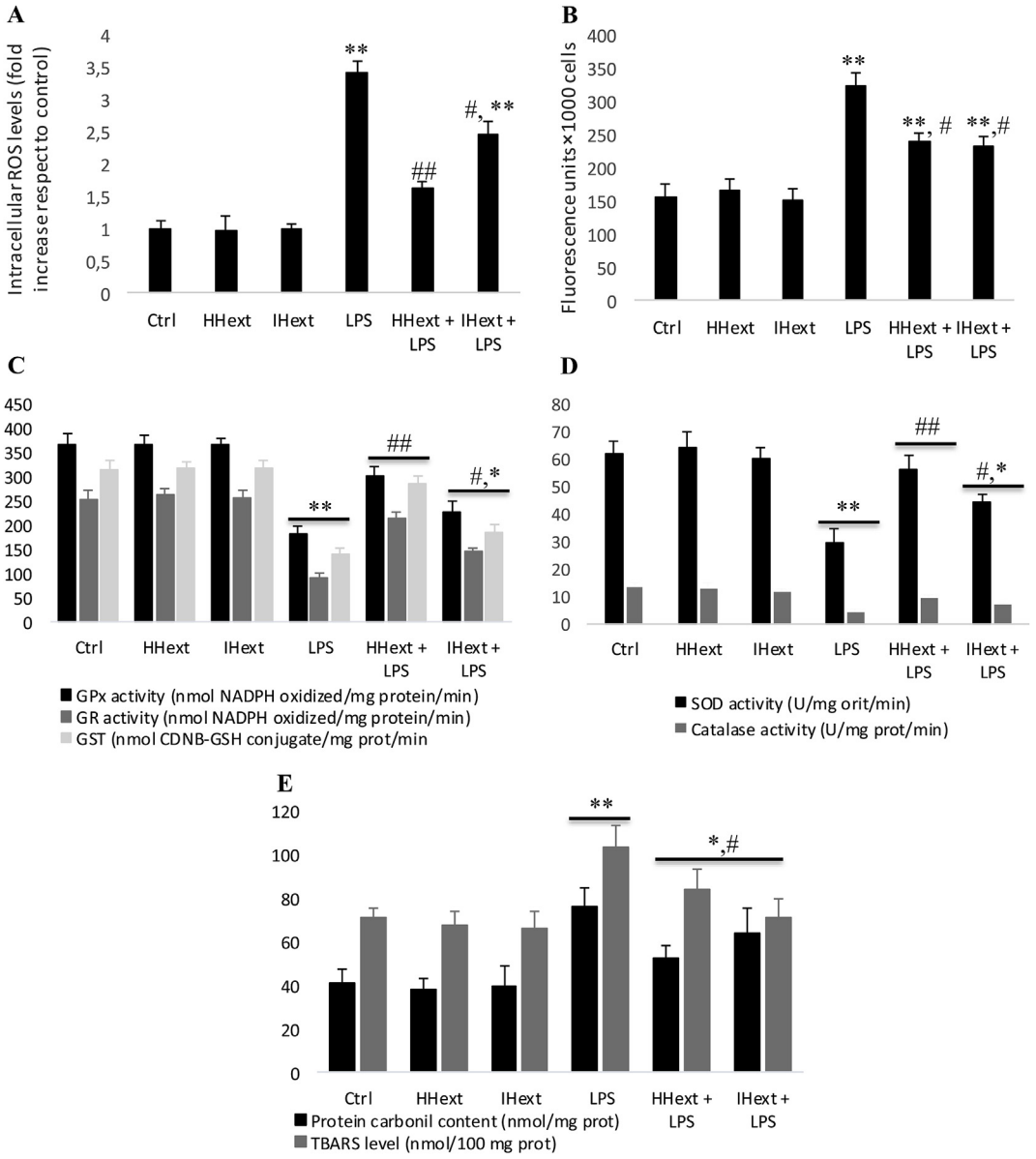

Fig. 4. Markers of antioxidant status and oxidative damage in RAW 264.7 macrophages submitted to different treatments: (i) RPMI 1640 medium only (Ctrl group), (ii) HHext or IHext extract for 24 h (HHext group and IHext group), (iii) LPS at 1 mg/mL for 24 h (LPS group), and (iv) HHext or IHext extract for 24 h and then with LPS at 1 mg/mL for 24 h (HHext + LPS or IHext + LPS group). **(A)** Intracellular ROS levels, **(B)** ATP levels, **(C)** GPx, GR and GST activity, **(D)** SOD and catalase activity, and **(E)** TBARS and carbonylated proteins content. Results are reported as mean ± SD of three experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, significant differences compared to control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, significant differences between LPS-treated group and HHext + LPS or IHext + LPS group.

The results here obtained are in line with those previously reported that evidenced the anti-inflammatory effect of the different bioactive compounds against inflammatory damage (Torres-Carro et al., 2017).

4. Conclusions

The horchata plants mixture contains a group of bioactive compounds able to reduce cell oxidative damage, through the increase in the levels of endogenous antioxidant enzymes and modulation of the production of pro-inflammatory and anti-inflammatory cytokines, activating the self-regulatory mechanisms of the inflammation processes, thus contributing to overall protection against tissue damage by an excessive inflammatory response. Among the bioactives present in the horchata mixture, phenolic compounds are prominent, being mostly represented by flavones and flavonols and chlorogenic acids. These compounds are not much affected by the heating treatment used to prepare the infusion, the common form of preparation of this Ecuadorian beverage, whereas other bioactive compounds also present in the original plants, such as vitamin C and carotenoids, are sensitive to high temperature or not well extracted in the water medium. All in all, the results obtained in this work revealed that the phytochemical composition was not much different in the horchata infusion and the hydroalcoholic extract, and that the infusion still maintains significant antioxidant and anti-inflammatory activities, which would support the protective effects on health traditionally attributed to the consumption of this beverage by the population.

Conflicts of interest

The authors declare no conflicts of interest.

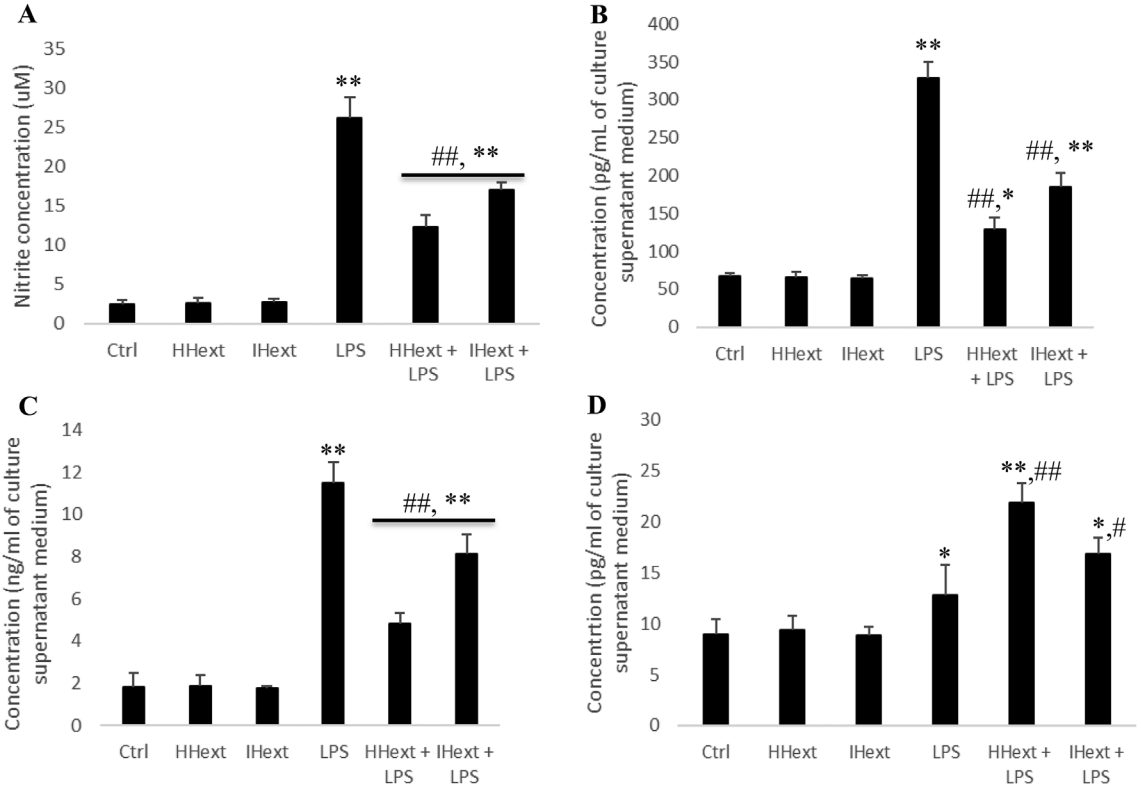

Fig. 5. Nitrite and inflammatory cytokine levels in RAW 264.7 macrophages submitted to different treatments: (i) RPMI 1640 medium only (Ctrl group), (ii) HHext or IHext extract for 24 h (HHext group and IHext group), (iii) LPS at 1 mg/mL for 24 h (LPS group), and (iv) HHext or IHext extract for 24 h and then with LPS at 1 mg/mL for 24 h (HHext + LPS or IHext + LPS group). **(A)** Nitrite content, **(B)** TNFα, **(C)** IL-6, and **(D)** IL-10. $^{*}P < 0.05$, $^{**}P < 0.01$, significant differences compared to control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, significant differences between LPS-treated group and HHext + LPS or IHext + LPS group.

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