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Antioxidant and anti-inflammatory activity characterization and genotoxicity evaluation of *Ziziphus mistol* ripe berries, exotic Argentinean fruit

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ABSTRACT

Ziziphus mistol Griseb. (Rhamnaceae), popularly known as "mistol," is widely distributed throughout Perú, Bolivia, Paraguay and Argentina. Its fruit is consumed in different forms in several Argentinean communities. The aim of this work is to quantify *Z. mistol* fruit macronutrients and phytochemicals as well as to determine its functional antioxidant and anti-inflammatory properties and toxicity after two different processes: boiling and hydroalcoholic extraction. Phytochemical recovery was variable depending on the extraction method used. All preparations showed antioxidant activity, but the ethanolic one (EME) was significantly more active than the aqueous one (AME) as hydrogen or electron donors with SC_{50} values between 1.45 to 6.31 μg GAE/mL and 7.38 to 64.77 μg GAE/mL, respectively. The aqueous extraction was significantly more active than EME on superoxide and hydroxyl radical scavenging. Polyphenols showed a dose–response relationship ($R^2 > 0.90$) with antioxidant capacity in the decoction and the alcoholic beverage. The maceration showed an inhibitory effect on lipoxygenase (LOX) activity with an inhibitory concentration (IC_{50}) value of 183.80 μg gallic acid equivalents (GAE)/mL but the decoction did not. On the other hand, extracts did not show any mutagenic effect. Therefore, mistol fruits consumption could be encouraged not only for its functional properties, but also because of the positive ecological impact of preserving biological diversity through the exploitation of native natural resources in a regional economy.

1. Introduction

The Great American Chaco, a vast region having an apparent ecological unit, is immersed in a severe degradation process of its natural resources and biodiversity due to deforestation, desertification and changes in land use (Giménez, Hernández, Gerez, & Ríos, 2007). The American Chaco forest is characterized by the presence of "red quebracho" (*Schinopsis quebracho-colorado*), "white quebracho" (*Aspidosperma quebracho-blanco*), and other tree species such as *Ziziphus mistol, Prosopis alba, Prosopis nigra, Prosopis kuntzei, Prosopis ruscifolia, Caesalpinia paraguariensis, Cercidium praecox,* and *Geoffroea decorticans* (Giménez et al., 2007). *Ziziphus mistol* Griseb. (Rhamnaceae), traditionally known as "mistol," is a thorny, deciduous, honey plant (10–15 m tall) widely distributed throughout Perú, Bolivia, Paraguay and Argentina. Its fruit is a reddish brown spherical drupe of approximately 10–17 mm in diameter, with a sticky, sweet pulp (Fig. 1A). Many traditional communities of Northwestern Argentina utilize its bark infusion or decoction in the treatment of dandruff (Bourdy, 2002; Martínez & Barboza, 2010) and seborrhea (Martínez & Barboza, 2010). Its bark and roots are used as dyes and soap, for the presence of saponins (Ragonese & Milano, 1984).

Its fruit has long been used in folk medicine in many medicinal preparations. "Mistol tea" (infusion prepared with the fruit) is taken against biliary colic, dysentery, "cold stomach" and "empacho", and as an antitussive and an antidote against snake and poisonous insect bites (Scarpa, 2004). The fruit is also roasted and ground to prepare "Mistol coffee", a homemade preparation popularly said to be good for respiratory system diseases, cardiac pathologies, hypertension and diabetes. It is also used as a natural energizer (Palacio, Carrizo, & Contreras, 2009). Moreover, the fruit has an exquisite taste and an attractive reddish-hazel color and is exploited in other food preparations such as "bolanchao" (Boelcke, 1989). Bolanchao (Fig. 1B and C) is prepared by grinding the fruit until getting a granulose paste which is pressed into balls that are coated with roasted "Algarrobo" flour (*Prosopis alba*, preferably). This is a convenient snack to carry for outdoor activities. Besides these traditional uses, mistol also makes a significant contribution to the environment by fixing carbon and protecting soils and biodiversity (Galindez et al., 2006).

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Fig. 1. (A) *Ziziphus mistol* fruits; (B) Bolanchao; (C) Closer look to "bolanchao", where fragments of mistol fruits can be appreciated as well as the coated of "Algarrobo" flour.

Epidemiological studies suggest that increased consumption of fruits, vegetables and minimally refined cereals is associated with a lower incidence of illnesses such as coronary heart disease, some forms of cancer, diabetes, and other aging-related diseases by reducing oxidative stress (Dragsted et al., 2006; Wang, Melnyk, Tsao, & Marcone, 2011). These properties are linked to the presence of vitamins, carotenoids, fiber, glutation, terpenoids and significant amounts of polyphenols, a group of phytochemicals recognized as the most abundant antioxidants in our diet (Lee, Koo, & Min, 2004; Manach, Scalbert, Morand, Rémésy, & Jimenez, 2004; Wootton-Beard, Moran, & Ryan, 2011). Natural antioxidants have currently attracted considerable interest among nutritionists, food manufacturers and consumers because of their potential therapeutic value and presumed safety, but it should be kept in mind that they are not necessarily less toxic than synthetic compounds (Vijaya Kumar Reddy, Sreeramulu, & Raghunath, 2010). Taking these factors into consideration in our continued quest to find new edible plants with functional properties and to promote their reproduction and preservation, we undertook the so far unexplored chemical and biological research of *Z. mistol* fruit. The aim of the present work was to determine the nutritional composition, evaluate the antioxidant and anti-inflammatory properties and genotoxicity of *Ziziphus mistol* fruit grown in Northwestern Argentina after being treated to processes that simulate domestic cooking.

2. Materials and methods

2.1. Samples

2.1.1. Plant material

Ziziphus mistol samples were collected in December 2008 in "Termas de Río Hondo", Santiago del Estero, Argentina. Fruit was air-dried and ground into powder with a particle size of 80 meshes for further analysis.

2.1.2. Sample processing

In order to emulate domestic preparations two extraction types (beverage and decoction) were used

- *Ethanolic mistol extraction (EME):* Dried fruit powder (10 g) was weighed and extracted with 100 mL 96° ethanol for 7 days at room temperature and centrifuged for 20 min at 10000 × *g*. The supernatant was then filtered through Whatman N° 4 filter paper, the volume was adjusted to 100 mL and used for all assays.
- *Aqueous mistol extraction (AME)*: 10 g of ground samples were decocted in 100 mL of distilled water for 20 min. The decoction was left to cool at room temperature, centrifuged for 20 min at 10000 × *g* and filtered through Whatman N° 4 filter paper. The volume was adjusted to 100 mL and used for antioxidant and characterization assays.
- *Acetone Water Mistol Extract (AWME):* Dried fruit powder (2 g) was extracted with acetone water (70:30, v:v) in an ultrasonic bath for 30 min at room temperature and then centrifuged at 13000 × *g* for 10 min. The suspension obtained was filtered and the remaining solids were extracted exhaustively with the same solvent system. All organic extracts were combined and the final volume adjusted to 100 mL. Then, a fraction was subjected to acid hydrolysis by evaporating the acetone and adding 4 N sulfuric acid to the aqueous fraction. The solution was maintained at 100 °C for 26 h.

2.2. Chemical composition determination

2.2.1. Sugar determination

The phenol–sulphuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) was used to determine total neutral sugars in aqueous and ethanolic preparations. Aliquots (0.8 mL) of different extract dilutions were taken and 0.04 mL of 80% phenol and 2 mL H_2SO_4 were added. After a 20 min of incubation at 100 °C, absorbance at 490 nm was measured on a Beckman-DU-650 spectrophotometer. Results were expressed as g of glucose/100 g dry weight.

Reducing sugars were measured using the Somogyi–Nelson method. Aliquots (0.1 mL) of different extract dilutions were taken and 0.5 mL of copper tartrate reagent (Somogyi, 1945) was added. The solution was heated at 100 °C during 15 min and 0.5 mL of arsenomolybolic acid reagent (Nelson, 1944) was added. Absorbance was measured at 520 nm. Results were expressed as g of glucose/100 g dry weight.

Glucose was determined by the glucose-oxidase method (Jorgensen & Andersen, 1973). Aliquots (0.3 mL) of different extract dilutions were taken and 0.02 mL of Na_2CO_3 (0.5 M) and 1 mL glucose oxidase–peroxidase reagent were added. The solution was maintained at 37 °C during 20 min and 1 mL of HCl (6 N) was added. Absorbance was measured at 530 nm.

Fructose was measured according to Roe (1934). Aliquots (0.5 mL) of different extract dilutions were taken and 0.5 mL of resorcinol thiourea reagent and 1.5 mL of HCl (conc.) were added. The solution was heated at 80 °C during 13 min and the absorbance was measured at 515 nm.

Sucrose was estimated by the resorcinol method (Cardini, Leloir, & Chiriboga, 1955). Aliquots of 1.5 mL of different extract dilutions were taken and 0.3 mL of distilled water and 0.2 mL NaOH (2 N) was added. The mixture was heated at 100 °C during 10 min. The same procedure described to determine fructose was performed.

Results were expressed as g of glucose, sucrose or fructose/100 g dry weight, respectively.

2.2.2. Protein determination

Soluble protein concentration in both preparations was determined by the method of Bradford (1976) using bovine serum albumin (BSA) as standard. Aliquots (0.1 mL) of different extract dilutions were taken and 5 mL of dye solution (Coomassie Brilliant Blue G 250) was added. After 5 min at room temperature the absorbance was measured at 595 nm. Results were expressed as mg of BSA/100 g dry weight. Total Protein (N × 6.25) was determined according to the AOAC (1998) methods.

2.2.3. Total polyphenols

Total polyphenols in mistol fruit preparations (EME, AME, AWME) were determined by Folin–Ciocalteau's reagent (Singleton, Orthofer, & Lamuela-Raventos, 1999). The reaction mixture contained 20 μL of each preparation, 2 mL of distilled water, 200 μL of Folin–Ciocalteau reagent and 800 μL of sodium carbonate (15.9% w/v). Absorbance was measured at 765 nm in a spectrophotometer UV–visible Beckman DU 650. Results were expressed as mg gallic acid equivalents/100 g dry weight (mg GAE/100 g DW).

2.2.4. Total flavonoids

Two different methods were used to assess the amount of flavonoids present in AME, EME and AWME by solubility problems.

The total flavonoid content in AME was determined with aluminum chloride ($AlCl_3$) according to a known method (Zhishen, Mengcheng, & Jianming, 1999). 0.1 mL of fruit extracts (AME) was added to 0.3 mL distilled water followed by $NaNO_2$ (0.03 mL, 5%). After 5 min at 25 °C, $AlCl_3$ (0.03 mL, 10%) was added. After a further 5 min, the reaction mixture was treated with 0.2 mL 1 mM NaOH. Finally, the reaction mixture was diluted to 1 mL with water and absorbance was measured at 510 nm.

The $AlCl_3$ method (Lamaison & Carnet, 1990) was used for the determination of the total flavonoid content of the fruit extract (EME and AWME). 0.5 mL of ethanolic 2% $AlCl_3.6H_2O$ was added to equal volumes of each extract. The mixture was shaken and the absorbance was read at 420 nm after 60 min incubation at room temperature. Flavonoid content was expressed as mg quercetin equivalents per 100 g dry weight (mg QE/100 g DW).

2.2.5. Proanthocyanidins determination

The total proanthocyanidins (PACs) content was determined with 4-dimethylaminocinnamaldehyde (DMAC) according to Prior, Wu, & Schaich (2010). To 150 μL of 80% ethanol (blank), standard, AME, EME or AWME, 450 μL of the DMAC solution (0.1% in acidified ethanol) was added. Absorbance was read at 640 nm after 20 min at 25 °C. Data were expressed as mg of procyanidin B2 equivalents per 100 g dry weight (mg PB2E/100 g DW).

2.2.6. Gallotanins determination

One mL of AWME was first hydrolyzed with 1 mL of 4 N H_2SO_4 at 100 °C for 26 h and the gallic acid released was determined with the rhodanine method of Inoue & Hagerman (1988). Twenty microliters of hydrolyzed AWME (HAWME) were made up to 200 μL with distilled water and 200 μL of AWME without hydrolyzing were dried under nitrogen and resuspended in 200 μL of 0.2 N H_2SO_4. Two hundred microliter of 0.2 N H_2SO_4 and 300 μL rhodanine (0.667% methanol) were added to the diluted extracts. After 5 min, 200 μL of 0.5 N potassium hydroxide and 4 mL distilled water were added. The mixtures were left for 10 min at room temperature and the absorbance at 520 nm was determined (Beckman DU-650 UV–vis spectrophotometer). AWME was evaluated in replicates and gallotannin concentrations were expressed as mg gallic acid equivalents per 100 g dry weight (mg GAE/100 g DW).

$$\text{Gallotanins (mg GAE)} = \text{GHAWME} - \text{GAWME}$$

where GHAWME is the amount of gallic acid present in HAWME and GAWME is the amount of gallic acid present in the sample without hydrolysis.

2.2.7. Total anthocyanins

Dried fruits (3 g) were extracted with 25 mL 1% HCl in methanol overnight at 5 °C without light. The solution obtained was then filtered through Whatman N° 1 filter paper and the remaining solids were extracted exhaustively with 100 mL of 1% HCl in methanol. All acidic–methanolic extracts obtained were combined and vacuum-concentrated by evaporating methanol in a rotary evaporator. They were resuspended with 25 mL MILLIQ water to obtain the anthocyanin extract (AE). Total anthocyanins were evaluated by the pH differential method (Lee, Durst, & Wrolstad, 2005). The AE in 25 mM potassium chloride solution (pH 1.0) and 400 mM sodium acetate buffer (pH 4.5) were measured simultaneously at 520 nm and 700 nm, respectively. The content of total anthocyanins was expressed as mg cyanidin-3-glucoside equivalents per 100 g of dry weight (mg C3GE/100 g DW).

2.2.8. Total alkaloids

Total alkaloids were measured by acid dye colorimetry using bromothymol blue (BTB) as the coloring agent (Önal, Kepekçi, & Öztunç, 2005). One milliliter of water, standard or AME was added to 2 mL of BTB reagent (4.78×10^{-4} M in phosphate buffer pH 5). After mixing, the ion-pair complex formed was extracted with 5 mL chloroform by shaking vigorously for exactly 1 min. Absorbance was measured at 414 nm against the blank prepared with water. Total alkaloids were calculated as μg of apomorfine chlorhydrate equivalents per 100 g of dry weight (μg ACE/100 g DW).

2.2.9. Total carotenoids

Carotenoid extraction was carried out according to Lee & Castle (2001). Samples (1 g) were extracted with 10 mL of hexane/acetone/ethanol (50:25:25, v/v). After centrifugation at $13000 \times g$ for 10 min at 4 °C, the top hexane layer containing the color was recovered and adjusted to 25 mL with hexane. Total carotenoid content was calculated according to De Ritter & Purcell (1981). The absorbance was measured at 450 nm and results were expressed as μg of β-carotene equivalents per 100 g of dry weight (μg β CE/100 g DW).

2.2.10. Vitamin C

Ascorbic acid (AA) was measured by the HPLC method. Briefly, dried fruits were ground in 100 mM phosphate buffer (pH 2.5) and then sonicated during 10 min. After centrifuging at $12000 \times g$ for 10 min, the supernatant was then filtered through a 0.22 μm filter (Millipore). HPLC analysis was carried out on a Waters liquid chromatography equipped with a Waters 1525 binary HPLC pump, and a Waters 2998 Photodiode Array Detector. The separation was performed at 30 °C using a C18 Security Guard Cartridge (Phenomenex) and a Waters XBridgeTM C18 4.6 × 150 mm, 5 μm particle size, end capped reversed-phase column (Waters), in isocratic mode with 1% acetic acid solution as mobile phase. The flow rate was 1.0 mL/min. The injection volume was 20 μL and detection was carried out at 244 nm.

Vitamin C was calculated and expressed as mg L-ascorbic acid per 100 g of dry weight (mg L-AA/100 g DW).

2.3. Measurement of antioxidant capacity

2.3.1. ABTS free radical scavenging activity

The antioxidant capacity assay was carried out by the improved $ABTS^{\bullet+}$ method as described by Re et al. (1999). $ABTS^{\bullet+}$ was generated by reacting 7 mM ABTS and 2.45 mM potassium persulfate after incubation at room temperature (23 °C) in the dark for 16 h. The $ABTS^{\bullet+}$ solution was obtained by diluting the stock solution to an absorbance of 0.70 at 734 nm in ethanol (for EME) or in buffer PBS pH 7.4 (for AME). $ABTS^{\bullet+}$ solution (1 mL) was added to AME and EME (1.04–12.34 μg GAE/mL) or to Trolox standard (final concentration 0–15 μM) in ethanol and mixed thoroughly. Absorbance was recorded at 734 nm after 6 min. The percentage of inhibition was measured by the following formula:

$$\%\ \text{inhibition} = (A_0 - A_s) / A_0 \times 100;$$

where A_0 is the absorbance of the control (blank, without extract) and A_s is the absorbance in presence of the extract. SC_{50} values denote the μg GAE/mL required to scavenge 50% ABTS free radicals.

2.3.2. DPPH free radical scavenging activity

The DPPH radical scavenging activity was measured according to Brand-Williams, Cuvelier, & Berset (1995). DPPH solution (1.5 mL of 300 μM in 96° ethanol) was added to 0.5 mL of AME and EME (containing 2.71–130.53 μg of GAE/mL) and shaken vigorously. It was kept at room temperature for 20 min and absorbance was measured at 515 nm. The percentage of radical scavenging activity (RSA%) was calculated using the following equation:

$$RSA\,\% = [(A_0 - A_s)/A_0]\; x\; 100$$

where A_0 is the absorbance of the control and A_s is the absorbance of the samples at 515 nm. SC_{50} values denote the μg GAE/mL required to scavenge 50% DPPH free radicals.

2.3.3. β-carotene bleaching assay

Antioxidant activity was determined according to the β-Carotene bleaching method following the procedure described by Ordoñez, Gomez, Vattuone, & Isla (2006). The initial absorbance at 470 nm was registered at zero time (t_0) and for 120 min. Antioxidant activity (AA %) was calculated as percent inhibition relative to control using the following equation:

$$AA\% = \left[\left(R_{control} - R_{sample}\right)/R_{control}\right] \times 100$$

where $R_{control}$ and R_{sample} are the bleaching rates of β-carotene in reactant mix without antioxidant and in presence of the extracts, respectively.

IC_{50} values denote the μg GAE/mL required to inhibit 50% β-carotene bleaching.

2.3.4. Superoxide radical scavenging

Superoxide radicals are generated in a phenazin methosulfate (PMS)-β-nicotinamide adenine dinucleotide (reduced form, NADH) system by oxidation of NADH and assayed by the reduction of nitroblue tetrazolium (NBT). In this experiment, the superoxide radicals were generated in 300 μL of sodium phosphate buffer (19 mM, pH 7.4) which contained 40 μL NADH (2 mM), 30 μL of NBT (500 μM), 40 μL of PMS (60 μM), AME and EME at different concentrations or distilled water and alcohol for controls. The color reaction was detected at 550 nm using a Microplate reader (Valentão et al., 2001). The scavenging ratio (%) was calculated using the following formula:

$$\text{Scavenging ratio}(\%) = (C - S)/C \times 100$$

where C is the absorbance of the control, and S the absorbance of the test sample. SC_{50} values denote the μg GAE/mL required to scavenge 50% of superoxide free radicals.

2.3.5. Hydroxyl radical scavenging

The deoxyribose assay as described by Chobot (2010) with a slight modification was applied to measure $HO^{\bullet}$ scavenger capacity. The reactants can only be dissolved in water or inorganic buffers, because the hydroxyl radical reacts with most of the organic solvents and substances employed for buffer preparations. Therefore, the aqueous extract was dissolved in a KH_2PO_4/KOH buffer solution (50 mM, pH 7.4) to yield final concentrations of 2.51 to 25.05 μg GAE/mL; to 250 μL of this solution, 50 μL of a 10.4 mM 2-deoxy-D-ribose solution in the same buffer system and 100 μL of an aqueous solution of $FeCl_3$ (50 μM) were added. In one series, those 100 μL contained 52 μM EDTA dissolved in buffer, which was premixed with the aqueous $FeCl_3$ solution (1:1 v/v). In the other series, the EDTA solution was replaced by the same volume of the buffer. In the first series, EDTA complexed the iron ions, preventing them from being chelated by the test compound; in the second series, the iron ions were complexed by the test compound. To start the Fenton reaction, various reactants dissolved in the above-mentioned buffer systems or in water were added: 50 μL of 10 mM aqueous solution of H_2O_2 and 50 μL of 1.0 mM ascorbic acid in buffer. The mixture was vortexed and incubated at 37 °C for 60 min. Thereafter, 10 μL of 2% ethanolic (BHT) butylated hydroxytoluene solution (w/v) followed by 500 μL of 1% 2-thiobarbituric acid dissolved in 3% trichloroacetic acid (w/v) was added to each vial to detect malonyl-dialdehyde, the decomposition product of 2-deoxy-D-ribose caused by the attack of hydroxyl radicals. The tubes were vortexed and heated in a water bath at 100 °C for 20 min. The reaction was stopped by transferring the tubes into an ice water bath for 3 min. To extract the reaction product of MDA and thiobarbituric acid, 700 μL of n-butanol was added, and the mixture was vigorously stirred. The absorbance of butanolic layers (600 μL), was determined at 532 nm. Reaction mixtures lacking the test compound were used as positive control (100% MDA). The negative control contained the full reaction mixture without 2-deoxy-D-ribose.

A modification was carried out without the addition of ascorbic acid, which was replaced by the same volume of the buffer. The negative control contained the full reaction mixture without 2-deoxy-D-ribose. The positive control was the H_2O_2/Fe^{3+}/ ascorbic acid system mixture lacking the extract (100% MDA).

Percent inhibition (I) of deoxyribose degradation was calculated according to the equation:

$$I\% = (A_0 - A_1/A_0) \times 100,$$

where A_0 is the absorbance of the positive control (100% MDA) reaction and A_1 is the absorbance of the extract tested. IC_{50} values denote the μg GAE/mL required to inhibit the degradation of 50% of 2-deoxy-D-ribose present.

2.4. Anti-inflammatory activity

2.4.1. Lipoxygenase enzyme assay

The UV absorbance-based assay of Sircar, Shwender, & Johnson (1983) and modified by Taraporewala & Kauffman (1990) was used. Lipoxygenase (LOX) activity was determined using a spectrophotometric method, based on the enzymatic oxidation of linoleic acid to the corresponding hydroperoxide. Absorption at 234 nm was recorded as a function of time for 3 min. The reaction mixture contained substrate (50 μM linoleic acid in 0.2 M borate buffer pH 9) and enzyme (0.9 nM soy LOX-1, Sigma-Aldrich). The assay to obtain the 100% of LOX activity was performed with DMSO as solvent control. Inhibitory assays were performed in presence of different concentrations of AME and EME. The test compound concentration causing 50% inhibition of hydroperoxide-release (IC_{50}) was calculated from the concentration–inhibition response curve by regression analysis. The

Table 1
Functional compounds concentration of mistol extracts.

Extracts	Proanthocyanidins g PACs/100 g DW	Phenolic compounds mg GAE/100 g DW	Flavonoids mg QE/100 g DW
AME[1]	84.54 ± 0.0^{b}	834.81 ± 104.29^{b}	318.62 ± 24.82^{a}
EME[2]	22.88 ± 4.64^{c}	797.20 ± 28.16^{b}	21.71 ± 0.90^{c}
AWME[3]	303.80 ± 10.34^{a}	1297.20 ± 143.40^{a}	91.00 ± 4.06^{b}

[1]Aqueous mistol extraction (AME),[2]Ethanolic mistol extraction (EME), [3]Acetonic water mistol extraction (AWME).
Proanthocyanidins content (PACs), gallic acid equivalent (GAE) and quercetin equivalent (QE).
Means ± SD followed by the same letter are not significantly different (Tukey's HSD, $p \leq 0.05$).

Table 2
Macronutrient concentration of mistol fruit.

Extracts	Total sugar	Reducing sugar g GE/100 g DW	Glucose	Fructose g FE/100 g DW	Sucrose g SE/100 g DW	Soluble proteins mg BSA/100 g DW
AME[1]	47.5 ± 2.19[a]	43.0 ± 7.71[a]	9.0 ± 0.81[a]	33.6 ± 2.31[a]	5.2 ± 0.34[a]	151.58 ± 19.64
EME[2]	34.2 ± 0.80[b]	30.0 ± 6.50[a]	7.5 ± 0.43[b]	18.7 ± 1.32[b]	4.1 ± 0.12[a]	<LD[3]

[1]Aqueous mistol extraction (AME), [2]Ethanolic mistol extraction (EME), [3]<LD = below limits of detection.
Means ± SD followed by the same letter are not significantly different (Tukey's HSD, $p \leq 0.05$).

extinction coefficient of 25 mM^{-1} cm^{-1} was used for hydroperoxide quantification. Caffeic acid was used as reference anti-inflammatory compounds.

2.5. Mutagenicity

2.5.1. Salmonella mutagenicity assay

The mutagenic effects of *Ziziphus mistol* extracts were evaluated on two *S. typhimurium* strains (TA98 and TA100). The plate incorporation assay was performed according to Maron and Ames (1983), by adding 0.1 mL of the overnight bacterial culture, 0.1 mL of extracts at different concentrations (6.25, 12.5 and 25 mg DW/plate) and 2 mL of top agar on minimal agar. In the case of metabolic activation, 0.5 mL S9 mixture was supplemented. His^+ revertants were counted after 72 h of incubation at 37 °C.

The positive controls employed were 4-nitro-o-phenylenediamine (4-NPD; Aldrich Chemical Co.), 20 μg/plate and 2-aminofluorene (2-AF; Merck), 10 μg/plate. As a solvent control 100 μL DMSO/plate was run concurrently with all experiments. Three plates at two separate experiments were used for each concentration tested and for positive and negative controls.

2.6. Statistical analysis

Sampling and analyses were performed in triplicate, and the data are presented as mean ± standard deviation. GraphPad Prism 5.0 software was used to perform analysis of correlation between two variants by Pearson test with the level of significance set at $p<0.05$ and of variance (ANOVA) with Tukey post test at a confidence level of 95%.

3. Results and discussion

3.1. Phytonutrient compounds in Ziziphus mistol fruit

Plant foods (fruits, vegetables, grains and grain derived food) are of great interest because they provide macro and micronutrients and bioactive components (phytochemicals or phytonutrients) (Manach, Scalbert, Morand, Rémésy, & Jimenez, 2004). Among them, phenolic compounds are very important by virtue of their antioxidant activity by radical scavenging, chelating redox active metal ions, inactivating lipid free radical chains and preventing hydroperoxide conversions into reactive oxyradicals (de Oliveira et al., 2009). Interestingly, the total polyphenol content of *Z. mistol* fruit extracted with 70% aqueous acetone (1195.80 ± 67.82 mg GAE/100 g DW) was not significantly lower than that of *Z. jujube* fruit (1320.00 ± 60.20 mg GAE/100 g) as reported by Sun, Liang, Shan, Viernstein, & Unger (2010). Condensed tannins were the dominant phenolics in the AWME (303.80 ± 10.34 mg PB2E/100 g DW), Table 1. Consumption of food, beverages, and spices containing flavan-3-ols and their oligomeric and polymeric derivatives has been shown to be associated with possible prevention of chronic diseases such as cancer, atherosclerosis, cardiovascular diseases, diabetes type II, infections, and photoprotection of the skin against UV radiation (Anderson et al., 2004; Heinrich, Neukam, Tronnier, Sies, & Stahl, 2006). Furthermore, proanthocyanidins are important for the quality of the products, especially for red wines and juices, because they are responsible for their bitterness, astringency and color properties. It seems more than likely that the interactions between salivary proteins and polyphenols, in particular tannin, are responsible for the astringency of foods and beverages (Clifford, 1986).

In our working conditions, gallic acid was not detected after acid AWME hydrolysis. Our results would thus indicate the absence of hydrolizable tannins in mistol fruit. Anthocyanins are usually responsible for the red, blue and purple colors displayed in many fruits, but they were not detected in *Z. mistol* fruit, while in *Z. jujube* fruit total anthocyanins content varied from 31.30 ± 7.16 to 79.80 ± 6.37 mg/100 g DW (Sun et al., 2010). Likewise, carotenoids, alkaloids and ascorbic acid were not detected in our samples of mistol fruit (data not shown).

The crude protein content of *Z. mistol* fruits was 3.06 ± 0.31 g/100 g DW, while in edible fruits from other plant species which grow in the same environments, such as *Prosopis alba* and *nigra,* protein content was around 4.2% (Cardozo et al., 2010).

Table 3
Antioxidant activity of two treatments simulating domestic cooking processing (food preparations) obtained from *Z. mistol* fruit expressed as μg GAE/mL.

Extracts	DPPH	ABTS	β-carotene	Superoxide anion	Hydroxyl radical
	SC_{50}	SC_{50}	IC_{50}	SC_{50}	SC_{50}
AME[1]	64.77 ± 4.51[bc]	7.38 ± 0.23[c]	10.87 ± 1.24[c]	134.98 ± 23.48[b]	14.13 ± 3.21[c]
EME[2]	6.31 ± 0.72[c]	1.45 ± 0.09[c]	4.49 ± 0.51[c]	476.81 ± 50.71[a]	–

[1]Aqueous mistol extraction (AME), [2]Ethanolic mistol extraction (EME). Means ± SD followed by the same letter are not significantly different (Tukey's HSD, $p \leq 0.05$).

Table 4
Mutagenicity of *Ziziphus mistol* extracts on *Salmonella* microsome assay (TA98 and TA100 strains).

Samples	Dose level (mg DW/plate)	TA100		T98	
		− S9	+ S9	− S9	+ S9
Negative Control[1]		164 ± 16[c]	135 ± 12[c]	44 ± 5[c]	64 ± 6[c]
AME	6.25	116 ± 28[c]	ND	48 ± 4[c]	ND
	12.5	122 ± 16[c]	143 ± 16[c]	50 ± 6[c]	62 ± 2[c]
	25	182 ± 00[c]	154 ± 18[c]	50 ± 6[c]	51 ± 2[c]
EME	6.25	ND	130 ± 28[c]	ND	45 ± 1[c]
	12.5	160 ± 0[c]	125 ± 21[c]	50 ± 12[c]	59 ± 12[c]
	25	76 ± 8[c]	120 ± 0[c]	27 ± 4[c]	37 ± 6[c]
Positive Control[2]		1055 ± 190[b]	1380 ± 78[a]	1438 ± 114[a]	865 ± 19[b]

[1]The number of spontaneous revertants was determined with the corresponding solvents. AME, aqueous mistol extraction. EME, ethanolic mistol extraction. [2]Positive controls employed were 4-nitro-o-phenylendiamine (4-NPD) at 20 μg/plate and 2-aminofluorene (2-AF) at 10 μg/plate, without and with S9 mix, respectively. ND, not determined values. Data are means ± SD of three plates at two separate experiments. Values followed by the same letter are not significantly different (Tukey's HSD, $p \leq 0.05$).

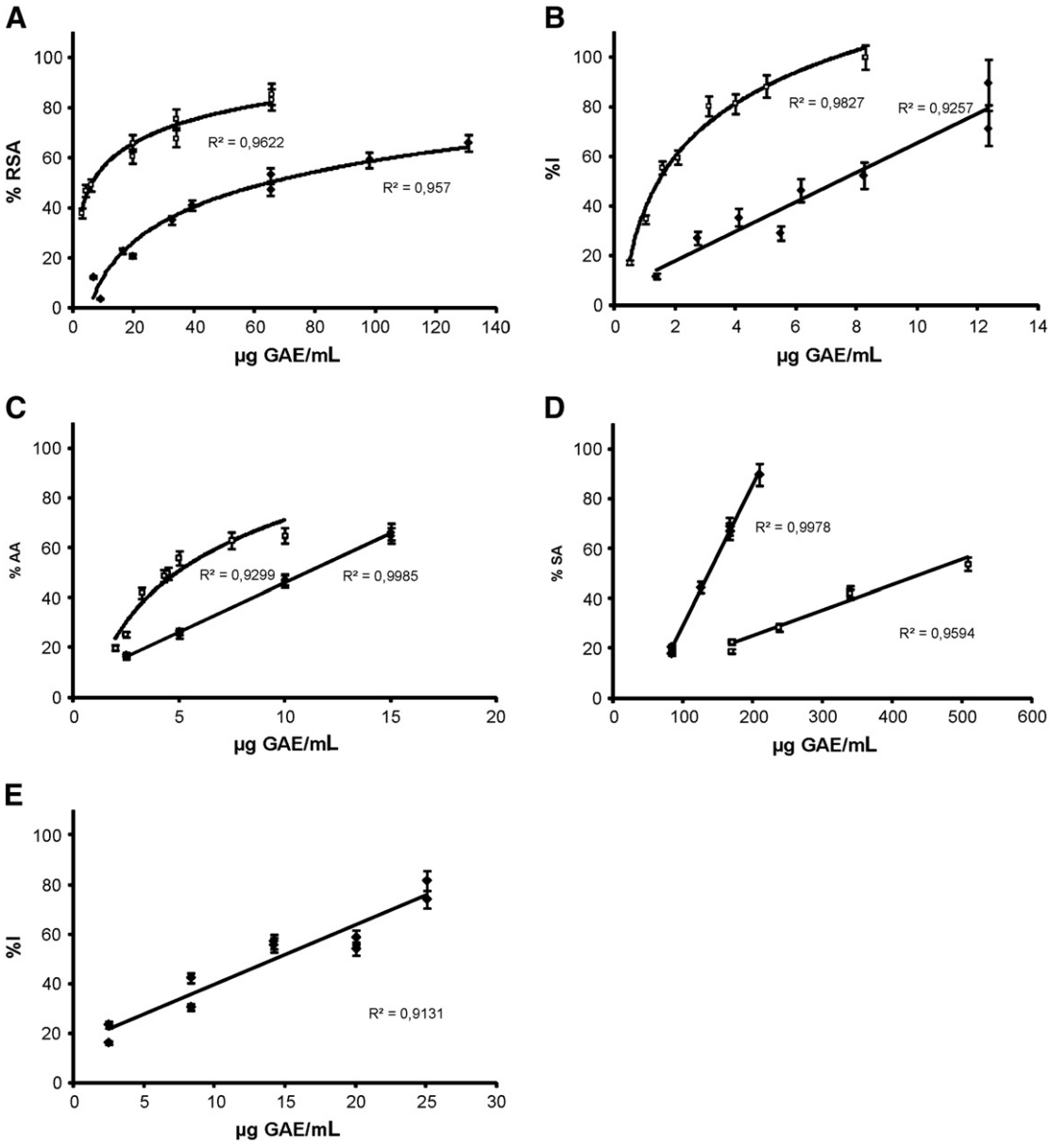

Fig. 2. Antioxidant activity of ethanolic mistol extract (□) and aqueous mistol extract (♦): (A) Percentage of radical scavenging activity (% RSA) on DPPH radical; (B) Percentage of inhibition (% I) of ABTS radical decoloration; (C) Percentage of antioxidant activity (AA%) relative to control using the β-carotene bleaching assay; (D) Percentage of scavenging activity (% SA) on superoxide radical; (E) Percentage of inhibition (% I) of 2-deoxy-ribose degradation. Data are expressed as means ± standard deviations.

3.2. Total phenolics, sugars and protein contents of two treatments simulating domestic cooking processing (food preparations) with mistol fruit

Processing fruit and vegetables affects their chemical composition and functional properties. For some vegetables, such as carrots, spinach, mushrooms, peppers, potatoes, sweet potatoes, cabbage, broccoli and tomatoes, antioxidant activity increased when these vegetables were boiled or steamed (Potsędek, 2007).

Fruits are most commonly consumed raw; however, their jams and liquors are also prepared to preserve them for longer periods of time.

Two preparations simulating domestic processing were obtained from the ripe fruit, an aqueous preparation with heating (decoction, AME) and an ethanolic extract (tincture, EME). Both preparations exhibited similar free total phenolics amounts (797.19 ± 28.16 and 834.81 ± 104.29 mg GAE/100 g DW for EME and AME, respectively), (Table 1) while flavonoid (Table 1) and sugar contents were significantly higher in AME than in EME (Table 2). Soluble proteins for AME were 151.58 ± 19.64 mg BSA/100 g DW while EME did not show detectable amounts. The increase in flavonoids and sugars can be due to their release from the food matrix after heating and disruption of the plant tissue. The conversion of insoluble phenolics into more soluble forms can also occur. Food processing, like the exposure to higher temperatures, can lead to cellular disruption and disassociation of some phenolic compounds and sugars from cellular structures such as lignin, tannins and polysaccharides (Bernhart & Schlich, 2005).

3.3. Antioxidant properties of two treatments simulating domestic cooking processing (food preparations) with mistol fruit

In vitro chemical assays, like DPPH and ABTS tests, are robust methods to quickly screen total or antiradical activities in extracts or pure compounds. Both preparations exhibited DPPH and ABTS reducing capacity (Table 3, Fig. 2A, B) with SC_{50} values between 1.45 and 64.77 µg GAE/mL.

All extracts were able to protect linoleic acid from oxidation with IC_{50} values of 4 to 10 μg GAE/mL (Fig. 2C). The ethanolic extract exhibited a significantly higher antioxidant activity in the ABTS, DPPH and β-carotene system than the aqueous extract.

Its superior activity, with respect to that observed for the water extract, might be associated to its higher phenolic aglicons content or the transformation of some phytochemicals after heating. As previously stated, food processing can lead to alterations in the amount and composition of polyphenols (flavonoids content in AME, Table 2). *Z. mistol* ethanolic extract, evaluated in this study, exhibited nearly 3-fold higher hydrogen or electron donor capacity than alcoholic extracts obtained from *Prosopis alba* and *nigra* pods (Cardozo et al., 2010).

Superoxide and hydroxyl radicals ($OH^{\bullet}$) have been observed to kill cells, inactivate enzymes and degrade DNA, cell membranes and polysaccharides. They occupy an exceptional position among ROS because of their extreme reactivity and oxidative potential (Wilson et al., 2006). Both extracts had a scavenging action on superoxide radicals with SC_{50} values of 134.98 ± 23.48 and 476.81 ± 50.71 μg GAE/mL for AME and EME, respectively. The aqueous extraction was 3.5 folds more active than the ethanolic one (Fig. 2D).

In the assay used to evaluate $OH^{\bullet}$ scavenger ability, AME showed a significant antioxidant effect ($P<0.0001$) by decreasing deoxyribose degradation in both systems, either with or without addition of EDTA. With EDTA added, AME was found to inhibit 2-deoxy-D-ribose degradation with an IC_{50} value of 14.13 μg GAE/mL (Fig. 2E). Hence, it can be concluded that AME has compounds with O_2^{-} and $OH^{\bullet}$ scavenger ability.

When iron is added to the assay mixture as $FeC1_3$ instead of ferric-EDTA, some of the Fe^{3+} ions bind to 2-deoxy-D-ribose, and hydroxyl radicals arise close to the 2-deoxy-D-ribose molecule. Accordingly, compounds with ligand properties compete for iron ions with the 2-deoxy-D-ribose molecules and thus decrease the 2-deoxy-D-ribose degradation that is caused by iron-catalyzed hydroxyl radical attack (Aruoma, Grootveld, & Halliwell, 1987).

The ability of a substance to inhibit 2-deoxy-D-ribose degradation under these reaction conditions is a measure of its ability to chelate iron ions and interfere with $OH^{\bullet}$ generation. Our results suggested that the iron ion chelating ability of AME contributed in a significant manner ($P<0.0001$) to the observed antioxidant effect, because 2-deoxy-D-ribose protection against the attack by hydroxyl radicals was more efficient in the medium without EDTA added (Fig. 3). Phenolic compounds, present in the extract, are well-known as efficient free radical scavengers and chelators of transition metals (Cheng & Breen, 2000). A decrease of the relative MDA concentrations in a dose-dependent manner in both variants of the assay was observed (Fig. 3).

Additionally, AME had a marked pro-oxidative effect at low concentrations in the variant without EDTA (Fig. 3B).The pro-oxidant effect was not seen at higher concentrations, perhaps because it was outweighed by the $OH^{\bullet}$ scavenging activity of the extract. Pro-oxidant effect was also absent when EDTA was present, thus suggesting that this effect is related to the presence of free Fe^{3+} ions in the medium.

In all cases, polyphenols showed a dose–response relationship ($R^2>0.90$) with antioxidant capacity in the decoction and the alcoholic beverage.

According to our results the extracts have higher antioxidant potency in one assay system than in the others (Table 3). This confirms that there is no universal method to measure the antioxidant capacity of all samples accurately and consistently. Clearly, matching radical source and system characteristics to antioxidant reaction mechanisms is critical in the selection of an appropriate antioxidant capacity assay assessing method (Prior, Wu, & Schaich, 2005).

3.4. Anti-inflammatory activity of two treatments simulating domestic cooking processing (food preparations) with mistol fruit

Products of the 5-LOX pathway are important mediators of inflammation. LOX and its reaction products are shown to play an important role in tumor formation and cancer metastasis. High expression of 5-LOX was found in prostate, lung, colon, breast, and other cancer cell lines (Wang & Dubois, 2010). Recently it has been shown that 5-LOX is a critical regulator for leukemia cancer stem cells in chronic myeloid leukemia (CML) (Chen, Hu, Zhang, Peng, & Li, 2009). Inhibitors of the 5-LOX pathway, therefore, have a therapeutic potential in a variety of inflammatory and allergic diseases as well as in cancer therapy. Soy LOX-1 is used as an in vitro biochemical model, since it resembles human LOXs in its substrate specificity and inhibition characteristics (Mahesha, Sridevi, & Appu Rao, 2007).

In our working conditions, only EME showed an inhibitory effect on LOX activity with an IC_{50} value of 183.80 μg GAE/mL while AME did not show an inhibitory effect on LOX activity in the concentrations

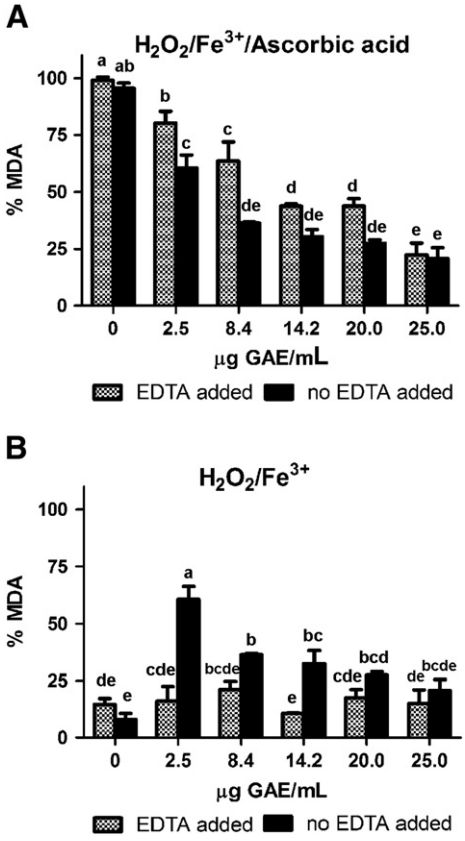

Fig. 3. Hydroxyl radical scavenging activity in various systems of the deoxyribose assay, expressed as % malonyldialdehyde (MDA), of different amounts (0–25 μg GAE/mL) of AME; N = 3. Means ± SD followed by the same letter are not significantly different (Tukey's HSD, $p \leq 0.05$).

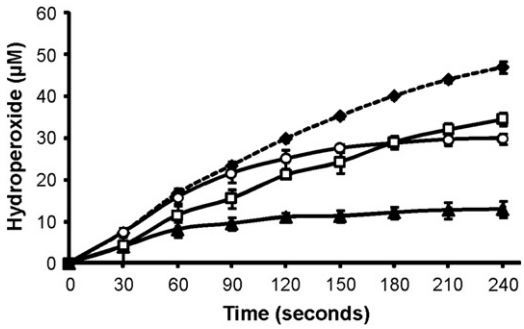

Fig. 4. Lipoxygenase activity without fruit extracts (--◆--) and in presence of different concentrations of EME: (-□-) 73.57 μg GAE/mL; (-○-) 147.16 μg GAE/mL; (-▲-) 255.43 μg GAE/mL.

tested (until 45.08 μg GAE/mL) (Fig. 4). These results suggest that bioactive compounds might be thermolabile.

LOX are sensitive to antioxidants, and the most common way of their action may consist in inhibition of lipid hydroperoxide formation due to scavenging of lipid oxy- or lipid peroxy-radicals formed in course of enzymic peroxidation. This could limit the availability of lipid hydroperoxide substrate necessary for the catalytic cycle of LOX.

Other probable action mechanism is presumably related to the ability of LOX inhibitors to chelate Fe^{3+}, since LOX contain a "non-heme" iron per molecule in the enzyme active site.

3.5. Mutagenicity activity

Many phytochemicals present in plant foods or beverages have antioxidant, anti-mutagenic, anti-carcinogenic and anti-inflammatory properties that might potentially be beneficial in preventing diseases and protecting genome stability. However, not all phytochemicals and not all actions of individual phytochemicals are necessarily favorable. Some have mutagenic and/or pro-oxidant effects, and can interfere with essential biochemical pathways (Ferguson, 2001). Hence, the mutagenicity of mistol preparations was analyzed (Table 4). Different doses of aqueous and ethanolic *Z. mistol* extracts did not show any mutagenic effect on TA98 and TA100 strains with and without metabolic activation (S9 mixture).

In brief, the absence of genotoxic response by the extracts against *Salmonella* is a positive step towards determining the safe use of the fruit in traditional medicine.

4. Conclusion

The present study indicates the presence of compounds in mistol fruit which are potent scavengers, inhibitors of lipid peroxidation and LOX-1. Our data highlights the good antioxidant and anti-inflammatory properties of ethanolic extracts from mistol fruit that make it of interest in preparing food supplements or additives with antioxidant and preservative properties.

It is worth mentioning that the total phenolic content of the extracts does not seem to determine their antiradical and anti-inflammatory activity. These results lay the groundwork for further studies on the molecular mechanisms underlying the biological profile of the extracts.

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