Journal Pre-proof

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PII: S2210-8033(19)30043-0

DOI: https://doi.org/10.1016/j.hermed.2019.100296

Article Number: 100296

Reference: HERMED 100296

To appear in:

Received Date: 16 May 2018

Revised Date: 8 February 2019

Accepted Date: 7 August 2019

Please cite this article as: Carabajal MPA, Isla MI, Borsarelli CD, Zampini IC, Influence of *in vitro* gastro-duodenal digestion on the antioxidant activity of single and mixed three "*Jarilla*" species infusions, *Journal of Herbal Medicine* (2019), doi: https://doi.org/10.1016/j.hermed.2019.100296

Influence of *in vitro* gastro-duodenal digestion on the antioxidant activity of single and mixed three "*Jarilla*" species infusions

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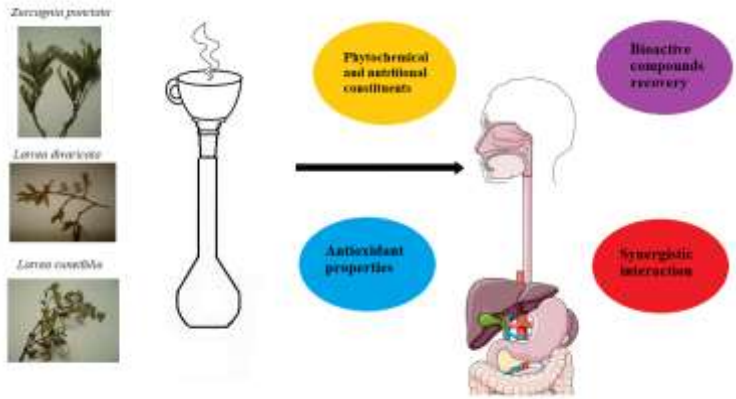
Graphical abstract



Highlights.

- Infusions from three "Jarilla" species and ternary mixtures from them were formulated
- The infusions were characterized through a functional and nutritional viewpoint
- After gastroduodenal passing, the infusions were still able to scavenge free radicals
- Bioavailability of three chemical markers was analyzed after the *in vitro* digestion
- The herbal combination HM2 exhibits synergism on the antioxidant potential analyses

Abstract

Zuccagnia punctata Cav. (Zp), *Larrea cuneifolia* Cav. (Lc) and *Larrea divaricata* Cav. (Ld), are shrubs commonly called "Jarillas" and used in Argentinian local communities as medicinal plants. In this work, phytochemical profile and antioxidant activity of infusions prepared from aerial parts and mixtures in different proportions, before and after a simulated gastro-duodenal digestion was determined. The main phytochemical groups, protein, sugar and mineral content were analyzed, whereas some chemical markers were quantified by HPLC-DAD. All infusions exhibited antioxidant activity measured by different mechanisms (total reducing power and free radical scavenging). Although the antioxidant potential was lower after passing through the gastro-duodenal digestion process, in some cases an enhancement or synergism was observed. The mixture infusion prepared from 50% Lc, 25% Ld and 25% Zp showed

higher antioxidant power after digestion and bioactive component recovery. These beverages could be important sources of antioxidant compounds for the prevention of chronic diseases associated with oxidative stress.

Keywords: Jarilla infusions; herbal mixture; antioxidant; gastroduodenal digestion; phytochemical profile

1. Introduction

The oxidative damage to intracellular lipids, proteins and DNA is related to the development of chronic degenerative pathologies, including neurodegenerative and cardiovascular diseases, cancer and the aging process itself. In this way, prevention has been established to be associated with a high intake of fruits, vegetables, cereal products, as well as herbs, spices, and particularly teas (Li *et al*., 2014; Samsonowicz *et al*., 2019). This practice provides a wide variety of phytochemicals that have been shown to have health benefits and antioxidant activity (Chen and Xu, 2019; Tsao and Deng, 2004).

Plants species with medicinal properties have been used for different purposes since ancient times (Farzaneh and Carvalho, 2015). Thus, there is an increasing interest in bioactive compounds derived from plants, mainly polyphenols, due to their pharmacological properties, apparent safety, and economic viability (Abbas *et al*., 2017).

Argentina is noted for its extraordinary biodiversity with numerous medicinal plants species. The northwestern region of the country is composed of several eco-regions, among which the Monte region (Cabrera, 1971) is a semiarid ecosystem with low and fluctuant temperature, low absolute humidity, and high solar radiation. The predominant community is the "Jarillal" a collection of plant species popularly known as "Jarillas", which are predominantly species of *Larrea* sp. *Larrea divaricata* Cav. and *Larrea cuneifolia* Cav. (Zygophyllaceae) which can be differentiated by their leaf characters; whilst the former has only two leaflets with a small filiform mucron, the latter exhibits a greater degree of leaflet fusion. *Zuccagnia punctata* Cav. (Fabaceae) is an endemic monotypic species widely distributed in Western Argentina (Cabrera, 1971). It bears some resemblance to the rightful "Jarillas" (*Larrea* sp.) with which it coexists. According to Morello (1951), it is a constituent species of "Jarillal" and confers

phytosociological unit to the Monte region. These shrubs are widely used in traditional medicine by rural communities, mainly as infusions, to treat different disorders such as rheumatism, inflammation of the respiratory and intestinal tract, gastric disturbances, microbial infections, among others (Del Vitto *et al*., 1997).

Several biological activities have been reported for these plants. *Z. punctata* is reported to have free radical scavenging capacity (Morán Vieyra *et al*., 2009), anti-inflammatory (Alberto *et al*., 2007), antiulcer, antigenotoxic, antibacterial and antifungal properties (Butassi *et al*., 2015; Isla *et al*., 2016). *L. cuneifolia* Cav. exhibited larvicidal activity against *Culex quinquefasciatus* larvae (Batallán *et al*., 2013); antibacterial and antioxidant activity (Carabajal *et al*., 2017). From *L. divaricata* Cav. tumouristic and immunomodulatory activities, cytotoxic effect and antioxidant capacity were demonstrated (Davicino *et al*., 2011). In addition, antimicrobial, anti-inflammatory and anti-ulcerogenic activity have been shown (Davicino *et al*., 2011).

Some combinations of plant extracts have been shown to be more effective than the use of a single plant species (Wagner, 2011). For multi-herb preparations, each component may enhance the others, the biological activity resulting in a combination whose effect is stronger than the sum of the parts (synergism). However, the combination can result in antagonism (a lower beneficial effect than the sum of its individual components) (Wagner, 2011). In this regard, three Argentinian “Jarillas” that have been used in popular medicine with the same purpose could improve their biological effect in herbal mixes, mainly by their high content and variety of polyphenols.

The therapeutic effect of polyphenols is well known to depend not only on intake levels but also on their bioavailability (BAv). As a first step to evaluate the bioaccessibility BAcs of these compounds, *in vitro* methods to simulate gastrointestinal

digestion can be used to ascertain the nutritional or functional quality of a nutrient or bioactive compound.

On this basis, the aim of this work was to compare the antioxidant activity of herbal teas prepared from the aerial parts of the three "Jarilla" species spread across Northwestern Argentina (i.e. *Z. punctata, L. divaricata*. and *L. cuneifolia*) as single extracts and mixed in different proportions before and after a simulated gastro-duodenal digestion *in vitro* process.

2. Material and Methods

2.1 Chemicals and reagents

All reagents and standards were of analytical grade. 4-dimethylamino-cinnamaldehyde (≥ 98.0%)**,** 2,4,6-tripyridyl-s-triazine (≥ 99.0%), 2-deoxy-D-ribose (≥ 99.0%), 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (≥ 98.0%), gallic acid (≥ 97.5%), L- ascorbic acid (≥ 99.0%), glucose (≥ 99.5%), proanthocyanin B2 (≥ 90.0%), bovine serum albumin (≥ 98.0%), nordihydroguaiaretic acid (≥ 90.0%), porcine pancreatic α-amylase (E.C. 3.2.1.1), porcine gastric mucosa pepsin (E.C. 3.4.23.1) and porcine pancreatic pancreatin (E.C. 232-468-9) were acquired from Sigma-Aldrich Co (Missouri, USA). Rhodanine (≥ 99.0%) and Folin–Ciocalteau reagent were obtained from Merck (Darmstadt, Germany). Quercetin (≥ 99.5%) was purchased from Fluka (Werdenberg, Switzerland), ethylenediaminetetraacetic acid (≥ 99%) was acquired from Cicarelli (Santa Fe, Argentina) and Bradford reagent was obtained from BioRad Laboratories Inc. (California, USA). The standard 2′,4′-

dihydroxychalcone (≥ 96.0%) was purchased from Indofine Inc. (New Jersey, USA) and 2′,4′-dihydroxy-3′-methoxychalcone was isolated according to Nuño *et al*. (2014).

2.2 Plant material

Aerial parts (leaves and stems) of *Z. punctata* Cav. (Zp), *L. cuneifolia* Cav. (Lc) and *L. divaricata* Cav. (Ld) were harvested from Amaicha del Valle, Tucumán, in April 2013. The plants were identified by the botanic specialist of INBIOFIV (Instituto de Bioprospección y Fisiología Vegetal) and voucher specimens were deposited at Fundación Miguel Lillo Herbarium (*Z. punctata*: LIL 612170; *L. cuneifolia*: LIL 614829 (A y B); *L. divaricata*: LIL 614299). The plant material was powdered and stored at room temperature in plastic flasks protected from light.

2.3 Herbal infusion preparation

Infusions were obtained following the usual domestic procedure by mixing 2 g of ground plant material and 200 mL of boiling distilled water. Three infusions were prepared from each plant species (Zp, Lc and Ld) and four ternary mixtures on different proportions:

Herbal mixture 1 (HM1): 1 g *Z. punctata* + 0.5 g *L. cuneifolia* + 0.5 g *L. divaricata*.

Herbal mixture 2 (HM2): 1 g *L. cuneifolia* + 0.5 g *Z. punctata* + 0.5 g *L. divaricata*.

Herbal mixture 3 (HM3): 1 g *L. divaricata* + 0.5 g *L. cuneifolia* + 0.5 g *Z. punctata*.

Herbal mixture 4 (HM4): 0.66 g *L. divaricata*+ 0.66 g *L. cuneifolia* + 0.66 g *Z. punctata*.

Herbal infusions were stored at 4°C in order to quantify their main chemical constituents.

2.4 Nutritional components

2.4.1 Sugar determination

The phenol–sulphuric acid method was used to determine total neutral sugars in herbal infusions (DuBois *et al.*, 1956). Aliquots (0.03 mL) of each infusion were diluted to 0.8 mL with distilled water. Then, 0.04 mL of 80% phenol and 2 mL H_2SO_4 were added. After 20 min incubation at 100 °C, absorbance at 490 nm was measured on a UV/visible spectrophotometer (Unicom 2400PC, China).

Reducing sugars were measured by using the Somogyi–Nelson method (Nelson, 1944; Somogyi, 1945). Infusion aliquots (0.05 mL) were diluted with distilled water to 0.5 mL and 0.5 mL of copper tartrate reagent (Somogyi, 1945) was added. The solution was then heated at 100°C for 15 min and 0.5 mL of arsenal molybdic acid reagent (Nelson, 1944) was added. Absorbance was measured at 520 nm.

Results were expressed as mg of glucose per cup (mg GE/cup), considering that a cup is equivalent to 200 mL of infusion.

2.4.2 Soluble protein determination

Soluble protein concentration in all preparations was determined according to Bradford (1976) by using bovine serum albumin (BSA) as a standard. Aliquots (0.1 mL) of infusion were taken and 0.8 mL of distilled water and 0.2 mL of dye solution (Coomassie Brilliant Blue G 250) were added. After 10 min at room temperature,

absorbance was measured at 595 nm. Results were expressed as mg of bovine serum albumin per cup (BSA/cup).

2.4.3 Mineral analysis

The analysis was carried out by quadrupole inductively coupled plasma mass spectrometry (Q-ICPMS). A Thermo-Elemental X7 series (Thermo Fisher Scientific, Bremen, Germany), equipped with an ASX-100 autosampler model (CETAC Technologies, Omaha, NE) was used (Instituto Superior de Investigación, Desarrollo y Servicios en Alimentos, ISIDSA, Córdoba, Argentina). Results were expressed as mg/ cup.

2.5 Phytochemical characterization

2.5.1 Soluble principle quantification

In order to determine soluble principles, the mixtures and single-plant infusions were dried by freeze-drying and the residues were weighed and stored at -20°C for further experiments. Results are expressed as mg per cup (mg/cup).

2.5.2 Quantification of the main phytochemicals

2.5.2.1 Total phenolic content

Total phenolic compounds were determined by the Folin-Ciocalteau method (Singleton *et al*., 1999). A volume of 10 µL of herbal infusion was mixed with 990 µL of distilled water, 100 µL of Folin Ciocalteau reagent and 400 µL of 15.9% sodium carbonate. The mixture was allowed to stand for 20 min at room temperature (23-26ºC).

The blue color developed was read at 765 nm. Results were expressed as mg of gallic acid equivalent per cup of infusion (mg GAE/cup).

2.5.2.2 Non-flavonoids phenolic compounds

Non-flavonoid phenolics were measured by the determination of total phenol content remaining after precipitation of flavonoids with acidic formaldehyde (Isla *et al*., 2014). An aliquot of each infusion (0.5 mL) was mixed with 0.5 mL HCl (1:3) and 0.25 mL of 8 g/L formaldehyde. The mixture was kept at room temperature for 24h and then centrifuged at 9000g for 5 min. The amount of non-flavonoid phenolics was determined in the supernatant according to Singleton *et al*. (1999). Results were expressed mg GAE/cup.

2.5.2.3 Flavone and flavonol content

Total flavone and flavonol content were measured by spectrophotometric assay based on aluminum chloride complex formation (Woisky and Salatino, 1998). A volume of 200 µL of infusion was mixed with 2250 µL of ethanol and 50 µL of 5% aluminum chloride was then added to the mixture. The mixture was kept at room temperature for 30 min and absorbance was measured at 415 nm. Results were expressed as mg quercetin equivalent per cup of infusion (mg QE/cup).

2.5.2.4 Hydrolysable tannin content

The rhodanine assay (Inoue and Hagerman, 1988) was used for the determination of gallotannin in the herbal infusions. For this purpose, 2 mL of infusion was hydrolyzed with 4 mL of 2 N H_2SO_4 at 100ºC for 26 h. 50 µL of non-hydrolyzed extract was diluted to 100 µL with 0.4 N H_2SO_4 and 100 µL of the hydrolyzed extract was mixed with 150 µL of rhodanine (0.667% methanol). After 5 min, 500 µL of 0.5 N potassium hydroxide and 1.75 mL distilled water were added. Both mixtures were kept for 10 min at room

temperature and absorbance was determined at 520 nm. Gallotannin concentrations were expressed as mg GAE/cup by difference between the amount of gallic acid present in hydrolyzed and non-hydrolyzed samples.

2.5.2.5 Condensed tannin content

Proanthocyanidin content was determined with 4-dimethylaminocinnam-aldehyde (DMAC) according to Prior *et al*. (2010) with slight modifications. Briefly, 50 µL of infusion was diluted with 80% ethanol to 300 µL and then 900 µL of DMAC solution (0.1% in acidified ethanol) was added. After 20 min at 25ºC, the absorbance was measured at 640 nm. Data were expressed as mg of procanidin B2 equivalents per cup (mg PB2E/cup).

2.6 RP-HPLC-DAD quantification method

Phenolic compounds present in Jarilla infusions were analyzed by reverse phase high performance liquid chromatography (RP-HPLC) fingerprints. The separation module consisted of a Waters 1525 Binary HPLC Pumps system with a 1500 Series Column Heater, a manual injection valve with a 20 µL loop (Rheodyne Inc., Cotati, CA) and a Waters 2998 photodiode array detector (PDA). An XBridge™ C18 column (4.6 mm×100 mm, 5µm; Waters corporation, Milford, MA) with a two-gradient solvent system was used. A system of solvent A (0.1% acetic acid in water) and solvent B (0.1% acetic acid in methanol) (conditions: 10–57% B from 0 to 45 min and 57–100% B from 45 to 65 min) was used. The flow rate was set at 0.5 mL/min. A solution of 3 mg/mL was used. Data collection was carried out with EmpowerTM2 software. Compounds were identified by comparing their retention times and UV–Vis spectra in the 220–500 nm range by using a spectra database of commercial standards. The main components 2′,4′-

dihydroxychalcone (DHC), 2′,4′-dihydroxy-3′-methoxychalcone (DHMC) and nordihydroguaiaretic acid (NDGA) were quantified by using plots relating areas and concentrations of commercial standards.

2.7 Measurement of antioxidant activity

2.7.1 ABTS radical cation decolorization assay

The antioxidant capacity assay was carried out by the $ABTS^{\bullet+}$ method as described by Re *et al*. (1999). The radical cation was generated by reacting 7 mM of 2,2′-Azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) and 2.45 mM potassium persulfate and then maintaining at room temperature for 16 h in the dark. The resulting $ABTS^{\bullet+}$ solution was diluted in ethanol to an absorbance of 0.700±0.02 at 734 nm. The assay was carried out on a microplate and 100 µL of diluted $ABTS^{\bullet+}$ solution was added to different extract concentrations (0-100 µg/mL) dissolved in dimethyl sulfoxide (DMSO). Absorbance was recorded at 734 nm in a microplate reader (Thermo scientific Multiskan GO, Finland) after 6 min. The inhibition percentage was measured by the following formula:

$$\%\ \text{Inhibition} = \frac{(A_C - A_S)}{A_C} \times 100$$

where A_C is the control absorbance and As is the sample absorbance. Green tea extract was used as a positive control. The results were reported as SC_{50} (µg/mL required to scavenge 50% of free radicals).

2.7.2 Ferric Reducing Antioxidant Power (FRAP)

The FRAP assay was performed according to Thaipong *et al*. (2006) with slight modifications. The stock solutions included 300 mM acetate buffer (3.1 g $C_2H_3NaO_2.3H_2O$ and 16 mL $C_2H_4O_2$), pH 3.6, 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) solution in 40 mM HCl, and 20 mM $FeCl_3.6H_2O$ solution. The fresh working solution was prepared by mixing 25 mL acetate buffer, 2.5 mL TPTZ solution, and 2.5 mL $FeCl_3.6H_2O$ solution and then warmed at 37°C before use. Aliquots (15 µL) of different extract concentrations were taken and 285 µL of the FRAP solution added and then left for 30 min in the dark. Readings of the colored product (ferrous tripyridyltriazine complex) were taken at 593 nm. Results are expressed in µmol of ascorbic acid equivalent per milliliter of infusion (µmol AAE/mL).

2.7.3 2-deoxy-D-ribose degradation assay

In order to evaluate the hydroxyl radical scavenging capacity of herbal infusions, the 2-deoxy-D-ribose degradation assay was applied as described by Chobot (2010), with slight modifications. After the Fenton reaction, 250 µL of 2-thiobarbituric acid (1% w/v) dissolved in trichloroacetic acid (3% w/v) was added to each vial to detect malondialdehyde (MDA). The tubes were vortexed and heated at 100 °C for 20 min. The reaction was halted by transferring the tubes into an ice water bath. Absorbance was determined at 532 nm. In one series, an ethylenediaminetetraacetic acid (EDTA) solution was replaced by a buffer solution in order to evaluate the capacity of the compounds present in the samples to chelate iron ions and, in other series, the reaction was carried out without the addition of ascorbic acid to determine pro-oxidative effect.

2.7.4 Synergism, additive and antagonism

The median effect principle was adopted to evaluate the interactions between different extracts (Chou, 2010). Data from this study were analyzed with CompuSyn Software. The combination index (CI) was calculated to identify synergistic (CI<1), additive (CI=1) and antagonistic interactions (CI>1).

2.8 Simulated gastroduodenal digestion

Gastro-duodenal (GD) digestion was performed according to Tenore *et al*. (2015), divided into three stages: salivary, gastric and duodenal digestion. Briefly, for the first stage, 20 mL of each infusion was mixed with 6 mL of an artificial saliva (89.6 g/L KCl, 20 g/L KSCN, 88.8 g/L NaH_2PO_4, 57.0 g/L Na_2SO_4, 175.3 g/L NaCl, 84.7 g/L $NaHCO_3$, 25.0 g/L urea and 290 mg α-amylase) at pH 6.8. The mixture was taken up to 40 mL, homogenized (180 rpm, Jeio Tech BS-11 shaking bath, USA) and incubated for 3 min at 37ºC. For gastric digestion, pepsin (14,800 U) dissolved in 0.1 M HCl was added. The pH was adjusted to 2.0 (with 6 M HCl) and the mixture was incubated at 37ºC for 2 h. Subsequently, in intestinal digestion, the pH was adjusted to 6.5 with 0.5 M $NaHCO_3$ and 5 mL of a mixture (1:1; v/v) containing pancreatin (8 mg/mL) and bile salts (50 mg/mL) dissolved in water were added. Lastly, the mixture was incubated at 37ºC for 2 h. Controls (ultrapure water) were run concurrently. The process was carried out in two separate experiments.

2.8.1 Total phenolic content after digestion

To determine total polyphenol content, the residues obtained from gastro-duodenal digestion were extracted with ethyl acetate, evaporated to dryness in a vacuum rotary evaporator and dissolved in methanol. Phenolics were determined as described above (see section 2.5.2.1), as well as the main components of infusions: NDGA, DHC and DHMC post-digestion recovery was determined by RP-HPLC-DAD quantification in order to evaluate their bioaccessibility (see section 2.6).

2.8.2 Antioxidant activity of herbal infusions after digestion

The residues obtained after simulated GD digestion were resuspended in DMSO and several concentrations were prepared to evaluate ABTS radical cation and hydroxyl radical scavenging activity as described above (see section 2.7.1 and 2.7.3, respectively).

In order to evaluate the reducing power after passing gastro-duodenal digestion, FRAP assay was carried out as described previously (see section 2.7.2).

2.9 Data analysis

Chemical compound determinations were carried out in triplicate and data were reported as mean ± SD. SC_{50} values were calculated from three independent assays performed in triplicate and all results are presented as mean values. One-way ANOVA was employed to determine whether "Jarilla" infusions differed significantly from each other. The significance level was established by using *Tukey's* test ($p \leq 0.05$). Results were analyzed by using Infostat software v.2008.e.

3. Results and discussion

3.1 Nutrient and mineral composition

Results regarding protein and sugar content of plant infusions and their mixes are described in Table 1. In general, per cup of infusion there was a higher content of carbohydrates than of soluble proteins. Zp infusion revealed the highest content in soluble proteins and total sugars, while Ld infusion contained mainly reducing sugars. Total sugar content in *Larrea* sp. infusions was higher than that obtained for hydroalcoholic extracts of those species (Moreno *et al*., 2018).

Among herbal mixtures, HM4 infusion (equal quantities of each plant species) gave the highest concentration of soluble proteins and the remaining mixtures had a similar content ($p \leq 0.05$). Carbohydrate content was not statistically different between the above-mentioned mixtures, whereas reducing sugars were higher in HM1 and HM3 (Table 1).

On account of the relevance of minerals in a daily diet (vitamin synthesis, enzyme activation, bone growth and maintenance of body functions) (Pereira *et al*., 2014) and their influence on the antioxidant potential of food and beverages (Samsonowicz *et al*., 2019), the content of some minerals in "Jarilla" infusions was evaluated. Regarding single plant infusions, Ld stood out showing the highest Mg and Ca concentration, while Lc infusion had more Na and K quantities than the other infusions (Table 2). The highest amounts of Na, K, Ca and Mg in comparison with other minerals were in agreement with previous studies, which indicated that these macro-elements were easily soluble and are also the most abundant in many medicinal plants and their infusions (Basgel and Erdemoglu, 2006; Gallaher *et al*., 2006; Łozak *et al*., 2002).

Zp infusion exhibited the highest Zn concentration (Table 2). Among single plant infusions, Ld showed the highest Cu concentration and it was the only one in which Fe

content could be quantified. All herbal mixture infusions are an additional supplementary source of K, Mg, Ca and Cu. Fe content could only be quantified in HM1 infusion. All these values were closely comparable to those reported in tea and other herbal samples previously published by many authors (Basgel and Erdemoglu, 2006; Łozak *et al*., 2002). The Zn and Cu concentration in these herbal infusions are higher than the values reported previously for boldo (*Pneumus boldus*), green tea (*Camellia sinensis*), lemongrass (*Cymbopogon citratus*), mate (*Ilex paraguariensis*) and strawberry fruits (*Fragaria spp.*) infusions (Milani *et al.*, 2015). Even though "Jarilla" infusions do not contain enough quantities of these elements to cover recommended daily dietary intake, they can be considered as an additional supplementary source of minerals in the daily human diet.

3.2 Phytochemical characterization of herbal infusions

Ld infusion had the highest total phenolic content (TPC), mainly flavonoid phenolics (Table 3). Zp infusion exhibited the highest quantities of flavones and flavonols, proanthocyanidins and it was the only extract with detectable quantities of hydrolysable tannins (Table 3). In regard to herbal mixtures, all of them showed similar total phenolic concentration. Nonflavonoid phenolic content was higher in HM3, and it was similar in the remaining mixtures. Flavonoid phenolic content was higher in HM1. Condensed and hydrolysable tannins and flavonoid (flavone and flavonols) content of the mixtures presented herein showed quantitative variation, HM1 being the sample with the highest values obtained. The phenolic concentration reported here as regards "Jarilla" infusions is higher than the one previously reported for mint (*Mentha piperita*), eucalyptus (*Eucalyptus globules*), chamomile (*Matricaria recutita*) infusions, among others (Atoui *et al*., 2005; Khokhar and Magnusdottir, 2002), and is similar to green and

black tea and other medicinal herbal infusions in other countries (Fu *et al*., 2011; Stodt and Engelhardt, 2013).

HPLC fingerprints of the studied herbal infusions from "Jarilla" were obtained and peaks with relatively high intensity and good resolutions were assigned as "characteristic common peaks" to represent the characteristics of each sample. Single-plant chromatograms are shown in Fig. 1. The presence of DHC and DHMC in Zp infusions and NDGA in Ld and Lc infusions could be determined by comparing their retention time and UV spectrum with those of chemical standards. Plant mixture chromatograms were very similar among them, and those chemical markers were also identified. Furthermore, they were quantified for the first time in "Jarilla" infusions from Northwestern Argentina, in order to be considered chemical markers for future quality control of these medicinal infusions. The content of DHC and DHMC (Fig. 2) did not differ significantly in Zp infusion (10.9±2.0 µg/mL and 10.1±1.2 µg/mL, respectively), whereas the content of the NDGA in Lc infusions differed from Ld, being superior in the former (53.2±3.8 µg/mL for Lc and 42.1±3.1 µg/mL for Ld). The NDGA content in *L. divaricata* infusion represents 1.5±0.1 % (w/w) of plant material. This value significantly exceeds the previously reported content by Turner *et al*. (2011) for an aqueous extract for the same species, collected in the province of Cordoba (Argentina).

In plant combinations, NDGA concentration (28.6 µg/mL on average) was higher than DHC (3.7 µg/mL, on average) and DHMC (5.2 µg/mL, on average) content. These are three of the major phytochemicals in "Jarilla" infusions and these compounds were further demonstrated to be responsible for different biological activities attributed to these medicinal plants (Anesini *et al*., 2001; Floriano-Sánchez *et al*., 2006; Morán Vieyra et *al*., 2009; Zampini *et al*., 2008; Zampini *et al*., 2012).

3.3 Antioxidant properties

Several methods are known to measure antioxidant capacity and are needed in order to evaluate different actions of substances with antioxidant properties. In Table 4, Zp and Lc infusions can be seen to be the most active single plant infusions in $ABTS^{\bullet+}$ and hydroxyl radical scavenging capacity assay, whereas Ld had the highest FRAP value (the highest total reducing power). Among herbal mixtures, HM1 and HM2 showed higher free radical scavenger activity (ABTS and $HO^{\bullet}$) than the other mixtures, while HM4 showed higher reducing power (Table 4). The antioxidant potential of "Jarilla" infusions was similar to that exhibited by black tea (*C. sinensis*) and even higher than that of Mint (*M. piperita*), Melissa (*Melissa officinalis*), Fennel (*Foeniculum vulgare*) and other herbal mixture infusions (Barroso *et al*., 2016).

Morán Vieyra *et al.* (2009) and Ávila *et al*. (2001) had already demonstrated that some phytochemicals present in *Z. punctata*, including flavones, flavanones and chalcones have free radical scavenging capacities and singlet oxygen quenching. Moreover, NDGA lignan exhibited free radical scavenging and catalase activity, as reported by Turner *et al*. (2011). These are important antioxidant mechanisms that could account for the potential of these medicinal infusions.

In the Fenton reaction, hydroxyl radicals were generated after reduction of Fe^{3+} by ascorbic acid (Chobot, 2010). The infusions could inhibit 2-deoxy-D-ribose degradation both with and without the addition of EDTA (Fig. 3). Moreover, none of them showed pro-oxidative effects in the concentration range that antioxidant properties do (data not shown).

In order to confirm what kind of interaction exists between herbal infusions in all the antioxidant mechanisms assayed, the median-effect principle was used (Chou, 2010). As a conclusion after CompuSyn software analysis (Table 4), plant combinations analyzed showed CI value between 0.6 and 1.54. In FRAP assay, there is mostly an additive-type interaction, and in free radical scavenging (ABTS and hydroxyl radical scavenging) there is a predominantly synergistic-type interaction among herbal combinations, HM2 and HM3 mixtures being the most remarkable.

3.4 Influence of a gastroduodenal digestion process on antioxidant capacity

Human gastrointestinal digestion is a complex process, influenced by several factors. Among the different attempts reported to simulate the *in vitro* process, static models are the most widespread. Although they do not reproduce the dynamic process involving the *in vivo* digestion, they present several advantages such as low cost, simplicity and reproducibility, making it convenient as a preliminary analysis. Herbal mixture infusions were subjected to a gastro-duodenal (GD) digestion in an *in vitro* process. The total phenolic compound recovery after digestion was 21% for HM1, 49.5% for HM2, 29% for HM3 and 31% for HM4, with respect to the beginning of the GD digestion. This reduction in TPC was in agreement with other beverages subjected to GD digestion in an *in vitro* simulation (Gullon *et al*., 2015; Pinto *et al*., 2017). Physicochemical changes in gastrointestinal tract (temperature, pH, enzymes) are known to affect the TPC bioavailability (Gullon *et al.*, 2015).

The passage through the intestinal environment conditions leads to phenolic compound hydrolysis and degradation, and/or their conversion into other unknown or undetected compounds under dramatic pH changes (Tenore *et al*., 2015). The digestive

stability of chemical markers in "Jarilla" infusions was investigated. The quantification of NDGA, DHC and DHMC in each herbal infusion and plant mixture infusions subjected to GD digestion is shown in Fig. 2. Lower recovery was obtained for NDGA, HM2 being the sample with the highest quantity after digestion. The chalcone content (DHC and DHMC) was not affected in Zp infusion, and it remained more stable in herbal mixtures, the HM2 group being the one with the highest percentage of recovery.

Since herbal infusions could exert the biological activity in the gastrointestinal compartment, their antioxidant potential was measured after digestion. Although some samples improved their activity in the ABTS assay after the digestion process (Ld, HM1, HM2, HM3 and HM4), the infusions partially lost their reducing capacities and hydroxyl radical scavenging activity, comparing with the values obtained before the digestion process (Table 4). These results would indicate that the antioxidant compounds in the mixture could be turned into other compounds with different biological activity. The herbal mixture HM2 had the highest antioxidant effect after digestion. These results are in accordance with the bioactive compound recovery after GD digestion. Total phenolic compounds and marker compounds (NDGA, DCH and DHMC) recovery after gastro-duodenal digestion were higher in the most active mixture (HM2) than in the other mixtures (Fig. 2, Table 3).

Moreover, after their passing through the gastroduodenal simulated conditions, lower CI values were obtained for HM2 on the antioxidant mechanism mentioned above, improving the synergism after the digestion process (Table 4).

3.5 Conclusion

This work constitutes an original contribution to the knowledge of the sugar, protein and mineral composition of the three species of "Jarilla" and four mixes. Herbal infusions can be considered as good supplementary mineral sources in human nutrition. Three chemical markers could be identified in the infusions with each plant species and in herbal mixtures (nordihydroguaiaretic acid, 2′,4′-dihydroxychalcone, 2′,4′-dihydroxy-3-metoxychalcone). It could be anticipated that "Jarilla" infusions have antioxidant activity achieved by free radical scavenging and reducing capacities. The infusions containing plant mixtures mostly exhibited a positive effect by increasing the antioxidant potential (synergy and additive action), highlighting HM2 which contained a higher proportion of *L. cuneifolia.*

After mixtures cross gastroduodenal tract conditions, between 50.5% and 79% of total phenolic were lost. However, the infusions were still able to scavenge free radicals. The plant combination HM2 was the most active and this could be partially attributed to the high NDGA, DHC and DMHC content present after the simulated GD digestion.

Conflict of Interest

The authors have declared that there is no conflict of interest.

Acknowledgements

This work was supported by the following Argentinian institutions: Secretaría de Ciencia, Arte y Tecnología (SCAyT) de la Universidad Nacional de Tucumán (UNT), Agencia Nacional de Promoción Científica y Técnica (ANPCyT), and Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET).

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Figure legends

Figure 1. HPLC fingerprints of *Z. punctata* (A), *L. cuneifolia* (B) and *L. divaricata* (C) infusions. On the right, the chemical structures of the main chemical markers from "Jarilla" infusions are depicted. 1: 2′,4′-dihydroxychalcone (DHC); 2: 2′,4′-dihydroxy-3′-methoxychalcone (DHMC)and 3: nordihydroguaiaretic acid (NDGA).

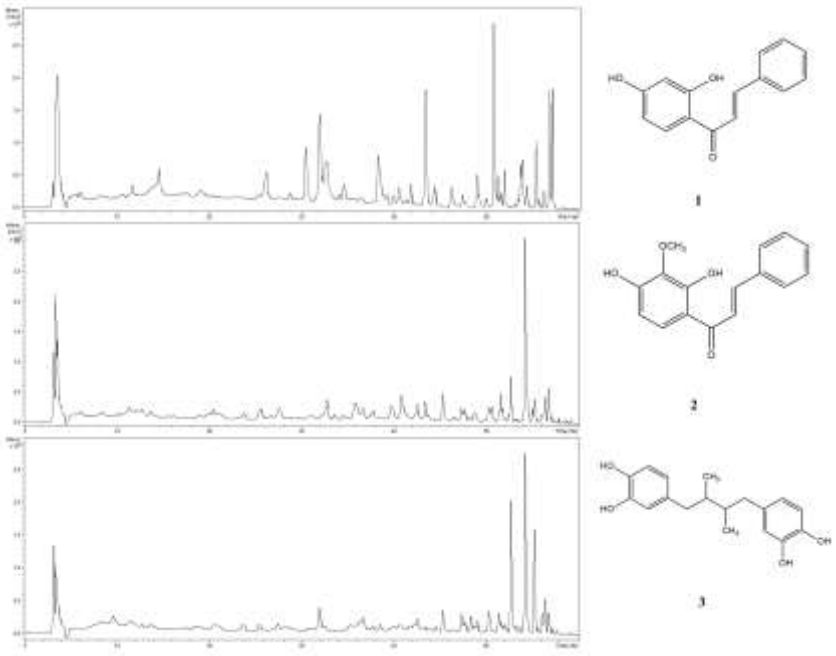

Figure 2. NDGA, DHC and DHMC concentration before and after the *in vitro* simulated digestion process. Values show mean ± SD for the experiment performed in duplicate.

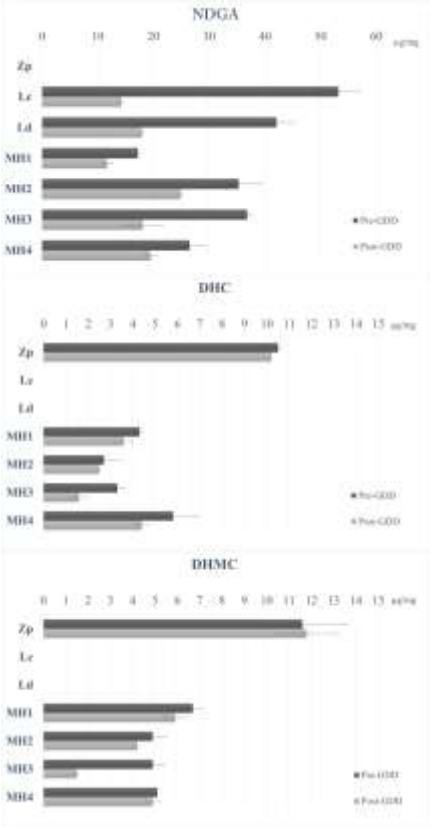

Figure 3. Scavenging activity of "Jarilla" tea on hydroxyl radical without the addition of EDTA, quantified in percentage of malondialdehyde (% MDA). Values show mean ± SD for the experiment performed in triplicate.

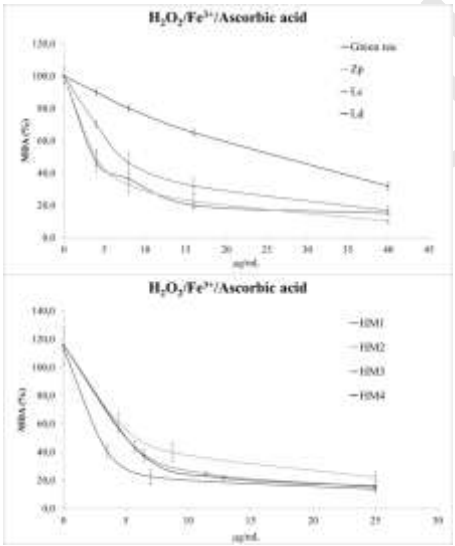

Table 1. Macronutrient concentration of "Jarilla" herbal infusions.

Extract	**Total Soluble Proteins** mg BSAE/cup	**Reducing sugars** mg GE/cup	**Total sugars** mg GE/cup
Zp	38.0±0.6^{a}	101.8±0.6^{bc}	144.6±8.5^{a}
Lc	34.7±0.1^{b}	101.4±5.3^{bc}	131.5±6.6^{ab}
Ld	35.5±1.4^{ab}	120.4±1.0^{a}	118.7±10.1^{bc}
HM1	37.5±1.4^{ab}	109.7±5.8^{b}	128.9±3.5^{abc}
HM2	37.2±0.3^{ab}	92.7±1.0^{c}	113.1±2.6^{c}
HM3	37.7±0.3^{ab}	111.0±4.9^{ab}	124.1±2,6^{bc}
HM4	38.7±2.2^{a}	99.1±1.2^{c}	116.5±3.4^{bc}

Means ±SD followed by the same letter in each column are not significantly different (Tukey's HSD, $p \leq 0.05$).

Table 2. Mineral composition of Jarilla infusions and their mixtures.

	Mineral concentration (mg/cup)							
Elements	**Zp**	**Lc**	**Ld**	**HM1**	**HM2**	**HM3**	**HM4**	**ADDI [1] mg/day (range)**
Na	0.620±0.017^{bc}	0.832±0.027^{a}	0.428±0.042^{bc}	0.295±0.003^{bc}	0.471±0.009^{bc}	0.248±0.002^{c}	0.610±0.015^{ab}	2200 (1100-3300)[2]
K	16.857±0.450^{d}	24.647±0.747^{a}	22.565±1.848^{a}	19.344±0.236^{bc}	23.286±0.499^{a}	17.125±0.010^{cd}	19.567±0.550^{b}	3800 (1900-5600)[2]
Mg	1.122±0.025^{d}	2.555±0.070^{b}	3.514±0.246^{a}	1.909±0.019^{c}	2.448±0.019^{b}	1.958±0.026^{c}	2.027±0.042^{c}	350 (300-400)[3]
Ca	2.008±0.039^{e}	6.444±0.152^{d}	26.429±1.834^{a}	8.596±0.258^{c}	10.440±0.247^{bc}	11.989±0.046^{b}	10.123±0.322^{bc}	1000 (800-1200)[3]
Fe	<LQM	<LQM	0.017±0.003^{b}	0.025±0.001^{a}	<LQM	<LQM	<LQM	15(8-18)[4]
Cu	0.005±0.000^{e}	0.006±0.000^{d}	0.013±0.002^{a}	0.010±0.000^{b}	0.008±0.000^{c}	0.011±0.000^{b}	0.006±0.000^{d}	0.9(0,7-0,9)[4]
Zn	0.034±0.001^{a}	0.016±0.001^{c}	0.018±0.001^{b}	0.017±0.001^{bc}	0.016±0.001^{c}	0.0120±0.000^{d}	0.016±0.000^{bc}	11(8-15)[4]

<LQM= below limit of quantification of the method.

[1] Average Daily Dietary Intake. Means ±SD followed by the same letter are not significantly different (Tukey's HSD, $p \leq 0.05$).

[2]Institute of Medicine, Food and Nutrition Board. 2004. Dietary Reference Intakes for Water, Potassium, Sodium, Chloride and Sulfate. National Academy Press, Washington, DC.

[3]Institute of Medicine, Food and Nutrition Board, 1997. Dietary Reference Intakes for Calcium, Phosphorus, Magnesium, Vitamin D and Fluoride. National Academy Press, Washington, DC.

[4]Institute of Medicine, Food and Nutrition Board, 2000. Dietary Reference Intakes for Vitamin A, Vitamin K, Arsenic, Boron, Chromium, Copper, Iodine, Iron, Manganese, Molybdenum, Nickel, Silicon, Vanadium and Zinc. National Academy Press, Washington, DC.

Table 3. Dry weight yield by freeze-drying and the main secondary metabolites of the herbal infusions studied.

	Total phenolics mg GAE/cup	**Nonflavonoid phenolics** mg GAE/cup	**Flavonoid phenolics** mg GAE/cup	**Flavone and flavonols** mg QE/cup	**Hydrolizable tannins** mg GAE/cup	**Proanthocyanidin** mg PB2E/cup
Zp	219.6±15.1^{bc}	26.3±3.8^{d}	171.6±6.9^{a}	19.7±0.3^{a}	3.1±0.2^{a}	88.7±2.4^{a}
Lc	205.0±3.4^{bc}	53.6±1.6^{bc}	107.2±2.8^{c}	9.7±0.9^{f}	<LD	30.1±1.0^{e}
Ld	255.8±16.3^{a}	76.1±2.7^{a}	117.0±5.0^{c}	15.0±0.1^{c}	<LD	17.4±0.3^{f}
HM1	235.8±6.1^{ab}	48±4.5^{c}	148.2±8.3^{b}	17.1±0.3^{b}	1.7±0.1^{b}	58.2±1.6^{b}
HM2	193.7±12.8^{c}	48±3.2^{c}	106.1±5.9^{c}	11.1±0.3^{e}	0.7±0.1^{c}	36.1±1.8^{d}
HM3	223.9±12.2^{bc}	59.7±4^{b}	114.9±7.3^{c}	15.5±0.4^{c}	0.7±0.1^{c}	40.2±1.1^{c}
HM4	208.3±6.3^{bc}	50.0±1.9^{c}	117.0±3.6^{c}	13.4±0.2^{d}	<LD	41.8±0.7^{c}

Means ±SD followed by the same letter in each column are not significantly different (Tukey's HSD, $p \leq 0.05$). <LD= below limit of detection.

Table 4. Antioxidant activity of Jarilla infusions and their mixtures through various mechanisms of antioxidant action.

		ABTS		FRAP		Hydroxyl Radical	
Infusions		**SC_{50}**	**CI**	**µmol AAE/ml**	**CI**	**SC_{50}**	**CI**
Zp	Pre-GDD	28.3±3.2	-	4.46±0.10	-	7.8±0.1	-
	Post-GDD	44.7±0.5	-	0.97±0.04	-	175.9±15.3	-
Lc	Pre-GDD	37.6±5.3	-	4.41±0.13	-	21.9±0.6	-
	post-GDD	41.3±1.8	-	0.81±0.04	-	299.5±5.7	-
Ld	Pre-GDD	76.3±2.1	-	4.92±0.06	-	47.7±2.7	-
	post-GDD	30.9±1.7	-	1.68±0.02	-	78.9±2.8	-
HM1	Pre-GDD	61.5±7.3	1.54±0.12	3.96±0.01	0.97±0.03	6.9±0.2	0.76±0.04
	post-GDD	41.2±0.3	1.10±0.04	1.29±0.09	1.74±0.14	150.3±4.7	5.73±0.50
HM2	Pre-GDD	45.1±1.0	0.85±0.02	3.07±0.05	1.37±0.08	8,3±0.6	0.60±0.08
	post-GDD	23.2±0.3	0.64±0.05	2.12±0.15	1.14±0.23	46.6±2.2	0.32±0.03
HM3	Pre-GDD	48.2±1.4	0.84±0.07	3.31±0.06	1.22±0.07	11.2±1.6	0.76±0.04
	post-GDD	41.5±2.7	1.28±0.11	0.95±0.07	1.65±0.12	124.2±19.8	4.35±0.04
HM4	Pre-GDD	53.7±4.8	1.14±0.23	4.53±0.04	0.96±0.03	17.5±0.2	1.13±0.06
	post-GDD	40.0±3.6	1.07±0.15	1.67±0.12	1.81±0.54	51.7±9.7	0.75±0.20

SC_{50}= Scavenging capacity 50; it denotes the µg /mL required to scavenge the 50% of free radicals

CI= Combination Index.
GDD= Gastroduodenal digestion