Journal Pre-proof

Antifungal, anti-inflammatory and antioxidant activity of bi-herbal phytotherapic using medicinal plants from Argentina highlands

María Alejandra Moreno, Iris Catiana Zampini, María Inés Isla

PII: S0378-8741(19)34245-X

DOI: https://doi.org/10.1016/j.jep.2020.112642

Reference: JEP 112642

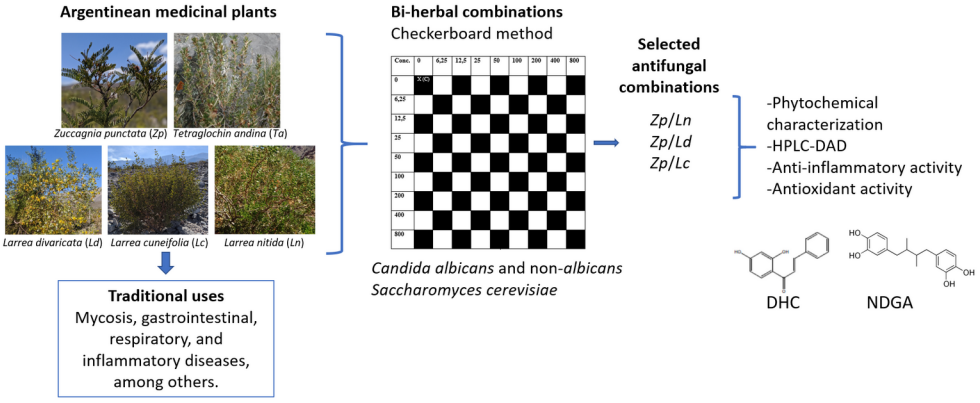
To appear in: *Journal of Ethnopharmacology*

Received Date: 23 October 2019

Revised Date: 31 January 2020

Accepted Date: 31 January 2020

Please cite this article as: Moreno, Marí.Alejandra., Zampini, I.C., Isla, Marí.Iné., Antifungal, anti-inflammatory and antioxidant activity of bi-herbal phytotherapic using medicinal plants from Argentina highlands, *Journal of Ethnopharmacology* (2020), doi: https://doi.org/10.1016/j.jep.2020.112642.



Antifungal, anti-inflammatory and antioxidant activity of bi-herbal phytotherapic using medicinal plants from Argentina highlands

María Alejandra Moreno[1], Iris Catiana Zampini[1,2], María Inés Isla[1,2#]

[1]*Laboratorio de Investigación de Productos Naturales (LIPRON), Instituto de Bioprospección y Fisiología Vegetal (INBIOFIV-CONICET-UNT).*

[2]*Facultad de Ciencias Naturales e Instituto Miguel Lillo, Universidad Nacional de Tucumán, San Lorenzo 1469. San Miguel de Tucumán, Tucumán, Argentina.*

Keywords: antifungal, anti-inflammatory, antioxidant, Argentinean medicinal plant extracts, synergism.

Abbreviation list: QE, quercetin equivalents; GAE, gallic acid equivalents; ABTS, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid); BHT, butylated hydroxytoluene; RBC, red blood cells; AAPH, 2,2'-azobis(2-methyl-propionamidine) dihydrochloride; NDGA, nordihydroguaiaretic acid; DHC, 2′,4′-dihydroxychalcone; TPC, total phenolic compound; NF-P, non-flavonoid phenols; FP, flavonoid phenolic; DW, dry weight; LOX, lipoxygenase.

[#]Corresponding author: Dra. María Inés Isla

INBIOFIV- CONICET

Universidad Nacional de Tucumán

San Lorenzo 1469

4000 - San Miguel de Tucumán. ARGENTINA

E-mail: misla@tucbbs.com.ar; misla@csnat.unt.edu.ar

Telephone: (+54)3814203062

Abstract

Ethnopharmacological relevance: The Argentinean medicinal plants *Larrea divaricata* Cav., *Larrea cuneifolia* Cav., *Larrea nitida* Cav., *Zuccagnia punctata* Cav. and *Tetraglochin andina* Ciald are used alone and in combined in traditional medicine by inhabitants from Argentinean northwestern to solve mycosis, vaginal infections, gastrointestinal, respiratory, and inflammatory process.

Aim of the study: To assess the effect of interactions between dry extracts of these five species of medicinal plants against yeast strains isolated from vaginal infections, select the most active mixtures and evaluate the anti-inflammatory and antioxidant activities.

Material and methods: Synergy between the plants extracts was studied using a broth microdilution assay by the checkerboard method against *Saccharomyces cerevisiae, Candida albicans,* and non *albicans* strains. The inhibitory effect on lipoxygenase enzyme and the antioxidant capacity in cell free and cell systems were studied. The chemical profile was evaluated by qualitative and quantitative screening, whereas chemical markers were quantified by HPLC-DAD.

Results: A synergistic antifungal effect was observed in some of the binary combinations. *Z. punctata/ L. divaricata, Z. punctata*/ *L. cuneifolia,* and *Z. punctata/ L. nitida* were the most active mixtures. Nordihydroguaiaretic acid and 2′,4′-dihydroxychalcone, two antifungal compounds present in these extracts, were identified and quantified by HPLC-DAD. Both the single extracts and the bi-herbal mixtures showed antioxidant activity (in cellular and in cell-free systems) and were active on pro-inflammatory enzymes (LOX).

Conclusions: Our results indicated that the most active combinations of these species extracts could be useful in the treatment of vaginal infectious diseases caused by *Saccharomyces cerevisiae* and *Candida* spp. strains and in the associated oxidative and

inflammatory processes. In addition, the results highlighted the potential phyto-therapeutic of total phytochemical compounds present in these medicinal plants

1. Introduction

Larrea divaricata Cav. (common names: “jarilla”, “jarilla hembra”, “chamanilla”, “jarilla del cerro”, “yarilla”), *Larrea cunefolia* Cav. (common names: “jarilla”, “jarilla crespa”, “jarilla macho”, “jarilla del campo”, “jarilla norte-sur”), *Larrea nitida* Cav. (common names: “jarilla”, “jarilla de la montaña”, “crespa”, “pispa”, “pispita”, “jarilla fina”), *Zuccagnia punctata* Cav. (common names: “jarilla pispito”, “pus pus”, “lata”, “jarilla macho”) and *Tetraglochin andina* Ciald (common names: “horizonte”, “canguia”, “rancha-rancha”, “kailla”, “añahuaya”) are medicinal plants that grown in arid and semiarid regions of Argentina, and are used to treat mycosis, gastrointestinal, respiratory, and inflammatory diseases according to ethnobotanical data in several local communities in Argentina (Carabajal et al., 2020; Moreno et al., 2018a). Jarillas aerial parts are collected, dried and commercialized in northern Argentina, mainly in the Calchaquies valleys (Fig. 1). A wide range of pharmacological activities for these species was previously described, indicating their potential use as an alternative or complementary medicine. In previous works, we standardized hydroalcoholic extracts of these five species by chemical composition and biological properties. Each of them indicated to be effective against *Candida albicans* and non-*albicans* strains isolated from vaginal infections as well as antibiotic resistant bacteria (in vivo and in vitro), showed antioxidant and anti-inflammatory activities (Moreno et al., 2015a; 2018a, b, c; Zampini et al., 2005, 2007, 2012). The pharmacognostic characterization of the vegetative organs of these species was also reported (Mercado et al., 2018; Moreno et al., 2015b; 2018a, b). In a previous work, chitosan microcapsules containing dry

extracts of these medicinal plants for infections vaginal were developed and compared with conventionally used tablets containing the same extracts (Moreno et al., 2018c). The purpose of this study was to determine the antifungal against strains of *Candida* spp. and *Saccharomyces cerevisiae*, anti-inflammatory and antioxidant activities of mixtures of these herbs. Herbal medicines are characterized using mixtures of two herbs (bi-herbs) or several herbs in a single formula, in which the pharmacological activities of one single herb is either potentiated and/or its adverse effects reduced by addition of other herbs (Wagner, 2011). This thus led to a more favorable response for some herbal combinations than for the constituent's herb used alone, which suggests that therapeutic effects of these herbal products may arise from synergistic actions. In these studies, synergism is assumed to occur if the effective concentration of components in combination is significantly reduced or the effects of components in combination are significantly increased with respect to that of each individual component (Torres et al., 2017). Combination therapy can be used to expand the antimicrobial spectrum, to prevent the emergence of resistant strains, to minimize toxicity and to obtain synergistic antimicrobial activity (Aiyegoro and Okoh, 2009). An increasing number of studies methods of synergistic method have been used, such as isobolographic analysis, ANOVA and interaction index (Wagner, 2011, Ma et al., 2009). In the present study, was determined the interaction index of binary combinations between these five medicinal plants by the checkerboard method.

2. Material and methods

2.1. Plant material

The plant parts used were leaves and stems (aerial parts), according to the traditional use. *Zuccagnia punctata* Cav. (*Zp*), *Larrea cuneifolia* Cav. (*Lc*) and *L. divaricata* Cav.

(*Ld*) were collected in April 2015 at Amaicha del Valle, Tucumán, Argentina at 2000 m.a.s.l. (65°43'36.119"W, 26°40'59.88"S,). *Larrea nitida* (*Ln*) was collected in April 2015 at Vinchina, La Rioja, Argentina at 3485 m.a.s.l. (68°41'33.74"W, 28°34'03.74"S). *Tetraglochin andina* Ciald, according to Acosta et al. (2016) and Cialdella and Pometti (2017), formerly *Tetraglochin cristatum* (Britton) Rothm (www.theplantlist.org) was collected in February 2015 in Huaca Huasi, Tucumán, Argentina (4300 m.a.s.l., 65°44.23'W, 26°39.35'S). The samples were identified by Dra. Soledad Cuello (botanic specialist), Laboratorio de Investigación en Productos Naturales (LIPRON-INBIOFIV-CONICET) and the taxonomic identification was confirmed by the Plant List (2013). Voucher specimens (*L. cuneifolia*: LIL 614829; *L. divaricata*: LIL 614299; *L. nitida*: LIL 615845; *Z. punctata* LIL 605935; *T. andina* LIL 610669) were deposited at the Herbarium of Fundación Miguel Lillo (Tucumán, Argentina). The samples (Figure 2) were dried in a forced air oven at 40°C and then they were ground.

2.2. Dry extract preparation

The powdered dried plant material (10 g) was macerated in 200 mL of 60° ethanol for 1 h with ultrasonic application five times for 10 min. Combined extracts were filtered, taken to dryness under reduced pressure and then lyophilized to afford the extracts. The extraction yield was determined. Dry extracts were kept at -20°C until their used. Then, the dry extracts were dissolved in ethanol 60° to carry out the phytochemicals and biological assays. They were mixed to obtain binary combinations as described in Table 1.

2.3. Phytochemical characterization

2.3.1. Qualitative phytochemical screening

Qualitative screening was carried out to determine the occurrence of flavonoids (Mojab et al., 2003), cardiac glycosides (Teke et al., 2010), coumarins (Teke et al., 2010),

tannins (Adegboye et al., 2008), free anthraquinones (Onwukaeme et al., 2007), saponins (Ayoola et al., 2008), terpenoids, steroids and alkaloids (Adegboye et al., 2008).

2.3.2. Quantitative phytochemical screening

Total phenolic compound (TPC) content was determined by Folin-Ciocalteau method. (Costamagna et al., 2013). Non-flavonoid phenols (NF-P) were determined in the supernatant by the Folin-Ciocalteau method after precipitation of the flavonoids with acidic formaldehyde (Isla et al., 2014). Flavonoid phenolic (FP) content was calculated by the difference between TPC and NF-P. Flavone and flavanone content was also determined (Costamagna et al., 2013).

2.4. Identification of phenolics by HPLC-DAD

The extracts were analysed by HPLC (Waters Corporation, Milford, MA, USA) coupled to a diode array detector (Waters 2998 photodiode array detector) in an analytical C18 column (XBridge) (4.6 x 150 mm, 5 μm; Waters Corporation, Milford, MA), using a manual injection valve with a 20 μL loop (Rheodyne Inc., Cotati, CA). The solvent system for the separation of components from extracts was composed of solvent A (9% acetic acid in water) and solvent B (methanol) (conditions: 25–45% B from 0 to 10 min, kept at 45% B from 10 to 20 min, increasing to 70% to 40 min, 75% to 50 min and 100% B to 55 min).

The flow rate was set at 0.5 mL/min. A solution of 2 mg/mL of each extract was used. Data collection was carried out with EmpowerTM 2 software. The identification of compounds present in the mixtures was carried out by comparing the retention times and spectral data (220–600 nm) of each peak with those of standards from Sigma-Aldrich (MO, USA) and Indofine Chemical Company, Inc.

2.5. *Biological activity studies*

2.5.1. Antimicrobial assays

2.5.1.1. Yeast strain and inoculum preparation

The yeast strains were provided by Instituto Dr. Carlos G. Malbrán, Buenos Aires, Argentina. The strains tested were *Candida albicans* (134333), *C. glabrata* (031646), *C. tropicalis* (1841), *Saccharomyces cerevisiae* (134528), *C. parapsilopsis* (DMic 134410) and *C. krusei* (DMic 134409). All the microorganisms were maintained in brain-heart infusion containing 30% (v/v) glycerol at -20°C. Before testing, the suspensions were transferred to Yeast Medium Agar (malt extract 0.3%, yeast extract 0.3%, peptone 0.5%, glucose 1%, agar 2%) and aerobically grown at 37°C during 24 h. Individual colonies were isolated and suspended in 2 mL of 0.9% NaCl solution. The inocula were prepared by adjusting the turbidity of the suspension to match the 0.5 McFarland scale to achieve the adequate inoculum in each case. The cell number was estimated using a serial dilution technique according to the recommendations of the M27-A3-S4 reference document of CLSI, 2012 (Clinical and Laboratory Standards Institute, 2012) for each assay.

2.5.1.2. Checkerboard method

The microdilution checkerboard method is the technique used most frequently to assayed antimicrobial combinations *in vitro*. MIC values of binary mixtures of hydroalcoholic extracts of five plant species against *Candida* were determined (CLSI, 2012). The concentration used in the combination for each extract ranged from 12.5 to 800 µg GAE/mL (Table 1), according to the MIC values previously reported for each extract (Moreno 2018a, b, c). The inoculum (200 µL) containing 0.5-2.5×10^3 CFU/mL was added to each well. Some wells were used in each plate as control: sterility (no inoculum added), microorganism viability (no extract added) and solvent effect (ethanol

60°). Plates were aerobically incubated at 37°C. After incubation for 48 h, fungal growth was evidenced by the presence of turbidity and a pellet on the well bottom. The lowest concentration of extract without macroscopically visible growth was determined as MIC values. The results of the combined effects of the extracts were categorized as synergism, addition, indifference or antagonism. In order to assess the activities of combinations, fractional inhibitory concentration (FIC) indices were calculated as FIC_A+ FIC_B, where FIC_A and FIC_B are the minimum concentrations that inhibited the fungi growth for samples A and B respectively:

FIC_A= MIC_A combination/MIC_A alone; FIC_B= MIC_B combination/MIC_B alone

The mean FIC index was calculated as FIC index= FIC_A + FIC_B, and the results were interpreted as synergistic (≤0.5), additive (>0.5 and ≤1.0), indifferent (>1.0 and <4.0) or antagonist effect (≥4.0).

The mixtures that showed synergistic or additive effect against most of the strains were selected for subsequent trials.

2.5.2. In vitro anti-inflammatory activity in cell-free systems

LOX activity was determined spectrophotometrically according to Torres Carro et al. (2017). The assays were performed with 50 µg/mL of each extract or binary herbal mixture. The effect was determined by calculating the percentage of inhibition of the hydroperoxide production obtained from the ΔOD (optical density) values at 234 nm after 3 min of incubation. Naproxen (50 µg/mL) was used as reference compound.

2.5.3. Antioxidant activity

For this study, different concentrations of each extract and of selected binary herbal mixtures were assayed by antioxidant activity in cell free and cell systems.

2.5.3.1. ABTS free radical scavenging activity

The antioxidant capacity was estimated using the technique described by Re et al. (1999). ABTS radical cation ($ABTS^{\bullet+}$) was produced by reacting ABTS solution (7 mM) with ammonium persulfate (2.45 mM) and the mixture was maintained in dark at room temperature for 12–16 h. Different concentrations of each ethanolic extract or bi-herbal mixture (1-25 µg/mL) were assayed. $ABTS^{\bullet+}$ solution (absorbance of 0.7±0.02 at 734 nm) was added to different concentrations of each sample and mixed thoroughly. Quercetin (0.5-5 µg/mL) and BHT (0.5-10 µg/mL) were employed as positive controls. The reactive mixture was allowed to stand at room temperature and absorbance was recorded at 734 nm, 1 min and 6 min after initial mixing. Dose–response curves were made, and results were expressed in terms of concentration of the extracts or bi-herbal mixtures, in micrograms per milliliter (µg/mL), that was necessary to scavenge 50% of $ABTS^{\cdot+}$ (SC_{50}) was determined.

2.5.3.2. Protection against oxidative hemolysis

The protection of oxidative hemolysis of RBC by the dry extracts or binary herbal mixtures (concentration range between 0.02 and 2.0 µg/mL) was determined according to Mendes et al. (2011), using AAPH solution. The extent of hemolysis was quantified spectrophotometrically at 545 nm. Percentage hemolysis was calculated and the IC_{50} values were determined as the concentration needed to protect the RBC from oxidative hemolysis in 50%. For the hemolysis control, ethanol 60° was used as a solvent control instead of the extracts. BHT and quercetin were used as reference compounds.

2.5.3.3. H_2O_2 scavenging assay

The capacity of the dry extracts or binary herbal mixtures to hydrogen peroxide scavenge was measure according to Fernando and Soysa (2015) with some modifications. The reaction mixture containing extracts or bi-herbal mixtures (50–250

µg/mL) and H_2O_2 was pre-incubated during 3 min at 37°C. Then, a solution of phenol (12 mM) and 4-aminoantipyrine was added to the reaction mixture. The amount of hydrogen peroxide was spectrophotometrically determined catalyzing its conversion by a horseradish peroxidase (HRP) in a colored quinone that was read at 504 nm. The SC_{50} values were determined as the concentration (µg/mL) necessary to scavenge 50% of H_2O_2. Quercetin and ascorbic acid were used as controls.

2.5.3.4. Hydroxyl radical scavenging assay

The experiment was performed based on the deoxyribose degradation assay developed by Chobot (2010). The reaction mixture contained extracts or bi-herbal mixtures (0.1–5 µg/mL) in KH_2PO_4/KOH buffer (pH 7.4), 50 mL of 10.4 mM 2-deoxy-D-ribose, 50 mL of 50 mM $FeCl_3$ and 50 mL of 52 mM EDTA. To start the Fenton reaction, 50 mL of 10 mM H_2O_2 and 50 mL of 1.0 mM ascorbic acid were added. The reaction mixture was incubated 1 h at 37°C. Then, 500 mL of 2-thiobarbituric acid (1%, w/v) dissolved in trichloroacetic acid (3%, w/v) was added. After 20 min at 100°C, the absorbance was read at 532 nm. The hydroxyl radical scavenging activity was expressed as SC_{50} values (µg/mL necessary to inhibit by 50% the degradation of 2-deoxy-D-ribose by the hydroxyl radicals).

2.6. Mutagenic activity. The Ames test

The assays were performed according to Maron and Ames (1983), with *Salmonella typhimurium* TA98 and TA100 strains using different concentrations for individual extracts and bi-herbal mixtures (until 500 µg per plate). Negative and positive controls were used simultaneously in each experiment. The positive control was 4-nitro-o-fenilendiamine (10 µl/plate, 1 mg/mL solution) for TA98 and TA100, and the negative control was DMSO (100 µl/plate). The revertant colonies of each plate were counted manually after 48 h of incubation at 37°C and the mutagenicity relation (His^+ revertant

per plate/ His^+ spontaneous revertant) was calculated. An extract or bi-herbal mixture was considered mutagenic if the number of revertants per plate was more than twice the number of colonies produced on the solvent control plates (spontaneous revertant frequency) or the mutagenicity relation ≥2.

2.7. Statistical analysis

All assays were conducted at least three times with three different sample preparations. Each experimental value is expressed as the mean±standard deviation (S.D.). The scientific statistic software InfoStat (Student Version, 2011) was used to evaluate the significance of differences between groups. Comparisons between groups were done using a Student's t-test. The criterion of statistical significance was taken as $p \leq 0.05$.

3. Results and discussion

Due to native people from highlands of Argentina nothwestern use jarillas and añaguaya extracts to solve mycosis, vaginal moco and inflammatory process (Carabajal et al., 2020), they were prepared from each species and then were analyzed and standarized according to their phytochemistry. In previous works were demonstrated the antifungal, antioxidant and anti-inflammatory activities of hydroalcoholic extracts of these plants species (Moreno et al., 2018a, b, c). In general, the native people in northwest of Argentina use mixtures of plant as medicine. In the present work, the antifungal activity of mixtures containing these extracts as phytotherapic ingredients was analized to know if its chemical components could interact between them to enhance their biological activity.

3.1. Antifungal activity

Vulvovaginal candidiasis (VVC) is estimated to be the second most common cause of vaginitis. Up to 75% of women suffer this infection during their lifetime, and 5–8% of

adult women have recurrent candidiasis (das Neves et al., 2008). This pathology is associated with inflammatory and oxidative processes (das Neves et al., 2008; Delgado Olivares et al., 2010). In recent years, resistance to traditional antifungal therapies (azoles and polyenes) has increased, and it is also known to be highly toxic and present many additional interactions and disorders. The lack of availability of conventional antifungals has led to search for alternatives among natural products (Tangarife-Castaño et al., 2011). These antifungal agents could be used as complementary therapy to conventional antifungals or as source for the search for new chemical structures with antifungal potential.

In this sense, it is important to find products without resistance to the treatment of *Candida* infection and with anti-inflammatory and antioxidant activities. Plant extracts (a single extract or extracts combination) could be considered as an alternative therapy because they are multi-component drugs with a binding ability for one or several targets by different action mechanisms (Wagner and Ulrich-Merzenich, 2009). Therefore, microorganisms are less likely to develop mechanisms of resistance to phyto-extracts.

The MIC values were determined to each extract, alone and in combination between them, and the FIC index were calculated (Table 1). The antifungal activity of the dry extracts was assayed *in vitro* against 6 yeast strains obtained from vaginal exudates of patients with vaginal yeast infections. They included three strains of *Saccharomyces cerevisiae*, three strains of *C. albicans*, three strains of *C. glabrata* and one strain of *C. tropicalis* (Table 1). Previously we reported that *S. cerevisiae* and *C. albicans* and non-albicans strains are azole-susceptible as fluconazole, voriconazole, itraconazole (85%) or resistant (R) (15%). These species were also susceptible to nystatin and amphotericin B (Moreno et al., 2018c).

The MIC values of each dry extract had been previously determined by the authors (Moreno et al., 2018a, b, c). However, a new MIC determination was carried out using several concentrations of plant extracts (12.5-800 μg GAE/mL) since these determinations were necessary to calculate the FIC index. The MIC values of the individual extracts are shown in Table 2. The dry extracts were effective against the assayed yeast strains in microdilution method. According with the MIC values, E-*Zp* and E-*Ld* and E-*Ln* could be considered strong antifungals in relation to classification of Tangarife-Castaño et al. (2011), while *Lc* and *Ta* could be considered moderate antifungals (Table 2).

A synergist effect was observed with the combination between *Zp*/*Lc* against *C. albicans*, *C. glabrata* and *S. cerevisiae* and between *Zp*/*Ln* and *Zp*/*Ld* against *C. glabrata* (FIC index= 0.5). An additive effect was observed with the combination of *Zp*/*Lc* against *C. tropicalis* and *Zp/Ln* against *C. albicans* and *C. tropicalis* (FIC index> 0.5 in both cases). Some combinations revealed an indifferent interaction, the FIC indices varying from 1.0 to 2.5 (Table 1). The less active mixture was *Ta*/*Ln*, which showed indifferent effect against all the yeasts tested (FIC between 1.1-2.2). The results indicate that combinations between *Z. punctata* and *Larrea* species are more efficient as antifungal than between *Larrea* species and between *Larrea* or *Z. punctata* with *T. andina*. The best combination was between *Zp* and *Lc* since it showed a synergistic or additive effect against all the tested strains, indicating that the interaction between chemical components contained in both plant species is more efficient as an antifungal.

The increase in antimicrobial capacity when herbal extracts are combined could be explained by the mechanism through which several bioactive constituents of plants affect various target sites and act cooperatively through synergy (Al-Bayati, 2008). This strategy is called multi-target effects. This approach is not exclusive for extract

combinations, but also for combinations between individual natural products or extracts with chemotherapeutics or commercial antibiotics (Hemaiswarya et al., 2008; Rodrigues et al., 2009; Wagner and Ulrich-Merzenich, 2009).

Previously, Butassi et al. (2015, 2019) showed the antifungal synergistic effect of combinations of *Z. punctata* and *L. nitida* dichloromethane extracts against *C. albicans* and *C. glabrata* species, and Carabajal et al. (2019) demonstrated the antioxidant activity of herbal teas combinations prepared from the aerial parts of *Z. punctata*, *L. cuneifolia* and *L. divaricata*. The evaluation of the therapeutic potential of Argentine native plants extracts could enhance the use of them in the treatment of several diseases, especially when there is synergy between them. In this work, the *Zp*/*Ln*, *Zp*/*Ld* and *Zp*/*Lc* mixtures were selected to continue the analysis by its synergistic or additive antifungal activity and effect against greater number of strains. The proportions chosen were *Zp*/*Ln* 1:1; *Zp*/*Ld* 1:1 and *Zp*/*Lc* 1:4. In previous works, we demonstrated that the chemical markers in these extracts are chalcones in *Zp* and nordihidroguaiaretic acid in *Lc, Ld* and *Ln* (Nuño et al., 2014; Moreno et al., 2015a, b, 2018b). The chalcones and nordihidroguaiaretic acid are actives compounds against *Candida albicans* (Nuño et al., 2014, Moreno et al., 2015a, Agüero et al., 2011).

3.2. Chemical composition

3.2.1. Phytochemical screening

The extracts and bi-herbal mixtures were standardized by chemical composition analysis. The qualitative analysis of all crude extracts showed positive reaction for triterpenoids/steroids, tannins and flavonoids. *T. andina* extract was also positive saponins.

The main groups of phenolic compounds present in bi-herbal mixtures (*Zp*/*Ld, Zp*/*Ln and Zp*/*Lc*) were quantified. The obtained results were compared with the content of the

same compounds previously determined in each individual plant species that were used to prepare the combinations (Moreno et al., 2018c). Figure 3 shows that no significative differences were observed in the content of total phenolics, flavonoid phenolic, non-flavonoid phenolic and flavones and flavanones between the bi-herbal mixtures and each individual plant extract.

3.2.2. Identification and quantification of phenolic compounds by HPLC-DAD

The mixtures of extracts were analysed by HPLC-DAD. The identity of each component was determined according with visible UV absorption spectra and retention times compared to commercial standards. In the mixtures of *Z. punctata* and *Larrea* species, NDGA was determined in peak 1 and DHC in peak 2. Table 3 shows the content of each of them in the single extracts and in the mixtures *Zp*/*Ld, Zp*/*Ln* and *Zp*/*Lc*. The content of NDGA in *Larrea* species was higher in *Ln* than *Ld* and *Lc.*

3.3. Anti-inflammatory activity

The traditional therapeutic approach toward inflammatory diseases focuses on targeting specific steps of particular pathways. In this sense, the complex composition of herbal extract or herbal mixtures would be useful to inhibit different signaling pathways or to inhibit one pathway at different levels to achieve a more efficient treatment of complex diseases such as vulvovaginitis, infection process associated to inflammatory pathology (Wagner et al., 2011; Meirer et al., 2014). The arachidonic acid (AA) pathway is one of the most important processes involved in the inflammatory response, with enzymes as phospholipase 2 ($sPLA_2$), cyclooxygenase (COX) and LOX, which lead to the production of group inflammatory mediators. The best approach to deal with inflammation would be to inhibit AA pathway at different levels in a way that would suppress the inflammatory response but at the same time maintaining physiological levels of mediators (Hwang et al., 2013). The more active extracts as antifungals (*Zp*,

Ld and *Ln*) inhibited the enzymes LOX of AA pathway. The inhibitory activity of binary combinations was in general higher than the levels exhibited by the individual plant species. All the combinations showed a level of inhibition of leukotriene synthesis similar or higher than those of the commercial anti-inflammatory drugs used as positive controls (Table 4). In a previous report was described the effect of NDGA on LOX (Blecha et al., 2007). Consequently, the anti-LOX activity of bi-herbal mixtures could be ascribed to nordihydroguiaretic acid of *Larrea* species. The inhibitory effect of DHC isolated from *Z. punctata* on COX-2 was previously reported (Alberto et al., 2007). Consequently, it is expected that the herbal mixtures containing *Zuccagnia punctata* also act at the level of COX-2 due to the presence of DHC.

Our results show that the mixtures tested could be potentially used to target the AA pathway, thus achieving a more efficient control of the production of pro-inflammatory mediators.

3.4. Antioxidant activity

Interactions between epithelial cells and yeasts in vaginal infections promote the release of free radicals and a local inflammatory response that results in mucosal damage (Delgado Olivares et al., 2010; Fischer, 2012). For this reason, a treatment with drugs with multiple effects, antifungal, antioxidant, and anti-inflammatory can be more effective. In this work, the antioxidant activity of extracts and binary mixtures was assayed in a free cell system and a cell system. In Table 4, SC_{50} and IC_{50} values obtained are showed.

In the ABTS test, it can be observed that the combinations were more active than the individual extracts. *Zp*/*Ld* was the most potent mixture with a SC_{50} value slightly lower than the other samples.

In the hydroxyl radical scavenging assay, no significant differences were observed between the individual extracts and the mixtures. However, in all cases, the potency was considerably higher than the natural antioxidant quercetin used as a reference. It is important to highlight the low values of SC_{50} obtained since the hydroxyl radical is one of the most reactive radicals, which can cause damage to biological membranes and react with almost any type of molecule (San-Miguel and Martín-Gil, 2009).

In the AAPH assay, the most active samples were *Zp*/*Lc, Zp*/*Ld, Zp*/*Ln* and the *Z. punctata* extract, being up to seven times more potent than the other assayed extracts. In addition, both the extracts and the mixtures showed lower IC_{50} values than the quercetin and BHT, demonstrating in all cases that they could protect the biological membranes from oxidation in low concentrations.

The samples were less active to H_2O_2 scavenging and the potency of the mixtures was similar to that of the individual extracts.

The antioxidant activity of the extracts and mixtures could be attributed in part to the nordihydroguaiaretic acid (Lee et al., 2003; Anesini et al., 2004; Guzmán-Beltrán et al., 2008) present in *Larrea* extracts, and to the presence in *Zuccagnia punctata* of 2′, 4′-dihidroxichalcone (Morán Vieyra et al., 2009; Nuño et al., 2014), pinocembrin (Vargas-Sánchez et al., 2015) and catechin (Aree and Jongrungruangchok, 2016; Grzesik et al., 2018), compounds with known antioxidant potency.

3.5. Mutagenicity test

The mutagenicity test using strains of *Salmonella typhimurium* TA98 and TA100 indicated that, up to a concentration of 250 μg/plate, the hydroalcoholic extracts of *Zp*/*Ld*, *Zp*/*Ln* and *Zp*/*Lc* mixtures do not induce an increase in the number of spontaneous revertants, showing in all cases MR <1.5 (Table 5). At the concentration of 500 μg/plate, the effect against either of the two strains could not be determined, given

that bacterial viability is affected at that concentration, due to the antimicrobial activity of the mixtures.

The results obtained would indicate that the selected binary mixtures are not genotoxic at the concentrations tested, as well as the individual extracts used for their preparation.

4. Conclusions

In summary, the mixtures of plant extracts *Zp*/*Ln*, *Zp*/*Ld* and *Zp*/*Lc* were the most active, demonstrating synergistic antifungal effect against *Candida albicans* and non-*albicans* and *Saccharomyces cerevisiae* strains. In addition, both extracts and mixtures showed antioxidant activity at low concentrations and were able to inhibit LOX, a proinflammatory enzyme.

The results obtained would indicate that the extracts (individual or combined) could be a good alternative therapy for the treatment of vaginal fungal infections associated with oxidative and inflammatory processes, however, *in vivo* tests are necessary to determine the effectiveness of their use. The mutagenic effect absence is a positive advance for determination of safety use of this medicinal plant species.

Acknowledgements

The research was supported by grants from Consejo de Investigación de la Universidad Nacional de Tucumán (PIUNT 2018 G637 Project), Argentina, Agencia Nacional de Promoción Científica y Técnica (PICT 2014, 3136 Project, PICT 2017, 4416 Project), and Consejo Nacional de Investigaciones Científicas y Técnicas (PUE 2018 0011 Project), Argentina.

References

Adegboye, M.F., Akinpelu, D.A., Okoh, A., 2008. The bioactive and phytochemical properties of *Garcinia kola* (Heckel) seed extract on some pathogens. Afr. J. Biotechnol. 7, 3934–3938.

Agüero, M.B., Svetaz, L., Sánchez, M., Luna, L., Lima, B., López, M.L., Zacchino, S., Palermo, J., Wunderlin, D., Feresin, G.E., Tapia, A., 2011. Argentinean Andean propolis associated with the medicinal plant *Larrea nitida* Cav. (Zygophyllaceae). HPLC-MS and GC-MS characterization and antifungal activity. Food Chem. Toxicol. 49, 1970–1978.

Aiyegoro, O.A., Okoh, A.I., 2009. Use of bioactive plant products in combination with standard antibiotics: implications in antimicrobial chemotherapy. J. Med. Plant. Res. 3, 1147–1152.

Al-Bayati, F.A., 2008. Synergistic antibacterial activity between *Thymus vulgaris* and *Pimpinella anisum* essential oils and methanol extracts. J. Ethnopharmacol. 116, 403–406.

Aree, T., Jongrungruangchok, S., 2016. Crystallographic evidence for beta-cyclodextrin inclusion complexation facilitating the improvement of antioxidant activity of tea (+)-catechin and (−)-epicatechin. Carbohydr. Polym. 140, 362–373.

Ayoola, G.A., Coker, H.A.B., Adesegun, S.A., Adepoju-Bello, A.A., Obaweya, K., Ezennia, E.C., Atangbayila, T.O., 2008. Phytochemical screening and antioxidant activities of some selected medicinal plants used for malaria therapy in southwestern Nigeria. Trop. J. Pharm. Res. 7, 1019–1024.

Alberto, M.R., Nieva Moreno, M.I., Zampini, I.C., Isla, M.I., 2007. Antiinflammatory activity of structuraly related natural flavonoids. BLACPMA 6(5), 308–309.

Anesini, C., Turner, S., Borda, E., Ferraro, G., Coussio, J., 2004. Effect of *Larrea divaricata* Cav. extract and nordihydroguaiaretic acid upon peroxidase secretion in rat submandibulary glands. Pharmacol. Res. 49, 441–448.

Blecha, J.E., Anderson, M.O., Chow, J.M., Guevarra, C.C., Pender, C., Penaranda, C., Zavodovskaya, M., Youngren, J., Berkman, C.E., 2007. Inhibition of IGF-1R and lipoxygenase by nordihydroguaiaretic acid (NDGA) analogs. Bioorg. Med. Chem. Lett. 17(14), 4026–4029.

Butassi, E., Svetaz, L.A., Ivancovich, J.J., Feresin, G.E., Tapia, A., Zacchino, S.A., 2015. Synergistic mutual potentiation of antifungal activity of *Zuccagnia punctata* Cav. and *Larrea nitida* Cav. extracts in clinical isolates of *Candida albicans* and *Candida glabrata*. Phytomedicine, 22, 666–678.

Butassi, E., Svetaz, L.A., Sortino, M.A., Quiroga, A.D., Carvalho, V.S.D., Cortés, J.C.G., Ribas, J.C., Zacchino, S.A., 2019. Approaches to the mechanism of antifungal activity of *Zuccagnia punctata-Larrea nitida* bi-herbal combination. Phytomedicine, 54, 291–301.

Carabajal, M. P. A., Perea, M. C., Isla, M. I., Zampini, I. C., 2020. The use of jarilla native plants in a Diaguita-Calchaquí indigenous community from northwestern Argentina: An ethnobotanical, phytochemical and biological approach. J. Ethnopharmacol. 112258.

Chobot, V., 2010. Simultaneous detection of pro- and antioxidative effects in the variants of the deoxyribose degradation assay. J. Agric. Food Chem. 58, 2088–2094.

CLSI., 2012. Reference method for broth dilution antifungal susceptibility testing of yeasts; 4th informational supplement. Clinical and Laboratory Standards Institute, Villanova PA.

Costamagna, M.S., Ordoñez, R.M., Zampini, I.C., Sayago, J.E., Isla, M.I., 2013. Nutritional and antioxidant properties of *Geoffroea decorticans*, an Argentinean fruit, and derived products (flour, arrope, decoction and hydroalcoholic beverage). Food Res. Int. 54(1), 160–168.

das Neves, J., Pinto, E., Teixeira, B., Dias, G., Rocha, P., Cunha, T., Santos, B., Amaral, M.H., Bahia, M.F., 2008. Vulvovaginal candidosis. Drugs 68, 1787–1802.

Delgado Olivares, L., Betanzos Cabrera, G., Sumaya Martinez, M.T., 2010. Importancia de los antioxidantes dietarios en la disminución del estrés oxidativo. Investigación y Ciencia 50(1), 10–5.

Fernando, C.D., Soysa, P., 2015. Optimized enzymatic colorimetric assay for determination of hydrogen peroxide (H_2O_2) scavenging activity of plant extracts. Methods X 2, 283–291.

Fisher, G., 2012. Chronic vulvovaginal candidiasis: What we know and what we have yet to learn. Australas. J. Dermatol. 53, 247–254.

Grzesik, M., Naparło, K., Bartosz, G., Sadowska-Bartosz, I., 2018. Antioxidant properties of catechins: Comparison with other antioxidants. Food Chem. 241, 480–492.

Guzmán-Beltrán, S., Espada, S., Orozco-Ibarra, M., Pedraza-Chaverri, J., Cuadrado, A., 2008. Nordihydroguaiaretic acid activates the antioxidant pathway Nrf2/HO-1 and protects cerebellar granule neurons against oxidative stress. Neurosci. Lett. 447, 167–171.

Hemaiswarya, S., Kruthiventi, A.K., Doble, M., 2008. Synergism between natural products and antibiotics against diseases. Phytomedicine 15, 639–652.

Hwang, S.H., Wecksler, A.T., Wagner, K., Hammock, B.D., 2013. Rationally designed multitarget agents against inflammation and pain. Curr. Med. Chem. 20(13), 1783–99.

Isla, M.I., Salas, A., Danert, F.C., Zampini, I.C., Ordoñez, R.M., 2014. Analytical methodology optimization to estimate the content of non-flavonoid phenolic compounds in Argentine propolis extracts. Pharm. Biol. 52(7), 835–840.

Lee, C.H., Jang, Y.S., Her, S.J., Moon, Y.M., Baek, S.J., Eling, T., 2003. Nordihydroguaiaretic acid, an antioxidant, inhibits transforming growth factor-β activity through the inhibition of Smad signaling pathway. Exp. Cell Res. 289, 335–341.

Ma, X.H., Zheng, C.J., Han, L.Y., Xie, B., Jia, J., Cao, Z.W., Li, Y.X., Chen, Y.Z., 2009. Synergistic therapeutic actions of herbal ingredients and their mechanisms from molecular interaction and network perspectives. Drug Discovery Today 14, 579–588.

Maron, D.M., Ames, B.N., 1983. Revised methods for the Salmonella mutagenicity test. Mutat. Res. 113, 173–215.

Meirer, K., Steinhilber, D., Proshak, E., 2014. Inhibitors of the arachidonic acid cascade: Interfering with multiple pathways. Basic Clin. Pharmacol. Toxicol. 114, 83–91.

Mendes, L., De Freitas, V., Baptista, P., Carvalho, M., 2011. Comparative antihemolytic and radical scavenging activities of strawberry tree (*Arbutus unedo* L.) leaf and fruit. Food Chem. Toxicol. 49(9), 2285–2291.

Mercado, M.I., Moreno, M.A., Ruiz, AI., Rodríguez, I.F., Zampini, I.C., Isla, M.I., Ponessa, G.I., 2018. Morphoanatomical and histochemical characterization of *Larrea* species from Northwestern of Argentina. Rev. Bras. Farmacogn. 28(4), 393–401.

Mojab, F., Kamalinejad, M., Ghaderi, N., Vahidipour, H.R., 2003. Phytochemical screening of some species of Iranian plants. J. Pharm. Res. 2, 77–82.

Morán Vieyra, F., Boggetti, H., Zampini, I., Ordoñez, R., Isla, M., Alvarez, R., De Rosso, V., Mercadante, A., Borsarelli, C., 2009. Singlet oxygen quenching and radical scavenging capacities of structurally related flavonoids present in *Zuccagnia punctata* Cav. Free Radical Res. 43, 553–564.

Moreno, A., Nuño, G., Cuello, S., Sayago, J., Alberto, M.R., Zampini, C., Isla, M.I., 2015a. Anti-inflammatory, antioxidant and antimicrobial activity characterization and toxicity studies of flowers of "Jarilla", a medicinal shrub from Argentina. Nat. Prod. Commun. 6, 991–994.

Moreno, M.A., Mercado, M.I., Nuño, G., Zampini, I.C., Cuello, A.S., Ponessa, G.I., Sayago, J.E, Isla, M.I., 2015b. Histochemical localization and characterization of chalcones on the foliar surface of *Zuccagnia punctata* Cav. Insights into their physiological role. Phytochem. Lett. 13, 134–140.

Moreno, M.A., Córdoba, S., Zampini, I.C., Mercado, M.I., Ponessa, G., Alberto, M.R., Nader-Macias, M.E.F., Sayago, J., Burgos-Edwards, A., Schmeda-Hirschmann, G., Isla, M.I., 2018a. *Tetraglochin andina* Ciald.: A medicinal plant from the Argentinean highlands with potential use in vaginal candidiasis. J. Ethnopharmacol. 216, 283–294.

Moreno, M.A., Córdoba, S., Zampini, I.C., Mercado, M.I., Ponessa, G., Sayago, J.E., Pino Ramos, L.L., Schmeda-Hirschmann, G., Isla, M.I., 2018b. Argentinean

Larrea dry extracts with potential use in vaginal candidiasis. Nat. Prod. Commun. 13(2), 171–174.

Moreno, M.A., Gómez-Mascaraque, L., Arias, M., Zampini, I.C., Sayago, J.E., Pino Ramos, L.L., Schmeda-Hirschmann, G., López-Rubio, A., Isla, M.I., 2018c. Electrosprayed chitosan microcapsules as delivery vehicles for vaginal phytoformulations. Carbohydr. Polym. 201, 425–437.

Nuño, G., Alberto, M., Zampini, I., Cuello, S., Ordoñez, R., Sayago, J., Baroni, V., Wunderlin, D., Isla, M.I., 2014. The effect of *Zuccagnia punctata* Cav, an Argentina medicinal plant, on virulence factors from Candida species. Nat. Prod. Commun. 9, 933–936.

Onwukaeme, D.N., Ikuegbvweha, T.B., Asonye, C.C., 2007. Evaluation of phytochemical constituents, antibacterial activities and effect of exudate of *Pycanthus angolensis* Weld Warb (Myristicaceae) on corneal ulcers in rabbits. Trop. J. Pharm. Res. 6, 725–730.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., Rice-Evans, C., 1999. Antioxidant activity applying an improved abts radical cation decolorization assay, Free Radic. Biol. Med. 26(98), 1231–1237.

Rodrigues, F., Costa, J., Coutinho, H., 2009. Synergy effects of the antibiotics gentamicin and the essential oil of Croton zehntneri. Phytomedicine 16, 1052–1055.

San-Miguel, A., Martin, F.J., 2009. Importancia de las especies reactivas al oxígeno (radicales libres) y los antioxidantes en clínica. Gac. Med. Bilbao 106(3), 106–113.

Tangarife-Castaño, V., Correa-Royero, J., Zapata-Londono, B., Durán, C., Stanshenko, E., Mesa-Arango, A.C., 2011. Anti Candida albicans activity, cytotoxicity and

interaction with antifungal drugs of essential oils and extracts from aromatic and medicinal plants. Infection 15, 160–167.

Teke, G.N., Kuiate, J.R., Kueté, V., Teponno, R.B., Tapondjou, L.A., Tane, P., Giacinti, G., Vilarem, G., 2010. Bio-guided isolation of potential antimicrobial and antioxidant agents from the stem bark of *Trilepisium madagascariense*. S. Afr. J. Bot. 77, 319–327.

The Plant List (2013). Version 1.1. http://www.theplantlist.org/ (accessed 20.04.15).

Torres, C.A., Nuñez, M.B., Isla, M.I., Castro, M.P., Gonzalez, A.M., Zampini, I.C., 2017. Antibacterial synergism of extracts from climbers belonging to Bignoniaceae family and commercial antibiotics against multi-resistant bacteria. J. Herb. Med. 8, 24–30.

Torres Carro, R., Isla, M.I., Thomas-Valdes, S., Jiménez-Aspee, F., Schmeda-Hirschmann, G., Alberto, M.R., 2017. Inhibition of pro-inflammatory enzymes by medicinal plants from the Argentinean highlands (Puna). J. Ethnopharmacol. 205, 57–68.

Vargas-Sánchez, R.D., Mendoza-Wilson, A.M., Balandrán-Quintana, R.R., Torrescano-Urrutia, G.R., Sánchez-Escalante, A., 2015. Study of the molecular structure and chemical reactivity of pinocembrin by DFT calculations. Comput. and Theor. Chem. 1058, 21–27.

Wagner, H., Ulrich-Merzenich, G., 2009. Synergy research: Approaching a new generation of phytopharmaceuticals. Phytomedicine 16, 97–110.

Wagner, H., 2011. Synergy research: approaching a new generation of phytopharmaceuticals. Fitoterapia 82, 34–37.

Zampini, I.C., Vattuone, M., Isla, M.I., 2005. Antibacterial activity against antibiotic-resistant gram negative human pathogenic bacteria of hydroxychalcone isolated from *Zuccagnia punctata* Cav. J. Ethnopharmacol. 102, 450–456.

Zampini, I.C., Cudmani, N., Isla, M.I., 2007. Actividad antimicrobiana de plantas medicinales argentinas sobre bacterias antibiótico-resistentes. Acta Bioquím. Clín. Latinoam. 41, 385–393.

Zampini, I.C., Villena, J., Salva, S., Herrera, M., Isla, M.I., Alvarez, S. 2012. Potentiality of standardized extract and isolated flavonoids from Zuccagnia punctata for the treatment of respiratory infections by Streptococcus pneumoniae: In vitro and in vivo studies. J. Ethnopharmacol. 140, 287-292

Figure legends

Figure 1. Local selling regional products and dried herbs, including jarillas species in Amaicha del Valle.

Figure 2. Plant species. A) *Zuccagnia punctata* Cav.; B) *Tetraglochin andina* Ciald. C) *Larrea divaricata* Cav.; D) *Larrea cuneifolia* Cav.; E) *Larrea nitida* Cav. The pictures were obtained by the authors during samples collection in the places described in Material and methods.

Figure 3. Content of phenolic compounds in individual extracts and binary mixtures. The values are presented as mean ± standard deviation of triplicates. The content of flavones and flavanols is expressed in mg QE/g DW, while the rest of the compounds is expressed in mg GAE/g DW. Different letters in the same column for each species indicated significant differences according to the Tukey test ($p \leq 0.05$). The error bars represent the standard error of the mean.

Table 1. FIC index of combined dry extracts on yeast clinical isolates from vaginal infections in humans.

	Lc/Zp	***Lc/Ta***	***Lc/Ln***	***Lc/Ld***	***Zp/Ta***	***Zp/Ln***	***Ld/Zp***	***Ld/Ln***	***Ta/Ld***	***Ta/Ln***
C. albicans	100/25* (0.500)^{s}	25/800 (2.062)^{i}	50/200 (2.125)^{i}	200/100 (1.500)^{i}	25/100 (0.500)^{s}	50/50 (1.000)^{a}	200/6.25 (2.062)^{i}	25/25 (0.500)^{s}	50/200 (2.125)^{i}	50/200 (2.125)^{i}
C. glabrata	100/12.5 (0.500)^{s}	100/25 (0.312)^{s}	100/25 (0.750)^{a}	50/25 (0.625)^{a}	12.5/100 (0.500)^{s}	12.5/12.5 (0.500)^{s}	12.5/12.5 (0.500)^{s}	12.5/25 (0.750)^{a}	50/100 (2.125)^{i}	50/100 (2.125)^{i}
C. tropicalis	200/25 (0.750)^{a}	200/50 (0.625)^{a}	25/50 (0.562)^{a}	200/200 (2.500)^{i}	100/25 (1.062)^{i}	50/12.5 (0.625)^{a}	50/50 (1.000)^{a}	100/12.5 (1.125)^{i}	12.5/50 (0.531)^{a}	50/200 (2.125)^{i}
S. cerevisiae	25/12.5 (0.500)^{s}	25/400 (1.250)^{i}	25/12.5 (0.500)^{s}	12.5/25 (0.625)^{a}	50/12.5 (1.031)^{i}	12.5/12.5 (0.500)^{s}	25/12.5 (0.750)^{a}	25/12.5 (0.750)^{a}	25/25 (0.562)^{a}	25/50 (1.062)^{i}
ATCC C. *parapsilosis*	100/50 (0.750)^{a}	400/25 (1.062)^{i}	25/50 (0.562)^{a}	25/50 (0.562)^{a}	100/12.5 (1.031)^{i}	25/25 (0.500)^{s}	50/25 (0.750)^{a}	25/50 (0.750)^{a}	25/100 (1.062)^{i}	25/100 (1.062)^{i}
ATCC *C. krusei*	12.5/25 (0.625)^{a}	200/25 (2.250)^{i}	50/50 (1.500)^{a}	25/12.5 (0.500)^{s}	50/12.5 (1.125)^{i}	50/25 (1.500)^{i}	25/12.5 (0.750)^{a}	50/25 (1.500)^{i}	25/25 (0.750)^{a}	200/12.5 (2.250)^{i}

*µg GAE/mL of each extract in the combination. FIC Index values are shown in parenthesis. [a] Synergistic effect (≤ 0.5); [b] Additive effect (> 0.5 and ≤ 1); [c] Indifferent effect (> 1 and < 4).
Zp: *Zuccagnia punctata; Ta: Tetraglochin andina; Ld*: *Larrea divaricata; Lc*: *Larrea cuneifolia*; *Ln*: *Larrea nitida.*

Table 2. Effect of individual extracts on different yeast strains isolated from vaginal infections.

Yeast Strain	Collection number	Phenotype of clinical isolates	MIC (µg GAE/mL)				
			Zp	***Ta***	***Ld***	***Ln***	***Lc***
C. albicans	134333	Flu^R,Am^S,Ny^S	100	400	100	100	400
C. glabrata	042030	Flu^S,Am^S,Ny^S	50	400	50	50	400
C. tropicalis	1841	Flu^S,$Am^S Ny^S$	100	400	100	100	400
S. cerevisiae	134544	Flu^R,$Am^S Ny^S$	50	400	50	50	100
ATCC *C. parapsilosis*	134410	Flu^S,$Am^S Ny^S$	100	400	100	100	400
ATCC *C. krusei*	134409	Flu^R,$Am^S Ny^S$	50	100	50	50	100

MIC values represent growth inhibition compared with control growth. Flu: fluconazole; Am: amphotericin B; Ny: nystatin. R: resistant; S: sensitive. *Zp*: *Zuccagnia punctata; Ta: Tetraglochin andina; Ld*: *Larrea divaricata; Ln*: *Larrea nítida; Lc*: *Larrea cuneifolia*.

Table 3. Quantification of chemical markers in extracts of *Larrea divaricata, Larrea cuneifolia, Larrea nitida, Zuccagnia punctata* and in combined extracts.

Sample	NDGA µg/mg DW	DHC µg/mg DW
E-*Zp*	-	40.13±0.10
E-*Ld*	134.32±0.10	-
E-*Ln*	268.12±0.10	-
E-*Lc*	158.69±0.05	-
M-*Zp*/*Ld* (1:1)	62.84±0.05	20.21±0.50
M-*Zp*/*Ln* (1:1)	143.03±0.03	22.44±0.10
M-*Zp*/*Lc* (1:4)	204.65±0.02	18.19±0.50

E: Extract; M: Mixture; *Zp*: *Zuccagnia punctata; Ld*: *Larrea divaricata; Ln*: *Larrea nitida; Lc*: *Larrea cuneifolia*. DW: dry weight. Values are reported as mean ± standard deviation of triplicates.

Table 4. Antioxidant activities of dry extracts, combinations and commercial reference drugs.

Sample	SC_{50} $ABTS^{\bullet+}$ (µg DW/mL)	SC_{50} $^{\bullet}OH$ (µg DW/mL)	SC_{50} H_2O_2 (µg DW/mL)	$IC_{50\ AAPH}$ (µg DW/mL)	LOX (% inhibition) *
E-*Zp*	9.13±0.50^{e}	0.98±0.05^{a}	184.84±15.20^{b}	0.14±0.01^{a}	95.25±4.20^{b}
E-*Ld*	6.72±0.20^{d}	1.18±0.10^{a}	175.02±14.50^{b}	0.55±0.05^{c}	94.41±3.40^{b}
E-*Ln*	12.14±0.60^{g}	1.11±0.07^{a}	192.12±14.00^{b}	0.56±0.02^{c}	94.17±3.20^{b}
E-*Lc*	10.29±0.50^{f}	1.73±0.10^{a}	191.81±13.00^{b}	0.30±0.02^{b}	53.37±2.70^{a}
M-*Zp/Ld*	3.81±0.26^{b}	1.28±0.10^{a}	186.70±15.50^{b}	0.100±0.005^{a}	99.5±4.50^{b}
M-*Zp/Ln*	5.11±0.26^{c}	0.89±0.08^{a}	182.82±12.60^{b}	0.078±0.002^{a}	99.3±4.30^{b}
M-*Zp/Lc*	4.91±0.27^{c}	1.46±0.11^{a}	173.16±13.20^{b}	0.08±0.01^{a}	97.92±5.10^{b}
Quercetin	1.41±0.08^{a}	30.00±2.00^{b}	38.00±3.20^{a}	0.90±0.08^{d}	
BHT	3.52±0.20^{b}	-	-	1.20±0.10^{e}	
					95.00±2.80^{b}

E: Extract; M: Mixture; *Zp*: *Zuccagnia punctata*; *Ld*: *Larrea divaricata*; *Ln*: *Larrea nitida*; *Lc*: *Larrea cuneifolia*; DW: dry weight; BHT: butylatedhydroxytoluene.

*LOX inhibition percentage with 50µg/mL of extract or herbal mixtures.

Values are reported as mean ± standard deviation of triplicates. Different letters in the same column for each plant species indicated significant differences according to Tukey's test ($p \leq 0.05$).

Table 5. Results obtained in the mutagenic activity evaluation assay against *S. Typhimurium* TA98 and TA100 strains.

Samples	µg GAE/ plate	TA98 (-) S9	T100 (-) S9	MR TA 98	MR TA 100
		Number of revertants		MR	
M *Zp/Ld*	500	ND	ND	ND	ND
	250	40±0	128±14	1.17	1.16
	125	43±5	136±14	1.26	1.24
M *Zp/Ln*	500	ND	ND	ND	ND
	250	34±3	123±12	1	1.12
	125	35±3	139±19	1.03	1.26
M *Zp/Lc*	500	ND	ND	ND	ND
	250	32±2	129±21	0.94	1.17
	125	35±3	128±26	1.03	1.16
Positive control[1]		1222±109	723±171		
Negative control[2]		34±2	110±16		

* MR: mutagenicity relation; ND: not determined. These concentrations affect the viability of the *Salmonella* strains. [1]Mean number of revertants induced by 4-nitro-o-phenylendiamine (10 µg/plate). [2]The number of spontaneous revertant colonies (means±SD) determined without the addition of the samples, only with the vehicle, DMSO. *Zp*: *Zuccagnia punctata; Ld*: *Larrea divaricata; Lc*: *Larrea cuneifolia*; *Ln*: *Larrea nitida*

Figure 1. Local selling regional products and dried herbs, including “jarillas” species in Amaicha del Valle.

Figure 2. Plant species. A) *Zuccagnia punctata* Cav.; B) *Tetraglochin andina* Ciald. C) *Larrea divaricata* Cav.; D) *Larrea cuneifolia* Cav.; E) *Larrea nitida* Cav. The pictures were obtained by the authors during sample collection in the places described in Materials and methods.

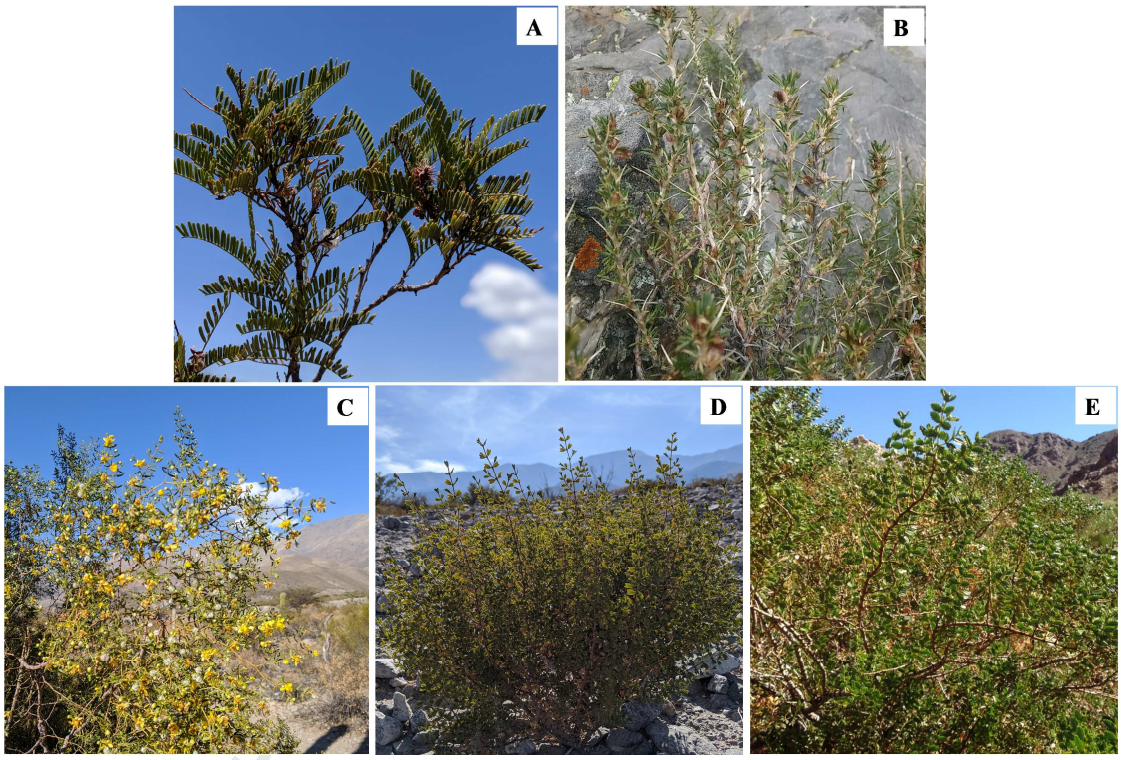
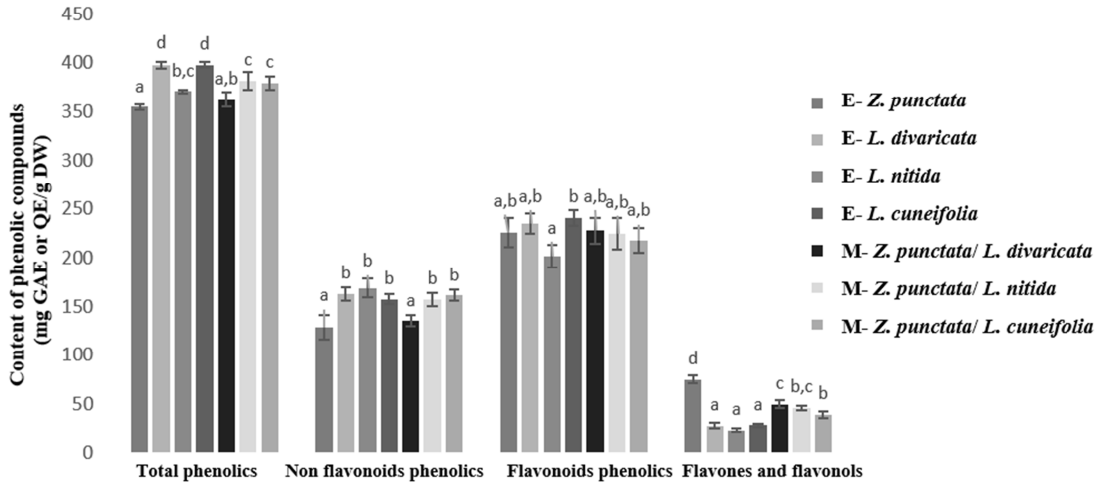


Figure 3. Content of phenolic compounds in individual extracts and binary mixtures. E: Extract; M: Mixture. The values are presented as mean ± standard deviation of triplicates. The content of flavones and flavanols is expressed in mg QE/g DW, while the rest of the compounds is expressed in mg GAE/g DW. Different letters in the same column for each species indicated significant differences according to Tukey's test ($p \leq 0.05$). The error bars represent the standard error of the mean.



Highlights

1. Plant species of Argentine highlands are used as antifungal in popular medicine
2. Extracts were effective against yeast strains isolated from vaginal infections
3. Bi-herbal mixtures showed synergistic or additive antifungal effect
4. Extracts and bi-herbal mixtures showed antioxidant and anti-inflammatory activity
5. Extracts and bi-herbal mixtures were standardized by chemical composition