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Electrosprayed Chitosan Microcapsules as Delivery Vehicles for Vaginal Phytoformulations

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Highlights

- Chitosan microcapsules and tablets with Argentinean plants extracts were developed
- Release of bioactive compounds from chitosan microcapsules was faster than from tablets
- The encapsulation process did not affect the antioxidant or antifungal activity
- The potential of chitosan-based delivery systems for phytomedicines was demonstrated

Abstract
The design of novel delivery systems to treat vaginal fungal infections is a topic of high interest. Chitosan, being itself antimicrobial and having good mucoadhesive properties, is an excellent candidate as a delivery matrix for active compounds. In this work, chitosan microcapsules containing dry extracts of Argentinean medicinal plants with proved biological properties (*Larrea divaricata*, *L. cuneifolia*, *L. nitida*, *Zuccagnia punctata* and *Tetraglochin andina*) were developed through electrospraying and compared with conventionally used tablets containing the same extracts. Total phenolics, loading efficacy, physical properties, morphology and particle size, molecular organization, water sorption capacity, release of bioactive compounds and biological properties were assessed. The encapsulation process or the inclusion in tablets did not degrade the bioactive compounds of the extracts. The release of phenolic compounds from chitosan microcapsules was faster than from tablets. The fingerprint of released phenolic compounds from microcapsules and tablets was similar to that from the dry extracts and the antioxidant and antifungal capacity remained unchanged. The FT-IR analysis suggested interactions between the chitosan and the extracts, which explained why the microcapsules kept the integrity in slightly acidic media. Increased solubility of the extracts when incorporated in the microcapsules was seen in simulated vaginal fluid, potentially increasing the bioavailability of bioactive compounds in the vaginal environment.

This work highlights the potential of the chitosan-based delivery systems for phytomedicines with antifungal and antioxidant activity to be used in vulvovaginal candidiasis.

**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; TPC, Total phenolic compound; NF-P, Non-flavonoid phenols; FP, Flavonoid phenolic; DMAC, 4-dimethylaminocinnamaldehyde; PB2E, procyanidin B2 equivalents; GE, glucose equivalent; DW, dry weight; SEM, scanning electron microscopy; PBS, phosphate buffer saline; SVF, simulated vaginal fluid; 1,2dHGPC, 1,2-diheptanoylthio-glycerophosphocholine.

**Key words:** anti-*Candida*, Argentinean dry plant extracts, microcapsules, vaginal tablets.

1. **Introduction**

In the last years, novel vaginal drug delivery formulations have focused attention to treat fungal infections. *Candida* species are the fourth leading agent of health care associated infections worldwide with the highest mortality rate of all nosocomial bloodstream infections, even exceeding those caused by *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Bandara, Matsubara, & Samaranayake, 2017).
The limited effectiveness of typical vaginal formulations like creams, suppositories or tablets are related to their low active residence time, small absorption area, barrier properties of mucosa and inadequate spreading of the formulation on vaginal surfaces (Marcie1lo, Rossi, Caramella, & Remuñán-López, 2017). To improve effectiveness, new delivery systems based on nano- or microparticles have been developed (Ensign, Cone, & Hanes, 2014; Wong, Dhanawat, & Rathbone, 2014; Li et al., 2015, 2017; Gómez-Mascaraque et al., 2016). These smaller carriers have several advantages, including high specific surface area related to their small size, improved stability of the therapeutic agents incorporated, enhanced bioavailability and controlled release properties, making them attractive for practical pharmaceutical applications.

Chitosan nano- and microparticles have high potential for vaginal drug delivery applications, because of the excellent properties of this carbohydrate polymer including good biocompatibility, low toxicity, mucoadhesivity and inherent antimicrobial properties, which have boosted a number of developments in this area (Andersen et al., 2017; Marciello et al., 2017). Chitosan has been reported to inhibit the adhesion of *C. albicans* to human vaginal epithelial cells (Knapczyk, Macura, & Pawlik, 1992; Li et al., 2009). Amongst the techniques that can be used to generate chitosan nano- and microparticles, electrospraying has gained increased attention. This technique can produce microencapsulation structures in a one-step process, under mild conditions, in the absence of organic/toxic solvents, limiting inactivation of the bioactive compounds and being adequate for both hydrophilic and hydrophobic drugs or ingredients with generally high loading efficiencies (Gómez-Mascaraque, Sánchez, & López-Rubio, 2016).

The side effects of antifungal agents currently used in commercial formulations (Sawant & Khan, 2017) and the very high recurrence rates observed (Bradshaw & Sobel, 2016), encourage alternative therapeutic approaches, which include the use of natural antifungal extracts.

Plants occurring in the semiarid and arid regions of Argentina have been widely used in traditional medicine to treat fungal and bacterial infections and as anti-inflammatory (Barboza, Cantero, Nuñez, Pacciaroni, & Ariza Espinar, 2009; Butassi et al., 2015; Quiroga, Sampietro, & Vattuone, 2001; Stege et al., 2006; Svetaz et al., 2010; Torres, Urbina, Morales, Modak, & Delle Monache, 2003; Vogt, Cifuente, Tonn, Sabini, & Rosas, 2013; Zampini, Cudmani, & Isla, 2007).

The hydroalcoholic extracts of the shrubs *Larrea divaricata, L. cuneifolia, L. nitida* (Zygophyllaceae), *Zuccagnia punctata* (Fabaceae) and *Tetraglochin andina* (Rosaceae) contain a distinctive array of phenolic compounds, which are species-dependent and show good to excellent antioxidant capacity and antifungal activity against *Candida albicans* and non-albicans isolated from vaginal infections (Agüero et al., 2011; Blecha et al., 2007; Carabajal, Isla, & Zampini, 2016; Martino et al., 2016; Moreno et al., 2018a, b). The proved antioxidant and antifungal
activity make these extracts excellent candidates to be used in vaginal formulations against candidiasis. The aim of the present work was to develop electrosprayed chitosan-based delivery systems for vaginal administration of selected medicinal plant extracts. The efficacy and release properties of the chitosan-based preparations from the above mentioned Argentinian species were compared with tablets containing the same extracts. The morphology, drug loading, water sorption ability and functional properties (antifungal and antioxidant activities) of these novel delivery systems were also characterized.

2. Materials and Methods

2.1. Reagents

Chitosan (reported degree of deacetylation 85 ± 2.5 % and an average molecular weight of 25 kDa) was purchased from Heppe Medical Chitosan GmbH. 2,2’-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) and 2,2’-azobis(2-methylpropionamidine) dihydrochloride (AAPH) were obtained from Sigma-Aldrich. Carbopol 934, hydroxypropylmethyl cellulose (molecular weight 26,000, viscosity 80-120 cP) and sodium carboxymethylcellulose (NaCMC, molecular weight 250,000, substitution degree 0.7) were purchased from Sigma-Aldrich. All solvents used were of analytical grade.

2.2. 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., Larrea nitida (Ln) was collected in April 2015 at Vinchina, La Rioja, Argentina at 3485 m.a.s.l. Tetraglochin andina Ciald (Ta) was collected from January to February 2015 in Huaca Huasi, Tucumán, Argentina (4300 m.a.s.l.). The plants are shown in Figure 1. 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 were dried in a forced air oven at 40°C.
2.3. Dry extract preparation

The powdered air-dried plant material (10 g) was macerated in 200 mL of 60° ethanol for 1 h with ultrasonic application five times for 10 minutes. Combined extracts were filtered, taken to dryness under reduced pressure and then freeze-dried to afford the extracts. The w/w extraction yield was determined. Dry extracts were placed in oxygen barrier bags and vacuum-packed (Multivac, D-8941, Germany) and stored at room temperature. The dry extract was dissolved in ethanol 60° to carry out the phytochemical and biological assays.

2.4. Microencapsulation of extracts by electrospraying
Chitosan solutions 5% (w/v) were prepared by dissolving the polysaccharide in 80% acetic acid (v/v) at room temperature under magnetic stirring. Dry plant extracts (10% w/w of the total solids content) were added to the chitosan solutions and the mixtures were stirred until complete dissolution. The final solutions were electrosprayed following the procedure optimized in a previous work (Gómez-Mascaraque et al., 2016). Briefly, samples were electrosprayed by using a home-made electrospinning/electrospraying apparatus, equipped with a variable high-voltage 0-30 kV power supply, at a steady flow-rate of 0.15 mL/h, an applied voltage of 17 kV and a syringe tip-collector distance of 10 cm. Processed samples were collected on a stainless-steel plate. The obtained dry powdered material was stored in a desiccator until use.

2.5. Design of tablets for vaginal delivery

Extract-free tablets (placebo) and dry extract-containing tablets were prepared. The composition of the tablets was as follows: carbopol 934P (100 mg), hydroxypropylmethyl cellulose (100 mg), carboxymethyl cellulose (50 mg), sodium bicarbonate (15 mg), citric acid (5 mg), magnesium stearate (2.5 mg), dry extract (1.5-10 mg). The tablets were compressed on a single punch tablet machine (Fareast, Shanghai, China) with a pressure of 14.3 kN.

2.6. Phytochemical analysis of dry extracts

Total phenolic (TP) content was determined by the Folin–Ciocalteau method (Costamagna, Ordoñez, Zampini, Sayago, & Isla, 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, Salas, Danert, Zampini, & Ordoñez, 2014). Flavonoid phenolic (FP) content was calculated by difference between TP and NF-P. Flavone and flavanone content was determined (Costamagna et al., 2013). The total condensed tannin (proanthocyanidins) and hydrolyzed tannins content was quantified according to Torres Carro, Isla, Ríos, Giner, & Alberto, 2015.

2.7. Identification of phenolic compounds

The Z. punctata extract was analyzed by HPLC-ESI-MS/MS to identify its constituents. Mass spectra were recorded using an Agilent 1100 (Agilent Technologies Inc., CA, USA) liquid chromatography system connected through a split to an Esquire 4000 Ion Trap LC/MS(n) system (Bruker Daltoniks, Germany). Ionization was performed at 3000 V assisted by nitrogen as nebulizing gas at 50 psi and as drying gas at 365°C and a flow rate of 10 L/min. Negative ions were detected using full scan (m/z 20-2200) and normal resolution (scan speed 10,300 m/z/s; peak
with 0.6 FWHM/m/z). The trap parameters were set in ion charge control (ICC) using manufacturer default parameters, and maximum accumulation time of 200 ms. The mass spectrometric conditions for analysis were: electrospray needle, 4000 V; end plate offset, -500 V; skimmer 1, 56.0 V; skimmer 2, 6.0 V; capillary exit offset, 84.6 V; capillary exit, 140.6 V. Collision induced dissociation (CID) spectra were obtained with a fragmentation amplitude of 1.00 V (MS/MS) using helium as the collision gas and was automatically controlled through SmartFrag option. The mixture was analyzed using a MultoHigh 100 RP 18-5µ (250 x 4.6 mm) column (CS-Chromatographie Service GmbH, Langerwehe, Germany) maintained at 25 °C. The HPLC-MS analyses were performed using a linear gradient solvent system consisting of 1% formic acid in water (A) and acetonitrile as follow: 15% to 25% B over 15 min, increasing to 30% B at 30 min, changing to 40% B at 37 min, 48% to 51% B from 40 to 70 min, 53% to 55% B from 75 to 78 min, 60% to 100% B from 81 to 85 min, maintained to 100% B from 90 to 95 min and returning to 15% B from 95 to 105 min. The flow rate was 0.5 mL/min and the volume injected was 20 µL. The compounds were monitored at 254 nm.

2.8. Characterization of microcapsules and tablets

2.8.1. Loading efficiency

To release all phenolic compounds included in the microcapsules and tablets, both formulations were macerated in 60° ethanol for 1 h with ultrasonic application (10 minutes, five times). Then, each one was centrifuged at 2,950 ×g for 5 min. The supernatants were gathered. Aliquots of the supernatant were taken for total phenolic compounds determination according to Costamagna et al., 2013. The loading efficiency (%) was determined as LE=100 x RPC/TPC, where LE: loading efficiency; RPC: released phenolic compounds; TPC: total phenolic compounds.

The phenolic compounds eluted were identified by HPLC-DAD and its chromatographic profile was compared with dry extract profiles. The HPLC system consisting 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) were used to analyze the extracts, microcapsules and tablets. An XbridgeTM 135 C18 column (4.6 x 150 mm, 5 µm; Waters Corporation, Milford, MA). The solvent system for the separation of components was composed of solvent A (0.1% acetic acid) and solvent B (0.1% methanol) (conditions: 10–57% B from 0 to 45 min and kept at 100% B from 45 to 60 min). The low rate was set at 0.5 mL/min. A solution of 2 mg DW/mL was used. Data collection was carried out with EmpowerTM 2 software. The identification of phenolic compounds 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 Fluka Chemical Corp. (USA). Experiments were performed in independent triplicates.

2.8.2. Evaluation of Physical Properties of the tablets

The weight, thickness, diameter, and hardness of 10 tablets of each formulation were measured. Hardness or tensile strength (TS) of the tablets was determined using a tablet hardness tester (VanKel 200, Benchsaver™ Series). TS was calculated as

\[ TS = \frac{2F}{\pi dt} \]

where \( F \) is the maximal diametrical crushing force, and \( d \) and \( t \) are the diameter and thickness of the tablet, respectively. The TS of each formulation was measured in triplicate. The disintegration test (DT) was performed according to the Real Spanish Pharmacopoeia, 1st edition, using a disintegration tester (Equipos Farmacéuticos®, Argentina) and purified water (as a solvent) at 37 °C. DT was defined as the interval required for the complete disappearance of a tablet or its particles from the tester net. DT was measured for six tablets of each formulation. The friability of the tablets was determined by a friability tester (Equipos Farmacéuticos®, Argentina) based on the Real Spanish Pharmacopoeia, 1st edition. These tablets were then accurately weighed (M1), placed in the drum of the machine, and rotated at 25 rpm for 100 times. Finally, the rotated tablets were accurately weighted (M2).

Friability (%) = \( \frac{(M1 - M2)}{M1} \times 100 \)

2.8.3. Morphological characterization of microcapsules and tablets

Morphological characterization of microcapsules and tablets was conducted by scanning electron microscopy (SEM) on a Hitachi microscope (S-4800). The samples were sputter-coated with a gold-palladium mixture prior to examination. Particle size distributions were obtained from the SEM micrographs from a minimum of 200 measurements per image in their original magnification using the FIJI software.

2.8.4. Fourier-transform infrared (FT-IR) analysis

Samples (ca. 1-2 mg) of the plant extracts as well as microcapsules and tablets containing the extracts were grounded and dispersed in about 130 mg of spectroscopic grade KBr. A pellet was then formed by compressing the samples at ca. 150 MPa. FT-IR spectra were collected in the
transmission mode using a Bruker (Rheinstetten, Germany) FT-IR Tensor 37 equipment. The spectra were obtained by averaging 10 scans at 1 cm$^{-1}$ resolution.

2.8.5. Swelling of microcapsules and vaginal tablets

The swelling ability of microcapsules and tablets was assessed by measuring their water uptake capacity following a method adapted from Bigucci et al., 2015. Samples (ca. 25 mg) were placed on circular pieces of filter paper (d = 50 mm), previously soaked with the swelling medium and placed on a cotton disc inside a Petri dish filled with 5 mL of the same medium. The swelling medium was made up of PBS adjusted with orthophosphoric acid to pH 4.5 to simulate vaginal conditions. At different time intervals, the filter paper plus the swollen microcapsules were weighed and then returned to the same Petri dish. Water uptake (WU) was calculated,

$$WU (\%) = \frac{W_t - W_0}{W_0} \times 100$$

where $W_0$ is the initial mass of dry microcapsules and $W_t$ is the mass of swollen microcapsules at time $t$. Experiments were performed in independent triplicates.

2.8.6. In-vitro release of extracts from microcapsules and tablets

The in-vitro release of the different plant extracts from chitosan microparticles was assessed following a method adapted from Gómez-Mascaraque, Lagarón, & López-Rubio, 2015. The microcapsules (5 mg/mL) were suspended in the same phosphate buffer used for the swelling assays (pH 4.5) and stored at 37ºC (i.e. physiological temperature). At different time intervals, dispersions were centrifuged at 2,950 × g and 37 ºC for 5 min using an Eppendorf 5804 R centrifuge (Hamburg, Germany). Aliquots (0.25 mL) of the supernatant were taken for sample analysis. The aliquot volume was then replaced by fresh release buffer. The extracted aliquots were analyzed by UV-Vis spectroscopy using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, USA) by measuring their absorbance at 280 nm. Calibration curves were previously obtained for the different extracts in the buffer solution ($R^2_{L.\divaricata} = 0.9958$, $R^2_{L.\cuneifolia} = 0.9998$, $R^2_{L.nitida} = 0.9996$, $R^2_{Z.punctata} = 0.9997$, $R^2_{T.andinum} = 0.9999$). As plant extracts are only partially soluble in aqueous buffer, calibration curves correlate the absorbance at 280 nm with the concentration of the soluble fraction extracted from each plant extract in the buffer. For comparison purposes, the release of the extracts was also evaluated from vaginal tablets. The tablets were introduced in the required volume of medium to achieve the same extract concentration used for microcapsules (i.e. 0.5 mg of extract/mL of release medium), and the
procedure previously described was followed. Experiments were performed in independent triplicates.

2.8.7. Release of phenolic compounds from microcapsules and tablets in simulated vaginal fluid

A medium was developed to simulate the fluid produced in the human vagina (das Neves et al., 2008). The simulated vaginal fluid (SVF) composition in g/L was as follows: NaCl, 3.51; KOH, 1.40; Ca(OH)_2, 0.222; bovine serum albumin, 0.018; lactic acid, 2.00; acetic acid, 1.00; glycerol, 0.16; urea, 0.4; glucose, 5.0 (das Neves et al., 2008). The mixture was adjusted to pH 4.2. The microcapsules and tablets were maintained at 37°C during 24 h in 9 mL of SVF since daily vaginal secretions were estimated in a range of 1–11 mL. Released TPC content was determined by the Folin–Ciocalteau method (Costamagna et al., 2013) at different time intervals. The relative percentage was calculated considering the phenolic compounds content of dry extracts used for the formulation of microcapsules and tablets as 100%. The phenolic compounds released were identified by HPLC-DAD. Experiments were performed in independent triplicates.

2.9. Biological activity determinations in extracts, microcapsules and tablets

To assess whether the polyphenols released from the microcapsules and the tablets during 24 h were active, the microcapsules and tablets were contacted with PBS and then centrifuged. The supernatant was freeze-dried and resuspended in 60º ethanol. The phenolic compounds content was determined to carry out the tests at the same concentration in which the extracts have activity (Moreno et al., 2018a, b).

2.9.1. Antimicrobial assays

Candida strain

Candida strains were provided by Instituto Nacional de Enfermedades Infecciosas-Administración Nacional de Laboratorios e Institutos de Salud (INEI-ANLIS) ‘Dr. Carlos G. Malbrán’, Buenos Aires, Argentina. Candida albicans (144783; 134333; 2089), C. glabrata (031646; 042030; 031982), C. tropicalis (1841), S. cerevisiae (134528; 134544; 124263), C. parapsilosis DMic 134410 and C. krusei DMic 134409 were used. All the microorganisms were maintained in brain–heart infusion containing 30% (v/v) glycerol at -20°C.

Inoculum preparation

The suspensions of each strain were transferred to Yeast Medium (malt extract 0.3%, yeast extract 0.3%, peptone 0.5%, glucose 1%, agar 2%) and maintained at 37°C during 24 h. Then, individual
colonies were isolated and suspended in 0.9% NaCl solution. The inocula were prepared by adjusting the suspension turbidity to match the 0.5 McFarland scale (1-5×10^6 cells/mL). The cell number was estimated using a serial dilution technique according to the recommendations of the M27-A3-S4 reference document of Clinical and Laboratory Standards Institute (CLSI, 2012) for each assay.

**MIC determination for yeast**

MIC values of dry extracts, microcapsules and tablets against *Candida* and *Saccharomyces* were determined by the macrodilution method (CLSI, 2012). The dry extracts (6.25–400 µg/mL) and the phenolic compounds eluted from microcapsules and tablets contained in a final volume of 1 mL were added to 9 mL of Sabouraud agar and then were put in Petri dishes. The inoculum (2µL) containing 0.5-2.5×10^3 CFU were seeded, and plates were aerobically incubated at 37°C. After incubation for 48 h, fungal growth was evidenced in each plate. The MIC (lowest concentration of extract without macroscopically visible growth) was determined.

**Microbiological control**

Tablets and microcapsules containing plant extracts were dissolved in sterile PBS at 37°C for 2h. Each sample aliquot was taken and spread with Drigalsky spatula in Petri dishes containing Mueller Hinton Medium (Britania) and Mold and Yeast Medium (Britania). The plates were incubated at 37°C and 28°C for 48 h, respectively. Sterility and solvent controls and controls of microcapsules and tablets without extracts were made. Microbial growth was analyzed after incubation.

2.9.2. Antioxidant activity

**Total antioxidant capacity assay**

The antioxidant capacity of the dry extracts, microcapsules and tablets (concentration range between 0.1 and 11 µg GAE/mL) was carried out by the improved ABTS radical cation (ABTS^+^) method as described by Costamagna et al., 2013. Results were expressed as the concentration of extract required to scavenge 50% of ABTS^+^ (SC_{50}). The negative control was performed with the vehicle (ethanol 60%). Butylated hydroxytoluene (BHT), quercetin and nordihydroguaiaretic acid (NDGA) were used as reference compounds.

**Protection against oxidative hemolysis**

The protection of oxidative hemolysis of red blood cells (RBC) by the dry extracts, microcapsules and tablets (concentration range between 0.1 and 2.3 µg GAE/mL) was determined.
according to Mendes, De Freitas, Baptista, & Carvalho, 2011, using azo compound solution [2,2’-Azobis(2-methylpropionamidine) dihydrochloride] (AAPH). The extent of hemolysis was quantified spectrophotometrically at 545 nm. Percentage of hemolysis was calculated and the IC$_{50}$ values were determined as the concentration needed to protect the RBC from oxidative hemolysis by 50%. For the 100% hemolysis control, ethanol 60° was used as solvent control instead of the extracts. BHT, quercetin and NDGA were used as reference compounds.

Statistical analyses
Analyses were conducted at least three times with three different samples. Each experimental value is expressed as the mean ± standard deviation (SD). The statistic software InfoStat (Student Version, 2011) was used to evaluate the significance of differences between groups. The one-way ANOVA with Tukey post-test at a confidence level of 95% was used for the comparisons between groups. The criterion of statistical significance was taken as $p \leq 0.05$.

3. Results and Discussion
3.1. Phytochemical analysis of dry extracts
Studies were performed with the dry hydroalcoholic extracts from the aerial parts of the five selected Argentine medicinal plants used in traditional medicine. Extracts contained a high level of total phenolic (TP between 354.7 to 397.9 mg GAE/g dry extract) and flavonoid phenolic compounds (FP between 201.6 to 260.4 mg GAE/g dry extract). The condensed tannin content ranged between 25.06 and 135.2 mg/g dry extract. Hydrolyzed tannin was detected in $Z$. punctata and $T$. andina. The results are summarized in Table 1.

In previous studies, it was determined the phytochemical composition of $Larrea$ species and $T$. andina by HPLC-ESI-MS/MS (Moreno et al., 2018a, b). The Tetraglochin extract was particularly rich in hydrolysable and condensed tannins, containing mainly mono-, di- and trigalloylhexosides, ellagitannins such as pedunculagin, gallo-ellagitannin, condensed tannins including (epi)catechin dimer and trimer as well as benzoic acids derived phenolics (Moreno et al., 2018a). The high tannin content agrees with the results obtained in the spectrophotometrical analysis (Table 1). Antioxidant, antimicrobial, anti-inflammatory, antitumor and hepatoprotective activities were previously demonstrated for ellagitannins (Moreno et al., 2018a). On the other hand, $Larrea$ species were richer in nordihydroguaiaretic acid (NDGA) and its derivatives, cyclolignans and epoxylignans, compounds with antiinflammatory, antitumoral, antifungal and antioxidant properties (Moreno et al., 2018b). The occurrence of chalcones in $Zuccagnia$ punctata was previously reported (Isla et al., 2016). In this paper, the profile of polyphenolic compounds in $Z$. punctata and $T$. andina was determined.
*punctata* was analyzed. Some 23 compounds were identified, including seven condensed tannins, seven flavonoid glycosides, one flavonoid, five phenolic acid derivatives and three chalcones (Table 2).

**Table 1.** Phytochemical composition of dry extracts from the selected Argentinean medicinal plants.

<table>
<thead>
<tr>
<th>Phytochemical content</th>
<th>L. cuneifolia</th>
<th>L. divaricata</th>
<th>L. nitida</th>
<th>Z. punctata</th>
<th>T. andina</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total free phenolics (mg GAE/g DW)</td>
<td>397.9±2.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>397.4±3.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>370.6±1.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>354.7±2.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>386.9±1.7&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Non-flavonoid phenolics (mg GAE/g DW)</td>
<td>157.5±5.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>162.6±6.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>169.0±10.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>128.9±12.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>126.5±2.3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Flavonoid phenolics (mg GAE/g DW)</td>
<td>240.4±7.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>234.8±9.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>201.6±11.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>225.8±15.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>260.4±4.0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Flavone and flavanone (mg QE/g DW)</td>
<td>28.5±1.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>27.9±2.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>23.5±1.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>74.9±4.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>16.1±1.2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Condensed tannins (mg PB2E/DW)</td>
<td>39.5±2.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>25.0±0.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>33.1±0.4&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>135.2±7.0&lt;sup&gt;d&lt;/sup&gt;</td>
<td>60.3±3.5&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Hydrolyzable tannins (mg GAE/g DW)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>6.6±0.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>26.3±0.2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

GAE: gallic acid equivalents, QE: quercetin equivalents, PB2E: procyanidin B2 equivalents; DW: dry weight; Values are reported as mean ± standard deviation of triplicates. Different letters in the same line indicated significant differences in the content of the phytochemical components according to Tukey’s test (p ≤ 0.05). ND: not detected.

**Table 2.** Identification of *Zuccagnia punctata* phenolics by HPLC-MS-MS in the negative ion mode

<table>
<thead>
<tr>
<th>Compound</th>
<th>Rt (min)</th>
<th>[M-H]</th>
<th>MS/MS</th>
<th>Identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.7</td>
<td>191</td>
<td>172 (80), 126 (100), 110 (44), 92 (86), 84 (92)</td>
<td>Quinic acid</td>
</tr>
<tr>
<td>2</td>
<td>7.9</td>
<td>343</td>
<td>191 (100)</td>
<td>Galloylquinic acid</td>
</tr>
<tr>
<td>3</td>
<td>10.6</td>
<td>577</td>
<td>559 (24), 451 (73), 425 (100), 407 (19), 289 (16)</td>
<td>Catechin dimer</td>
</tr>
<tr>
<td>4</td>
<td>11.2</td>
<td>495</td>
<td>343 (100), 191 (6)</td>
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</tr>
<tr>
<td>5</td>
<td>12.0</td>
<td>577</td>
<td>559 (19), 451 (70), 425 (100), 407 (14), 289 (8)</td>
<td>Catechin dimer</td>
</tr>
<tr>
<td>6</td>
<td>12.3</td>
<td>865</td>
<td>739 (26), 713 (26), 695 (68), 577 (100)</td>
<td>Catechin trimer</td>
</tr>
<tr>
<td>7</td>
<td>13.8</td>
<td>289</td>
<td>245 (100)</td>
<td>Catechin</td>
</tr>
<tr>
<td>8</td>
<td>15.5-15.8</td>
<td>729</td>
<td>577 (100)</td>
<td>Catechin dimer gallate</td>
</tr>
<tr>
<td>9</td>
<td>22.4</td>
<td>609</td>
<td>301 (100)</td>
<td>Q-rutinoside</td>
</tr>
<tr>
<td>10</td>
<td>23.7</td>
<td>761</td>
<td>609 (18), 459 (18), 301 (100)</td>
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<td>11</td>
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<td>Q-hexoside</td>
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<tr>
<td>12</td>
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<td>615</td>
<td>301 (100)</td>
<td>Q-hexoside gallate</td>
</tr>
<tr>
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<td>30.0-31.1</td>
<td>585</td>
<td>301</td>
<td>Q-derivative</td>
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<tr>
<td>14</td>
<td>34.0-35.0</td>
<td>419</td>
<td>257 (31), 239 (66), 213 (100)</td>
<td>Trihydroxydihydrochalcone hexoside</td>
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<tr>
<td>15</td>
<td>44.0</td>
<td>579</td>
<td>271 (100)</td>
<td>Naringenin rutinoside</td>
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<tr>
<td>16</td>
<td>46.0-46.4</td>
<td>561</td>
<td>269 (100), 213</td>
<td>Galangin dirhamnoside</td>
</tr>
<tr>
<td>17</td>
<td>47.9-48.5</td>
<td>343</td>
<td>179 (100)</td>
<td>1-Methyl-3-(3′,4′-dihydroxyphenyl)-propyl caffeate</td>
</tr>
<tr>
<td>18</td>
<td>52.6-53.2</td>
<td>327</td>
<td>179 (100), 135 (14)</td>
<td>1-Methyl-3-(4′-hydroxyphenyl)-propyl caffeate</td>
</tr>
<tr>
<td>19</td>
<td>55.3</td>
<td>255</td>
<td>227 (23), 213 (29), 211 (31)</td>
<td>Pinocembrin</td>
</tr>
</tbody>
</table>
| 20       | 59.8    | 311   | 163 (100) | 1-Methyl-3-(3′,4′-dihydroxyphenyl)-
Table 1. Mass data of subfractions identified in the Zuccagnia punctata extract.

<table>
<thead>
<tr>
<th>Extract Subfraction</th>
<th>Mass (m/z)</th>
<th>Intensity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>21</td>
<td>66.8</td>
<td>241</td>
</tr>
<tr>
<td></td>
<td></td>
<td>197 (42), 149 (48), 109 (33)</td>
</tr>
<tr>
<td>22</td>
<td>68.5-70.2</td>
<td>239</td>
</tr>
<tr>
<td></td>
<td></td>
<td>197(92), 153(10), 135(26)</td>
</tr>
<tr>
<td>23</td>
<td>73.0-73.9</td>
<td>311</td>
</tr>
<tr>
<td></td>
<td></td>
<td>179(100), 135(4)</td>
</tr>
</tbody>
</table>

propyl coumarate
2',4'-Dihydroxydihydrochalcone
2',4'-Dihydroxchalcone
1-Methyl-3-(phenyl)-propyl caffeate

Figure 2. HPLC-MS chromatogram of *Zuccagnia punctata* extract. Detection: all MS, negative ion mode.

3.2. Physical and morphological Characterization of Tablets.

Bioadhesive tablet formulations of ketoconazole for vaginal delivery based on Carbopol 934P, in combination with HPMC at different ratios, sodium bicarbonate and citric acid were formulated and assayed previously by other authors (Wang & Tang, 2008). The design of bioadhesive tablets for vaginal delivery using Carbopol 934P, in combination with HPMC and an effervescent mixture of sodium bicarbonate and citric acid to promote disintegration and delivery of medicinal plants from Argentina was described for the first time in this paper. The tablets formulated had an average weight of 272.5±10 mg, 12±0.1 mm diameter and 2.0±0.1 mm thickness. They showed a loading efficiency between 80 to 90% in all cases. However, extract accumulations could be visually observed in the surface of vaginal tablets, suggesting that the miscibility of the extracts with the tablet ingredients was low. All tablets showed adequate disintegration properties (DT between 20±4 min). The TS values were around of 1.34 ± 0.13 MPa. The friability percentages were low, between 0-0.71%. These values are acceptable according to the European Pharmacopoeia, United States Pharmacopoeia and Argentine Pharmacopeia. According to our results all loaded inserts, could be handled without damage during vaginal application. The presence of extracts in the tablets produced a rough and less porous surface rather than smooth as unloaded tablets. SEM images showed that the particles in all tablets containing plant extracts were non-uniform with rod-shape and round-shape (Figure 3).
3.3. Morphological characterization of the microcapsules and loading efficiency

The electrospraying technique has previously yielded smaller particles than other common encapsulation techniques such as spray-drying despite requiring higher polymer concentrations for processing (Gómez-Mascaraque & López-Rubio, 2016). The morphology of the chitosan-encapsulated extracts obtained by electrospraying is shown in Figure 4. The microcapsules structures containing different extracts exhibited by SEM a similar morphology to that previously observed for unloaded and (−)-epigallocatechin gallate-loaded chitosan particles prepared using the same electrospraying conditions (Gómez-Mascaraque et al., 2016). In this work, extracts containing complex polyphenols mixtures were encapsulated, which probably produced less homogeneous microcapsules, as deduced from the particle size distribution graphs which showed a much greater variation in capsule diameter than that observed when the pure polyphenolic compound was encapsulated. Depending on the extract incorporated, variations in size and morphology of the capsules were observed. Smaller particles were obtained for L. cuneifolia extract. As the encapsulation matrix and processing parameters were the same in all cases, the differences could be attributed to changes in the chitosan solution properties upon addition of the extracts (Chakraborty, Liao, Adler, & Leong, 2009). In all cases a loading efficiency was between 75 and 90%. The chromatography profile of each of them was similar to dry extracts of each plant species used in this study (profiles not shown).
**Figure 4.** SEM images and particle size distribution graphs of chitosan nano- and microparticles containing extracts from *Zuccagnia punctata* (a), *Tetraglochin andina* (b), *Larrea cuneifolia* (c), *L. divaricata* (d) and *L. nitida* (e). Scale bars correspond to 2 µm.

### 3.4. Fourier transform-infrared (FT-IR) analysis of microcapsules and tablets

The FT-IR spectra of the *L. divaricata* extract in its free form and encapsulated within electrosprayed chitosan microstructures, together with the unloaded chitosan microcapsules is shown in Figure 5A. The FT-IR spectra of all other extracts are shown as Supplementary Material. The FT-IR spectrum of electrosprayed chitosan showed a broad band with a maximum at 3400 cm$^{-1}$, attributed to the –OH and –NH stretching vibrations. Other characteristic bands of this polysaccharide were observed at 2929 cm$^{-1}$ (C–H bonds stretching), 1633 cm$^{-1}$ (amide I, C=O stretching), 1560 cm$^{-1}$ (–NH$_2$ bending), 1410 cm$^{-1}$ (–CH$_3$ bending) and 1078 cm$^{-1}$ (C–O stretching of sugar rings) (Azevedo, Santha Mariappan, & Kumar, 2012; Gómez-Mascaraque, Méndez, Fernández-Gutiérrez, Vázquez, & San Román, 2014). The spectra of the plant extracts were characterized by the presence of absorption bands at 3395 cm$^{-1}$, 2960-2930 cm$^{-1}$, 1730-1700 cm$^{-1}$ and in the 1600–800 cm$^{-1}$ region attributed to the stretching, bending and deformation vibrations of polyphenolic compounds (Sivam, Sun-Waterhouse, Perera, & Waterhouse, 2012).

The incorporation of plant extracts within the chitosan particles caused some changes in the relative absorbance of the characteristic bands of chitosan, due to the contribution of the extracts to the infrared spectra. In all cases, a decrease in the relative intensity of the bands attributed to the –OH and –NH stretching band at 3400 cm$^{-1}$ and the amide I and –NH$_2$ bending bands at 1633 and 1560 cm$^{-1}$, respectively, was observed. This could also point out to interactions between the chitosan matrix and the polyphenolic extracts, which would be mainly mediated by hydrogen bonding or hydrophobic interactions, as generally accepted for polysaccharides-polyphenols interactions (Gómez-Mascaraque et al., 2017; LeBourvellec et al., 2012; 2004). A shift towards lower wavenumbers of the C-O stretching band in the sugar rings suggested a change in the polymer conformation upon incorporation of the extracts, suggesting intermolecular interactions between the polysaccharide and the extracts. Figure 5B shows the FT-IR spectra of the placebo tablets and the tablets containing *L. divaricata* extract as a representative example. The spectra of the tablets containing the other extracts are included as Supplementary Material. Given the complex composition of the extracts, it is difficult to unambiguously describe the spectral bands of each component so broad bands observed represent the contributions of various compounds.
3.5. Swelling of microcapsules and tablets

The water uptake (WU) capacity of encapsulated plant extracts in simulated vaginal fluid was calculated according to Eq. (1). The obtained profiles are shown in Figure 6A. All chitosan-based encapsulation structures presented a fast swelling upon contact with the aqueous buffer due to the hydrophilic nature of the polysaccharide, reaching WU values as high as 80-90% (i.e. water accounted for up to 80-90% of the total weight of the hydrated microcapsules). Small differences were observed among the samples, suggesting that the extent to which the incorporation of extracts affected the polymer conformation (see previous section) and the extent of interactions between matrix and polyphenol-rich extracts varied among the different extracts, having an impact on the swelling capacity of the chitosan matrix.

A)
Figure 6. Swelling profiles in PBS buffer (pH = 4.5) of the extract-loaded and unloaded: A) microcapsules and B) tablets.
The unloaded chitosan microcapsules were also evaluated for comparison purposes. While the WU capacity of the extract-loaded samples remained almost constant after the initial rapid swelling, the values of the WU start to decrease after 1h for unloaded chitosan, as the weight of the hydrated microcapsules was found to decrease (Fig. 6A). A similar weight loss had been previously observed for chitosan-based hydrogels during swelling experiments and was attributed to the dissolution of a fraction of chitosan molecules in the buffer (Azevedo et al., 2012). Hence, the presence of the different plant extracts within the chitosan matrix was found to stabilize the microhydrogels formed thereof, delaying the dissolution of the polysaccharide (a lower weight loss was observed in the extract-containing microcapsules as observed in the inset from Figure 6A). The stabilization of carbohydrate matrices by polyphenols has been described and attributed to the presence of physical interactions between both components (Floriano-Sánchez et al., 2006). It is worth noting that, even though chitosan is soluble in acidic media, extract-loaded microcapsules could not be completely dissolved in acidic buffer (results not shown), corroborating the previous hypothesis.

In the case of the tablets (Figure 6B), swelling was much more gradual considering the different specific surface areas of both delivery vehicles (being much higher in the case of the microcapsules) and the fact that the material was compacted in a press, producing a denser material with slower water diffusion properties.

3.6. In-vitro release of plant extracts from microcapsules and tablets in a buffer system and in simulated vaginal fluid (SVF)

The release of plant extracts from electrosprayed chitosan microcapsules in aqueous buffer (PBS, pH = 4.5) was studied and compared with the release of the same plant extracts from conventional vaginal tablets. The amount of released extract was significantly higher from chitosan electrosprayed microcapsules than from tablets for all assayed plant extracts. In chitosan microcapsules, a very fast release of phenolic compounds in the first 4-5 hours was observed, which is the typical behavior of the release from hydrophilic matrices, due to the fast matrix swelling upon contact with an aqueous release medium (Atay et al., 2017). The level of released phenolic compounds from conventional vaginal tablets until 5 h was much lower than for microcapsules. The dispersion of the extracts was considerably better in electrosprayed microcapsules than in tablets, explained by the different preparation method, which affected their exposure to the release medium, especially due to the larger surface area of microparticles. Both the solvent used for preparing encapsulation solutions and the interweaving of polysaccharide chains with the polyphenol molecules must have facilitated their subsequent release in the aqueous
medium upon hydration of chitosan microcapsules, leading to a very fast release in the first 4-5 hours. In contrast, extract accumulations could be visually observed in conventional vaginal tablets, suggesting a poor miscibility of the extracts with the tablet ingredients. These extract clusters remained insoluble even after the disintegration of the tablets (they could still be visually observed in the release suspension). In brief, the encapsulation of extracts within chitosan electrosprayed particles increased their solubility (and thus their release) in aqueous medium compared to their formulation in traditional vaginal tablets in the first hours. However, after 24 h, 100% of the extract incorporated was released from the tablets except for *T. andina* (80%).

It is interesting to note that the release from the chitosan microcapsules was higher than expected, probably due to the previous solubility of the extracts in the acid media which apparently made some of the phenolic compounds more accessible in the release media. In fact, it has been previously reported that the properties of chitosan promote a closer contact between the phytopharmaceutical form and the vaginal tissue, thus increasing the residence time of bioactive compounds at the site of administration (Albertini et al., 2009; Martin-Villena et al., 2013; Caramella et al., 2015).

To predict the content of phenolic compounds released from microcapsules and tablets using *in-vitro* conditions which mimicked the *in-vivo* scenario, both vaginal formulations were also included in SVF for 24 h. Under our experimental conditions, the released total phenolic percentages were in all cases in the range of 90 to 100% for microcapsules and tablets (Figure 7 A and B). Our results have shown a good release of bioactive compounds in SVF for all assayed plant extracts. HPLC-DAD was used to analyze the individual phenolic compounds from polyphenols released from the microcapsules and tablets, to ascertain whether encapsulation or the inclusion in tablets affected their stability. The chromatography profile of each of them was similar to dry extracts of each plant species used in this study (profiles not shown).
Figure 7. Released phenolic percentages in simulated vaginal fluid from A) microcapsules and B) tablets.
3.7. Biological activities from plant extracts released from microcapsules and tablets

3.7.1. Antifungal activity

Adequate treatment of fungal infections such as candidiasis is difficult as the fungi are eukaryotic organisms with a structure and metabolism like that of eukaryotic host. Furthermore, long-term treatments with commonly used antifungal drugs, such as polyenes and azoles, have toxic effects and result in strain resistance. For this reason, it is necessary to find new natural products with antifungal activity. The antifungal activity of dry extracts of five medicinal plants used in Argentina as antifungal agents, either alone or incorporated within microcapsules and tablets, was assayed in vitro against 10 yeast strains obtained from vaginal exudates of patients with yeast infection. They included three strains of *Saccharomyces cerevisiae*, three strains of *C. albicans*, three strains of *C. glabrata* and one strain of *C. tropicalis*. Some *S. cerevisiae* and *C. albicans* and non-*albicans* strains were azole-susceptible (85%) or resistant (15%). The *Candida* and *Saccharomyces* species were also susceptible to nystatin and amphotericin B. Dry extracts and the extracts eluted from microcapsules and tablets were effective against *C. albicans* and non-*albicans* in macrodilutions assays. The MIC values on *C. albicans* of phenolic compounds eluted from microcapsules and tablets containing *Z. punctata*, *L. divaricata* and *L. nitida* extracts were lower than MIC values of the ones containing *L. cuneifolia*, and *T. andina* (Table 3). The MIC values of phenolic compounds eluted from microcapsules and tablets were like the MIC values of the corresponding dry extract used to obtain vaginal formulations. The highest activity of *Z. punctata* microcapsules and tablets on *Candida* species could be attributed to the content of 2′,4′-dihydroxychalcone and 2′,4′-dihydroxy-3′-methoxychalcone, two compounds with demonstrated antifungal activities (Isla et al., 2016; Nuño et al., 2014). These compounds were also effective inhibitors in biofilm formation as well as on pre-formed *Candida* biofilm and yeast germ tube formation. Furthermore, chalcones can inhibit exoenzymes, which are responsible for the invasion mechanisms of *Candida*. All these effects could moderate colonization, thereby suppressing the pathogen invasive potential (Nuño et al., 2014). All the formulations obtained from *L. divaricata* were more active on *S. cerevisiae* than the ones from *T. andina*. The products with *T. andina* contained a higher level of hydrolysed tannins of type of pedunculangins and casuarins (Moreno et al., 2018a). The hydrolysed tannins are compounds with demonstrated anti *Candida* activity. There are some evidences that the mentioned compounds affect fungal cells through interaction with the lipid bilayer of the cell membrane. They could act by disrupting the membrane integrity or by intercalation into cell wall (Martins et al., 2015; Sieniawska & Baj, 2017). Furthermore, the tablets and microcapsules with *Larrea* species mainly contained lignans (Moreno et al., 2018b).
Our results are consistent with powerful fungicidal activity in all cases, both in the extracts and in the phyto-formulations. Tangarife-Castaño et al. (2011) suggested a classification system for antifungal activity in plant derivatives based on MIC values as strong inhibitors (MIC of < 0.5 mg/mL); moderate inhibitors (MIC of 0.6-1.5 mg/mL); and weak inhibitors (MIC of > 1.6 mg/mL). Therefore, Zp, Ln, Lc, Ld and Ta extracts as well as their phytoformulations could be considered as strong natural antifungals.
Table 3. Effect of dry extracts (E), extract released from the microcapsules (M) and tablets (T) on yeast isolated from clinical vaginal infections in humans.

<table>
<thead>
<tr>
<th>Yeast</th>
<th>Collection number</th>
<th>L. cuneifolia MIC (µg GAE/mL)</th>
<th>L. divaricata MIC (µg GAE/mL)</th>
<th>L. nitida MIC (µg GAE/mL)</th>
<th>Z. punctata MIC (µg GAE/mL)</th>
<th>T. andina MIC (µg GAE/mL)</th>
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</thead>
<tbody>
<tr>
<td>C. albicans</td>
<td>144783</td>
<td>400</td>
<td>400</td>
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<td>C. albicans</td>
<td>134333</td>
<td>400</td>
<td>400</td>
<td>400</td>
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<td>C. albicans</td>
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<td>400</td>
<td>400</td>
<td>400</td>
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<td>100</td>
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<td>C. glabrata</td>
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<td>400</td>
<td>400</td>
<td>400</td>
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<td>100</td>
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<td>042030</td>
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</tbody>
</table>

MIC values represent growth inhibition as compared with control growth. E: extract; M: microcapsules; T: tablets.
3.7.2. Antioxidant capacity

*Candida*-vaginal epithelial cell interactions in vaginal infections promote the liberation of free radicals and a local inflammatory response that results in mucosal damage (Fisher, 2012). For this reason, a treatment with drugs with multiple effects, anti-*Candida*, antioxidant and anti-inflammatory can be more effective. The antioxidant activity of *L. cuneifolia*, *L. divaricata*, *L. nitida*, and *T. andina* extracts in ethanol 60° in free cell systems was previously reported (Moreno et al., 2018a, b). In this work, the antioxidant activity of extracts was confirmed and the antioxidant activity of *Z. punctata* extract, other Argentina medicinal plant, was assayed in a free cell system and a cell system. The antioxidant capacity of polyphenolic compounds enriched extracts was compared with the activity of released phenolics from microcapsules and tablets containing the medicinal plant extracts. The *T. andina* extract was more active as ABTS$^{•+}$ scavenger than *L. divaricata*, followed by *Z. punctata*, *L. cuneifolia*, and *L. nitida* with SC$_{50}$ values of 1.68±0.2; 2.68±0.10; 3.2±0.2; 4.10±0.2 and 4.50±0.3 µg GAE/mL, respectively (Table 4). The antioxidant activity of *T. andina* ethanolic extract could be attributed to the content of caffeoyl quinic acid, HHDP, bis HDDP hexosides (pedunculangins) and galloyl HHDP hexosides (casuarins) (Sieniawska & Baj, 2017).

In the assay of the oxidative hemolysis inhibition, *Z. punctata* exhibited a stronger inhibitory effect on lipoperoxidation of red blood cells followed by *L. cuneifolia*, *L. nitida*, *L. divaricata* and *T. andina*, with IC$_{50}$ values of 0.05±0.004; 0.12±0.01; 0.21±0.01; 0.22±0.02; 0.23±0.01 µg GAE/mL. The phytoformulations were able not only to reduce ABTS but also to prevent and limit the release of lysosomal enzymes from human red blood cell to the extracellular matrix indicating that they could be able to prevent damage on neutrophils membrane reducing the inflammatory response with similar potency to the medicinal extracts. The antioxidant capacity in all cases was higher than those of commercial natural and synthetic antioxidant compounds used in pharmaceutical products (Table 4). It has been reported that phenolic compounds were able to bind covalently to membrane proteins and by hydrogen bonding with the polar head groups of phospholipids, resulting in the protection of erythrocyte (Bonarska-Kujawa et al. (2012, 2015). This interaction provides a certain level of protection for erythrocyte hemolysis and its membrane skeletal damage against free radicals.
Table 4. Antioxidant activities of dry extract, microcapsules and tablets containing dry extracts and commercial reference drugs.

<table>
<thead>
<tr>
<th>Plant Species</th>
<th>CD&lt;sub&gt;50&lt;/sub&gt; ABTS&lt;sup&gt;•⁺&lt;/sup&gt; (µg/ml)</th>
<th>CI&lt;sub&gt;50&lt;/sub&gt; AAPH (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Larrea cuneifolia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>4.10±0.20&lt;sup&gt;f,g,h&lt;/sup&gt;</td>
<td>0.12±0.01&lt;sup&gt;a,b&lt;/sup&gt;</td>
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<tr>
<td>M</td>
<td>4.20±0.20&lt;sup&gt;f,g,h&lt;/sup&gt;</td>
<td>0.13±0.01&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
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<tr>
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<td>0.14±0.01&lt;sup&gt;a,b,c,d&lt;/sup&gt;</td>
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<tr>
<td>Larrea divaricata</td>
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<tr>
<td>E</td>
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<td>0.22±0.02&lt;sup&gt;b,c,d,e&lt;/sup&gt;</td>
</tr>
<tr>
<td>M</td>
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<td>0.24±0.02&lt;sup&gt;d,e&lt;/sup&gt;</td>
</tr>
<tr>
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<td>0.23±0.01&lt;sup&gt;c,d,e&lt;/sup&gt;</td>
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<td>Larrea nitida</td>
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<td>0.21±0.01&lt;sup&gt;b,c,d,e&lt;/sup&gt;</td>
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<td>0.26±0.02&lt;sup&gt;e&lt;/sup&gt;</td>
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<td>T</td>
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<td>0.23±0.01&lt;sup&gt;c,d,e&lt;/sup&gt;</td>
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<tr>
<td>Zuccagnia punctata</td>
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<tr>
<td>E</td>
<td>3.20±0.2&lt;sup&gt;c,d,e&lt;/sup&gt;</td>
<td>0.05±0.004&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>M</td>
<td>3.80±0.3&lt;sup&gt;e,f,g&lt;/sup&gt;</td>
<td>0.09±0.003&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>T</td>
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<td>0.08±0.003&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>E</td>
<td>1.68±0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.23±0.01&lt;sup&gt;c,d,e&lt;/sup&gt;</td>
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<tr>
<td>M</td>
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<td>0.29±0.02&lt;sup&gt;e&lt;/sup&gt;</td>
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<tr>
<td>T</td>
<td>1.90±0.3&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>0.28±0.01&lt;sup&gt;e&lt;/sup&gt;</td>
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<td>Reference compounds</td>
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<tr>
<td>BHT</td>
<td>3.50 ±0.20&lt;sup&gt;d,e,f&lt;/sup&gt;</td>
<td>1.20 ± 0.10&lt;sup&gt;e&lt;/sup&gt;</td>
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<tr>
<td>Quercetin</td>
<td>1.40 ± 0.10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.90 ± 0.08&lt;sup&gt;f&lt;/sup&gt;</td>
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<tr>
<td>NDGA</td>
<td>2.60 ±0.20&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>1.12 ± 0.05&lt;sup&gt;e&lt;/sup&gt;</td>
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</table>

E: extract; M: microcapsules; T: tablets. BHT: butylated hydroxytoluene; NDGA: Nordihydroguaiaretic acid. Values are reported as mean ± standard deviation of triplicates. Different letters in the same column for each plant species indicated significant differences in the antioxidant activity according to Tukey's test (p ≤ 0.05).

Conclusions

This work demonstrates that the dry extracts of *L. cuneifolia*, *L. divaricata*, *L. nitida*, *Z. punctata* and *T. andina* could be used to treat vulvovaginal candidiasis as they have shown strong antifungal and antioxidant capacity. The development of chitosan
microcapsules and tablets as vaginal formulations containing the plant extracts, did not affect the biological properties of the extracts. A different release rate of bioactive compounds from microcapsules and tablets was observed in the first hours, being faster in chitosan microcapsules. This fact could be attributed to the higher specific surface of the electrosprayed microcapsules. Moreover, the solubility of the selected extracts was favored by microcapsule formation, thus potentially increasing the bioavailability of the active compounds in the vaginal environment. The encapsulation of the whole dry extracts could be a practical and more economical way than isolated bioactive compounds encapsulation.

Conflict of interest
The authors declare they have no conflict of interest.

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