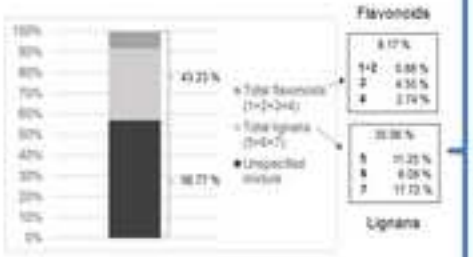
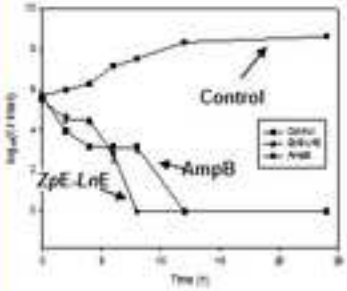
Graphical Abstract

***Zuccagnia punctata* exudate/ *Larrea nitida* exudate (*Zp*E-*Ln*E) synergistic anti- *C. albicans* fungicide combination**



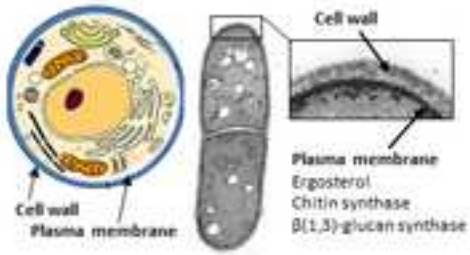
	Alone	Combination	DRI
*Zp*E MIC (µg/ml)	70	19	~4
*Ln*E MIC (µg/ml)	136	47	~3
*Zp*E MFC (µg/ml)	140	38	~4
*Ln*E MFC (µg/ml)	272	94	~3

Fungicidal action against *C. albicans*



Modes of action

Inhibition of fungal cell targets



- Inhibition of ergosterol biosynthesis
X Binding to membrane ergosterol
X Inhibition of membrane chitin synthase and β(1,3)-glucan synthase
X Inhibition of cell wall synthesis

↓

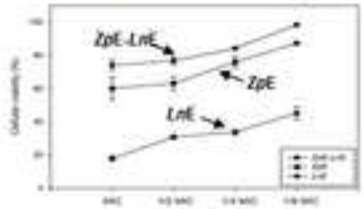
Fungal cell death

Inhibition of virulence factors

X Adhesion to eukaryote cells
X Secretion of phospholipases
X Secretion of proteinases
- Germ tube formation
- Haemolysins

Toxicity

Huh7 cells viability



Approaches to the mechanism of antifungal activity of *Zuccagnia punctata-Larrea nitida* bi-herbal combination

Estefania Butassi[1], Laura A. Svetaz[1], Maximiliano A. Sortino[1,2], Ariel D. Quiroga[3,4], Vanessa S. D. Carvalho[5], Juan C. G. Cortés[5], Juan C. Ribas[5], Susana A. Zacchino[1,*]

[1]Área Farmacognosia, [2]Area Micología, [3]Area Morfología, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario (UNR), [4]Instituto de Fisiología Experimental (IFISE), Consejo de Investigaciones Científicas y Técnicas (CONICET-UNR), Suipacha 531, 2000 Rosario, Argentina; [5]Instituto de Biología Funcional y Genómica, Consejo Superior de Investigaciones Científicas (CSIC)/Universidad de Salamanca, 37007 Salamanca, España

Corresponding author: Susana A. Zacchino
E-mail: szaabgil@gmail.com
Tel. +54 341-4375315

Abstract

Background

Anti-*Candida* synergism studies of four *Zuccagnia punctata-Larrea nitida* (*Zp*E-*Ln*E) bi-herbal combinations with the statistical-based MixLow method, allowed the finding of the most anti-*C. albicans* synergistic mixture, whose composition was previously quantified by a valid method, according to European Medicines Agency (EMA). These previous findings constitute an important basis for the continuation of the studies on this combination, with the aim of developing in the future a new bi-herbal combination.

Purpose

The purpose of this research was to advance in the study of the fungicidal properties, mechanism of action as well as the cytotoxic properties of the *Zp*E-*Ln*E combination.

Materials and methods

Minimum Fungicidal Concentration (MFC) of *Zp*E-*Ln*E was assessed with the microbroth dilution method of the Clinical and Laboratory Standard Institute (CLSI) and the rate of killing (time-kill) was determined by counting the colonies in the range 0-24 h. For studies of mechanisms of action, morphological studies using confocal and fluorescence microscopy on the yeast model *Schizosaccharomyces pombe* were performed. Then studies that target the fungal membrane (exogenous ergosterol assay and quantification of ergosterol) and the fungal cell wall (cellular sorbitol assay and enzymatic β1,3-D-glucan synthase (GS) and chitin synthase (ChS) assays) were conducted. In addition, the capacity of *Zp*E-*Ln*E for inhibiting *Candida* virulence factors such as adherence to buccal epithelial cells (BECs), germ tube inhibition, and inhibition of

phospholipases, proteinases and haemolysins secretion, were assessed with previously reported methods. Finally the effect of *Zp*E-*Ln*E and each *Zp*E or *Ln*E alone on cell viability against human hepatoma cell line Huh7 was determined. Cells were treated with different concentrations of *Zp*E, *Ln*E and *Zp*E-*Ln*E for 24 h, and the cell viability was determined with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide MTT assay.

Results

*Zp*E-*Ln*E was fungicidal rather than fungistatic, killing *C. albicans* at MFC = 2x MIC (Minimum Inhibitory Concentration) after 8 h. *S. pombe* cells treated with *Zp*E-*Ln*E at 1/2x MIC showed scorched, vacuolized, swollen and deformed cells in phase contrast images compared to the control cells without *Zp*E-*Ln*E, likely due to a damage in the plasma membrane or in the cell wall. In addition, *Zp*E-*Ln*E showed to act by binding to the ergosterol of the fungal membrane in the exogenous ergosterol assay, but not by inhibiting any step of the ergosterol biosynthesis (demonstrated by not decreasing the total ergosterol content). With respect to the interaction of *Zp*E-*Ln*E with the fungal cell wall, *Zp*E-*Ln*E did not show a higher MIC in the presence of the osmotic stabilizer sorbitol and showed a low or moderate capacity of inhibiting the GS and ChS enzymatic activities respectively, compared to the inhibitory capacity of papulacandin B (PapB) and nikkomycin Z (NikZ) respectively. Therefore, the *Zp*E-*Ln*E combination alters the plasma membrane structure and inhibits the cell-wall synthesis. Regarding *Zp*E-*Ln*E's effect on virulence factors, the combination significantly decreased the capacity of adhesion of fungal cells to eukaryotic BECs, although it did not significantly inhibit the germ tube formation up to 4x MIC. In addition, *Zp*E-*Ln*E completely inhibited the secretion of phospholipases and proteinases but not of haemolysins. The studies of toxicity of the *Zp*E-*Ln*E combination on Huh7 cells demonstrated that *Zp*E-*Ln*E possess very low toxicity, which showed to be much lower than that of the extracts alone.

Conclusions

The most synergistic combination *Zp*E-*Ln*E showed to be fungicidal and kill *C. albicans* in a shorter time than amphotericin B (AmpB). Regarding its mode of action, *Zp*E-*Ln*E appeared to act by binding to the ergosterol and altering the fungal membrane but not by inhibiting some steps of the ergosterol biosynthesis. Also cellular and enzymatic assays suggested that the combination would act simultaneously by inhibiting the synthesis or assembly of the polymers of the fungal wall. In addition, the results demonstrated that *Zp*E-*Ln*E would play a role in the virulence of *C. albicans* such as inhibiting the adhesion of fungal cells to eukaryotic BECs and the secretion of phospholipases and proteinases. However, it did not significantly inhibit neither the germ tube formation nor the haemolysins secretion. Interesting enough, *Zp*E-*Ln*E possessed very low toxicity. The

fungicidal properties of *Zp*E-*Ln*E against *C. albicans*, added to the evidences of the mode of action and its low toxicity add important information for the development of this combination as a new antifungal bi-Herbal Medicinal Product containing Argentinean plants. It would be a good candidate for replacing the toxic commercial antifungal agents and due to the easy availability of both plant species, the combination could be used mainly by the low-income population.

Keywords

Mode of antifungal action; *Zuccagnia punctata-Larrea nitida* combination; fungal cell-wall; fungal membrane; virulence factors; synergism.

Abbreviations

Ad: Additivism (or no interaction)
AmpB: Amphotericin B
An: Antagonism
BECs: Buccal Epithelial Cells
CCC = Centro de Referencia en Micología (CEREMIC, Rosario, Argentina)
CFU: Colony-forming unit
ChS: Chitin synthase
CLSI: Clinical and Laboratory Standards Institute
DCM: dichloromethane
DNDGA: 3´-deoxy-nordihydroguiaretic acid
DRI: Dose-Reduction Index
EMA: European Medicines Agency
GlcNAc: N-acetylglucosamine
GS: β(1,3)-glucan synthase
HPLC-DAD: High Resolution Liquid Chromatography-Diode Array Detection
Itra: Itraconazole
*Ln*E: *Larrea nitida* DCM exudate
Lϕ: Loewe index
Φ: Affected fraction or inhibition percentages
MES: 2-[N-morpholino]ethanesulfonic acid
MFC: Minimum Fungicidal Concentration
MIC: Minimum Inhibitory Concentration
MNDGA: 3´-Methyl-nordihydroguiaretic acid
MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NDGA: Nordihydroguiaretic acid

NikZ: Nikkomycin Z

PapB: Papulacandin B

PBS: Phosphate Buffered Saline

Pz: Precipitation or halo zone

Pz index: diameter of the colony / total diameter of colony plus Pz

S: Synergism

SDA: Sabouraud-dextrose agar

SDB: Sabouraud-dextrose broth

TCA: Trichloroacetic acid

*Zp*E: *Zuccagnia punctata* DCM exudate

*Zp*E-*Ln*E: Most synergistic bi-herbal combination of *Zp*E and *Ln*E

INTRODUCTION

The use of bi-herbal combinations for the treatment of a disease is a common practice in traditional medicine (Wagner and Ulrich-Merzenich, 2009) in the belief that they may achieve a better therapeutic effect (synergism) than when used independently (Sibandze et al., 2010). Within an Argentinean Strategic Plan (MINCyT, 2012) that included the developing of Argentinean Herbal Medicinal Products (alone and in combination), we previously assessed the antifungal behavior of bi-herbal combinations of *Zuccagnia punctata* Cav. (Fabaceae) (common names ‘‘jarilla macho’’, ‘‘jarilla de la puna’’, ‘‘laca’’ or ‘‘pus-pus’’) with *Larrea nitida* Cav. (Zygophyllaceae) (common names “jarilla de la sierra” and “jarilla fina”) DCM exudates (*Zp*E and *Ln*E) (Butassi et al., 2015).

In the previous study, four *Zp*E-*Ln*E combinations, prepared with plants collected in four different months of a year, were tested at a fixed ratio in a ray design against *Candida albicans* and *Candida glabrata.* The nature of the interaction [synergism (S), antagonism (An) or additivism or no interaction (Ad)] was assessed with the rigorous statistical-based MixLow method that generates a Loewe index (Lϕ) (Boik et al., 2008) for each combination at different ‘inhibition percentages’ [also expressed as ‘affected fractions’ (ϕ)]. Values of Lϕ = 1 indicates Ad; Lϕ > 1 indicates An and Lϕ < 1 indicates S (Boik et al., 2008).

The most synergistic combinations that showed a 95 % fungal growth inhibition (ϕ = 0.95) were chemically characterized by quantifying the selected markers of both extracts, following the European Medicines Agency (EMA) guidelines (EMA, 2010).

Due to *C. albicans* remains to be the most common species causing invasive candidiasis worldwide including Latin American countries (Pfaller and Diekema, 2007;

Pfaller et al., 2010), we decided to deepen the study of the most synergistic mixture of *Zn*E-*Ln*E against this clinically important species.

*Zp*E-*Ln*E was first investigated for its capacity of killing the fungus (fungicidal properties) as well as for the time that needs to kill it considering that only fungistatic activities were studied in the previous work (Butassi et al., 2015). Then, the mixture was subjected to studies of mechanisms of action, which is an important next step in the development of any herbal medicinal product.

The classical modes of action of the antifungal agents in current clinical use (Mathew and Nath, 2009) are the binding to the ergosterol of fungal membrane (polyenes) or the inhibition of some steps of its biosynthesis (allylamines, azoles), and the inhibition of the β1,3-D-glucan synthase (GS) complex responsible for the synthesis of the β1,3-D-glucan, main and essential polymer of the fungal cell wall (echinocandins).

To orient ourselves in the study of the possible mechanism of action of *Zp*E-*Ln*E, the fungal morphological changes caused by the antifungal combination were analyzed, since it is well known that the type of fungal morphological abnormalities produced by an antifungal agent often provides insight into the mechanism of antifungal action (Gunji et al., 1983; Fukushima et al., 1993). Then, based on these images, cellular and enzymatic assays that target the fungal cell wall as well as the fungal membrane were performed.

In addition, alternative targets such as virulence factors (Gauwerky et al., 2009) were used to investigate the possible mechanisms of action of the *Zp*E-*Ln*E combination. Adherence to host tissues, conversion of unicellular yeasts into filamentous forms and secretion of phospholipases, proteinases and haemolysins are some of the virulence factors that cause disease in the host and deal with the degree of fungal pathogenicity (Gauwerky et al., 2009).

At last, the cytotoxicity of the combination compared with that of each component on its own was assessed in order to have a view on the safety of the bi-herbal combination.

Materials and methods

Source and c*hemical characterization of ZpE-LnE*

The *Zp*E-*Ln*E combination used in this paper was the anti *C. albicans* most synergistic combination found in the previous work, there identified as ZpE-LnE May. It showed a Lϕ = 0.62 and 95 % of *C. albicans* growth inhibition (ϕ = 0.95) (Butassi et al., 2015).

The selection and quantification of markers in *ZpE-LnE* [performed with HPLC-DAD with a validated method according to EMA guidelines (EMA, 2010)] were previously reported (Butassi et al., 2015). In brief, the chalcones galangin (**1**) and pinocembrin (**2**)

and the flavonoids 2,4-dihydroxychalcone (**3**), 2,4-dihydroxy-3-methoxy chalcone (**4**) were selected as the markers for *Zp*E, while the lignans nordihydroguiaretic acid (NDGA) (**5**), 3´-deoxy-nordihydroguiaretic acid (DNDGA) (**6**) and 3´-methyl-nordihydroguiaretic acid (MNDGA) (**7**) were the markers for *Ln*E (Fig. 1). The HPLC profiles are provided as Supplementary material (Fig. S1). *Zp*E-*Ln*E had a total concentration of 65.96 µg/ml composed by 18.84 µg/ml of *Zp*E and 47.12 µg/ml of *Ln*E. The markers' quantification showed that the flavonoids represent ~8 % [(5.39 µg/ml/65.96 µg/ml) x100] and the *Ln*E lignans ~36 % [(23.63 µg/ml /65.96 µg/ml) x 100]. Among flavonoids, 2,4-dihydroxychalcone (**3**) was in the highest amount (4.5 %) followed by 2,4-dihydroxy-3-methoxy chalcone (**4**) (2.6 %) and galangin + pinocembrin (**1**+**2**) (0.9 %). Within *Ln*E lignans, a prevalence of MNDGA (**7**) (18%) followed by NDGA (**5**) (12 %) and then DNDGA (**6**) (6 %) was observed (Fig. 2).

Fungicidal studies

Strains and culture conditions

C. albicans CCC 125 [same strain as that used in the previous study (Butassi et al., 2015)] [CCC = Centro de Referencia de Micología (CEREMIC, Rosario, Argentina)] was grown on Sabouraud-chloramphenicol agar for 48 h at 30 ºC and maintained on Sabouraud-dextrose agar (SDA) at 30 ºC (Laboratorios Britania, Buenos Aires, Argentina). The fungal inoculum was obtained according to the reported procedures of the Clinical and Laboratory Standard Institute (CLSI, 2008) and adjusted to 1-5 $\times 10^3$ colony forming units (CFU)/ml.

MFC assessment

MFC of *Zp*E-*Ln*E against *C. albicans* was performed as follows: after determining the Minimum Inhibitory Concentration (MIC) with the CLSI microbroth dilution method M27-A3 (CLSI, 2008), an aliquot of 5 µl was withdrawn from each clear well of the MIC microtiter tray, plated onto a 150 mm SDA plate, and then incubated at 30-32 °C during 24 h. The fungicidal endpoint (MFC) was defined as the lowest *Zp*E-*Ln*E concentration at which 99.9 % of the final inoculum is killed in the SDA plates. Amphotericin B (AmpB) (Sigma-Aldrich, St Louis, MO, USA) was used as standard fungicidal drug.

Time-kill studies

Using a broth-based method, the rate of killing of a *C. albicans* CCC 125 fixed inoculum (5x 10^5 CFU/ml) was determined for a control tube (with fungus, no antifungal agent) and for tubes containing *Zn*E-*Ln*E and AmpB (positive standard) each at its MFC at 0, 4, 8, 12 and 24 h of incubation at 30-32 °C. A 100 µl aliquot of each tube was taken directly from the test solutions at each period and diluted to 1/10, 1/100, 1/1000 or 1/10000. About 30 µl of each dilution was plated onto a SDA plate. After 24 h of

incubation at 30-32 °C, the CFU *per* ml were counted (log CFU/ml) (Klepser et al., 1998). All kill-curves were performed in duplicate with the SigmaPlot software 11.0 (Systat Software, San José, CA, USA).

Studies of mechanisms of antifungal action

Morphological studies using confocal and fluorescence microscopy

Fission yeast *S. pombe 972* h^- (wild type), from culture collection of one of the authors (JC Ribas, IBFG, CSIC, Salamanca, Spain) was selected for morphological studies. The fission yeast model *S. pombe* presents a rod shape and divides by medial fission. These characteristics make *S. pombe* a very good yeast model for morphological studies because any alteration of the cell wall will result in clear and strong changes in the cell morphology (Cortés et al., 2016).

Early logarithmic phase *S. pombe* cells grown in YES (Yeast Extract with Supplements) at 28 °C, were treated with *Zp*E-*Ln*E at a sub-inhibitory concentration (1/2x MIC) and compared with untreated cells (control). Then, the treated and untreated cells were concentrated (1000 g, 1 min) and visualized under the microscope by phase contrast and fluorescence microscopy by adding a solution of the fluorochrome Calcofluor White (CW, Fluorescent Brightener 28 or CW F3543, Sigma-Aldrich) at 50 µg/ml final concentration (from a stock of 10 mg/ml in water or PBS) and by using the appropriate UV filter (Leica filter cube type A, excitation filter BP 340-380, Dichromatic Mirror 400, and Suppression Filter LP 425). Images were obtained using a Leica DM RXA fluorescence microscope (Leica, Wetzlar, Germany), a PL APO 63×/1.32 oil PH3 objective, a digital camera (DFC350FX; Leica), and CW4000 cytoFISH software (Leica) (Muñoz et al, 2013). Images were processed with Adobe Photoshop CS2 software. All the analyses were repeated in three to four independent experiments and representative images of the analyzed phenotype were selected from the experiments.

Fungal membrane as the target

Exogenous ergosterol effect assay

MICs of *Zp*E-*Ln*E mixtures either in the absence or in the presence of different concentrations (50, 100 and 200 µg/ml) of ergosterol (Sigma-Aldrich) added to the assay medium, were determined following the guidelines of CLSI (2008) against *C. albicans* CCC 125*.* AmpB that is known for its capacity of binding to ergosterol, was used as a control positive drug. MICs were determined after 48 h of incubation (Escalante et al., 2007).

Quantification of ergosterol

Total intracellular sterols were extracted as reported by Arthington-Skaggs et al. (1999) with slight modifications. Briefly, a single *C. albicans* CCC 125 colony from an overnight SDA plate culture was used to inoculate 50 ml of Sabouraud-dextrose broth (SDB) (Difco, Detroit, MI, USA) containing 65.96 µg/ml of *Zp*E-*Ln*E (MIC of the most synergistic combination) (Butassi et al., 2015). Itraconazole (Itra) (Sigma-Aldrich), that inhibits ergosterol biosynthesis, was used as standard drug. The cultures were incubated for 24 h with shaking at 30-32 °C. The stationary-phase cells were harvested by centrifugation at 1000 g (Presvac DCS-16 model centrifuge, New York, NY, USA) for 5 min and washed once with sterile distilled water. The net wet weight of the cell pellet was determined. Three ml of 25 % alcoholic KOH solution (25 g of KOH and 35 ml of sterile distilled water, brought to 100 ml with 100 % ethanol), were added to each pellet and vortexed for 1 min. Cell suspensions were transferred to sterile borosilicate glass screw-cap tubes and were incubated in an 85 °C water bath for 1 h. Following incubation, tubes were allowed to cool to room temperature. Sterols were then extracted by addition of a mixture of 1 ml of sterile distilled water and 3 ml of *n*-heptane, followed by vigorous stirring in a vortex for 3 min. The heptane layer was transferred to a clean borosilicate glass screw-cap tube. Prior to analysis, 20 ml of sterols extract were diluted 5-fold in 100 % ethanol and scanned in a range between 240 and 300 nm in a UV/Visible Spectrophotometer (model Libra S12, Biochrom Ltd., Cambridge, UK).

Ergosterol content was calculated as a percentage of the wet weight of the cell by the following equations: % ergosterol + % 24(28)DHE = [($A_{281.5}$/290) x *F*]/pellet weight, % 24(28)DHE = [(A_{230}/518) x *F*]/pellet weight, and % ergosterol = [% ergosterol + % 24(28)DHE] - % 24(28)DHE, where *F* is the factor for dilution in ethanol and 290 and 518 are the *E* values (in %/cm) determined for ergosterol and 24(28)DHE, respectively. A flat line would be indicative of absence of detectable ergosterol in extracts. A dose-dependent lowering in the height of the absorbance peaks corresponded to decreased ergosterol concentration.

Fungal cell-wall as the target

Sorbitol cellular assay

The MIC of *Zp*E-*Ln*E against *C. albicans* CCC 125 was determined either in the absence or in the presence of 0.8 M sorbitol (Sigma-Aldrich) added to the assay medium, following the guidelines of CLSI (2008). MICs were read at 7 days (Frost et al., 1995). The drug papulacandin B (PapB) (Novartis, Basel, Switzerland), known inhibitor of the fungal cell wall β1,3-D-glucan synthesis, was used as standard positive drug.

Enzymatic assays related to the fungal cell wall

Enzyme extract preparation

Cell extracts of *C. albicans* CCC 125 were obtained as described by Martins et al. (2011). Early logarithmic phase cells grown in 100 ml YES medium were collected, washed once with buffer A [50 mM Tris-HCl pH 7.5, 1 mM EDTA and 1 mM β-mercaptoethanol], suspended in 100 µl of the same buffer *per* g of cells (wet weight) containing 50 µM GTPγS to preserve enzyme activity, and broken with glass beads in a FastPrep FP120 apparatus (BIO 101, Thermo Savant Inc., New York, NY, USA) (one 15s pulse at speed = 6). Broken material was collected and cell debris was removed by low speed centrifugation (5000 g, 5 min at 4 °C). The supernatant was centrifuged at 48000 g for 30 min at 4 °C and the pellet was re-suspended in buffer A containing 33 % glycerol and 50 µM GTPγS (at a concentration of approximately 2 to 5 mg protein/ml) and stored at -80 °C.

β1,3-D-glucan synthase (GS) *assay*

The standard GS assay mixture contained 5 mM UDP-D- [^{14}C]glucose ($4x10^4$ cpm/200 nmol), 150 µM GTPγS, 0.75% bovine serum albumin (BSA), 2.1 mM EDTA, 75 mM Tris-HCl (pH 8.0), 7.5 % (v/v) glycerol, and 5 µl enzyme extract (approximately 10-15 µg of protein in 5 µl of enzyme extract) in a total volume of 40 µl. Two µl of DMSO or the corresponding mixture of *Zp*E-*Ln*E (kept in a stock solution of 50 mg/ml in DMSO at -20 °C), were added to each reaction. The reaction mixture was incubated for 90 min at 30 °C and stopped by addition of 1 ml of 10 % trichloroacetic acid (TCA). All reactions were carried out in duplicate. PapB was used as standard positive drug.

Chitin synthase (ChS) *assay*

ChS activity was measured as previously described (Choi and Cabib, 1994) with a slight modification as follows: for the proteolytic activation step of ChS, reaction mixtures were prepared with 0.5 M 2-[N-morpholino]ethanesulfonic acid (MES) at pH 6.5, 40 mM $MgCl_2$, 5 mM UDP-[U-^{14}C]GlcNAc ($2x10^4$ cpm/50 nmol), 2 µl of trypsin at the optimal concentration for enzyme activation (0.005 µg/µl) and 10 µl of enzyme extract (20-30 µg protein) in a total volume of 46 µl. Two µl of DMSO or the combination of *Zp*E-*Ln*E (kept in a stock solution of 50 mg/ml in DMSO at -20 °C), were added to each reaction. The reaction mixture was incubated for 15 min at 30 °C. Proteolysis was stopped by adding 2 µl of trypsin inhibitor solution at a concentration 1.5 times that of the used trypsin solution and cooling the tubes on ice. GlcNAc was added to a final concentration of 32 mM, followed by incubation for 90 min at 30 °C and stopped by addition of 1 ml of 10% TCA. All reactions were carried out in duplicate. NikZ was used as standard positive drug.

Inhibition of Candida albicans virulence factors

Adherence to Buccal Epithelial Cells assay

The adherence method used was described by Kimura and Pearsall (1978). Buccal Epithelial Cells (BECs) were collected from healthy human subjects by gently rubbing the inside of the cheeks with sterile swabs that were then agitated in 6 ml of Phosphate Buffered Saline (PBS). BECs were washed twice in PBS to remove unattached microorganisms and re-suspended in the same buffer to a final concentration of $5x10^5$ cells/ml counted in a Neubauer chamber.

C. albicans CCC 125 was grown for 24 h at 30-32 °C on SDB. Cells in the budding yeast phase were washed twice in PBS and re-suspended in the same buffer to a final concentration of 2.5×10^7 cells/ml counted in a Neubauer chamber.

For the assay, 0.5 ml of BECs, 0.5 ml of fungal inoculum and 1 ml of SDB (control), or 1 ml of SDB containing *Zp*E-*Ln*E at its MIC, were mixed in tubes and incubated on a shaker at 37 °C for 1 h. AmpB (at MIC = 1 µg/ml) was used as standard positive drug. BECs were collected on hydrophilic polyvinylidene fluoride 0,47 µm pore size filters (Merck Millipore, Billerica, MA, USA) and washed with 70 ml of PBS to remove unattached fungi. Thereafter, the filter was removed carefully with a forceps and placed firmly on a glass slide with the BECs against the glass surface. After 10 s, the filter was removed gently, leaving the BECs adhered to the glass slide. The preparations were air-dried, fixed with heat and stained with Gram dye. The number of adhered yeast cells was quantified by light microscopy (Eclipse E100, Nikon Corp., Tokyo, Japan) at 40x magnification. One hundred BECs were observed for adherent yeast cells.

Germ tube inhibition assay

This assay was performed according to Pinto and co-workers (Pinto et al., 2008) with modifications. Cell suspensions from overnight SDB cultures of *C. albicans* CCC 125 were adjusted to obtain a density of $1x10^6$ CFU/ml. From this cell suspension, 100 µl was added to tubes containing 200 µl of human serum and 100 µl of SDB (control) or 100 µl of SDB with the combination in the range 1/8-4x MIC. AmpB (1.6 µg/ml) was used as the standard positive drug. The tubes were then incubated at 37 °C for 3 h and 200 cells from each one were counted with a Neubauer chamber. The percentage of germ tubes was determined by using the following formula.

Germ tube formation % = (n° germ tubes in treatment/n° germ tubes in control) x 100

Results are presented as means ± standard deviations (SD) of three separate experiments. Germ tubes were considered positive when they were at least as long as the diameter of the blastospores. Protuberances showing a constriction at the point of connection to the mother cell, typical for pseudohyphae, were excluded (Pinto et al., 2008).

Inhibition of phospholipases secretion

At first, the phospholipase activity of several *C. albicans* strains [CCC 125, CCC 130-15, CCC 132-15, CCC 182-13, CCC 193-13 and CCC 131-15 (provided by CEREMIC)] was detected by using the egg yolk agar plate method of Price et al. (1982). Control medium consisted of SDA containing 1 M NaCl, 0.005 M $CaCl_2$ and 8 % of sterile egg yolk, while test media contained in addition *Zp*E-*Ln*E at 1x MIC. Petri dishes were filled with 20 ml of medium, and then 10 µl of cell suspension (1x 10^6 cells/ml) were placed in the plate after the agar had set. The plates were incubated at 37 °C for four days, and then, they were examined for the presence of a precipitation or halo zone (Pz) around the colony. The presence of a Pz indicated expression of phospholipases. The assay was conducted in triplicate for each isolate tested. The inhibitory activity of phospholipase secretion can be determined according to the 'Pz index', i.e., colony diameter/total diameter of the colony plus Pz (Treviño-Rangel et al., 2013) (see below).

Inhibition of proteinases secretion

Extracellular proteinase secretion of *C. albicans* isolates [CCC 125, CCC 130-15 and CCC 131-15 (provided by CEREMIC)] was analyzed in terms of Bovine Serum Albumin (BSA) degradation according to the technique described by Staib (1966). The control medium consisted of 1 % agar, 0.1 % KH_2PO_4, 0.5 % $MgSO_4$, 1 % glucose and 0.2 % BSA (as the sole nitrogen source), while test media contained in addition the combination *Zp*E-*Ln*E at 1x MIC. Petri dishes were filled with 20 ml of medium, and then 10 µl of fungal inoculum (1x10^6 cells/ml) were placed into the plate after the agar had set. The plates were incubated at 37 °C for five days and then were examined for the presence of Pz around the colony. The presence of Pz indicated expression of proteinases. The assay was conducted in triplicate for each isolate tested. The inhibitory activity of proteinase secretion can be determined according to the Pz index (see below).

Haemolysins secretion inhibition

Haemolysins production of *C. albicans* isolates [CCC 125, CCC 130-15, CCC 132-15, CCC 131-15, CCC 129-15 (provided by CEREMIC)] was evaluated with a blood plate assay as described by Sachin et al. (2012). The control medium was sugar-enriched human blood (2 % peptone, 1 % agar, 7 % fresh human blood, 3 % glucose), while test media contained control medium added with *Zp*E-*Ln*E at 1x MIC. Petri dishes were filled with 20 ml of medium, and then 10 µl of fungal inoculum (1x10^6 cells/ml) were placed in the plate after the agar had set. The plates were incubated at 37 °C for 48 h and then were examined for the presence of a Pz around the colony. The presence of a Pz indicated expression of haemolysins. The assay was conducted in triplicate for each isolate tested. The inhibitory activity of haemolysins secretion can be determined according to the Pz index.

Pz index

Phospholipases, proteinases and haemolysins secretion (in control and test plates) was measured in terms of the Pz index that is the quotient between the diameter of the colony (a)/total diameter of colony plus Pz (b) (Fig. 3), according to the method described by Price et al. (1982). The control plate shows a Pz index < 1.00 and the plate in the presence of phospholipases, proteinases and haemolysins inhibitors shows a Pz index = 1.00).

Cytotoxic effect of ZpE-LnE

The measurement of the cytotoxic properties of *ZpE-LnE* was based on the reduction of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, Sigma-Aldrich) by the mitochondrial dehydrogenase of viable Human hepatoma Huh7 cells to give a blue formazan product which can be spectrophotometrically quantified. Huh7 cells were seeded in 96-well plates at a density of 5000 cells/well. After 24 h of attachment, cells were treated for 72 h with different concentrations of the most synergistic combination *Zp*E-*Ln*E and with each extract alone. After treatment, MTT was added to the culture medium to assess its metabolization, as previously described (Ferretti et al., 2016). Absorbance of the metabolite produced from viable cells was measured at 540 nm (reference filter 650 nm) in a DTX 880 multimode detector (Beckman Coulter Inc., Fullerton, CA, USA). Results were expressed as percentage of absorbance from control cells.

Statistical analysis

Statistical analyses were performed by using the computer program GraphPad Prism 2.01 (GraphPad Prism Software Inc., San Diego, CA, USA). Minimum, median and maximum values, means and SD were determined. Differences between the treatment groups were analyzed by using Kruskall-Wallis and Dunn tests; p values < 0.05 were considered significant.

Results and discussion

Fungicidal activities of ZpE-LnE

*Zp*E-*Ln*E has previously shown (Butassi et al., 2015) a MIC of 65.96 µg/ml (18.84 µg/ml of *ZpE* + 47.12 µg/ml of *Ln*E) against *C. albicans* CCC 125 while the MICs of *Zp*E and *Ln*E each alone were much higher of 70.36 µg/ml and 136.22 µg/ml, respectively (Table 1). Considering that a fungicide rather than fungistatic agent assures the suppression of the fungus after the drug is removed, MFC and time-kill kinetics studies were performed on *Zp*E-*Ln*E.

MFC determination.

The MFC of *Zp*E-*Ln*E against *C. albicans* was performed with the CLSI-microbroth dilution method M27-A3 (CLSI, 2008) and defined as the lowest concentration at which 99.9 % of the final inoculum is killed under standardized set of conditions detailed in Materials and methods.

Results showed that *Zp*E-*Ln*E was fungicidal rather than fungistatic, killing *C. albicans* at MFC= 131.92 µg/ml (2x MIC) (37.68 µg/ml *Zp*E + 94.24 µg/ml *Ln*E), whereas the doses of each alone were also much higher (Table 1).

The fact that *Zp*E-*Ln*E showed fungicidal rather than fungistatic activity is a highly important feature for its development as a bi- Herbal Medicinal Product. In addition, it is interesting to note that the synergistic combination produced the killing of *C. albicans* at much lower concentration than each extract acting alone. This can be corroborated by calculating the Dose-Reduction Index (DRI) that is defined as the quotient between MFC value of each extract, on its own (*Zp*E and *Ln*E) and within the combination (*Zp*Ec and *Ln*Ec) [DRI_{ZpE}= *Zp*E/*Zp*Ec and DRI_{LnE} = *Ln*E/*Ln*Ec] (Table 1). Results showed that within the combinations, *Zp*Ec and *Ln*Ec kill at a concentration 3.73 times lower (DRI_{ZpE} = 3.73) and 2.89 lower (DRI_{LnE} = 2.89) respectively than when acting alone. The higher the DRI, the greater the benefit of the combination with respect to the single components.

Time-kill assay

*Zn*E-*Ln*E [at its MFC = 131.92 µg/ml (2x MIC)] was tested by the rate of killing a fixed *C. albicans* inoculum (1-5x 10^5 CFU/ml) at the incubation intervals 0, 4, 8, 12 and 24 h by counting the survivor CFU/ml as explained in Materials and methods. AmpB at MFC was used as standard positive drug.

Plots of the $\log_{10}$ CFU/ml *versus* time for *Zp*E-*Ln*E in comparison with the fungicide drug AmpB along with the drug-free growth control, are presented in Fig. 4. Interesting enough, a marked decrease in the $\log_{10}$ CFU/ml (> 99.99 %) compared with the starting inoculum activity was observed at 8 h for *Zp*E-*Ln*E combination while AmpB required 12 h to reach a similar decrease.

These results showed that *Zp*E-*Ln*E and AmpB (both at their respective MFC) kill *C. albicans* after 8 and 12 h respectively, making the *Zp*E-*Ln*E combination interesting for further development.

*Studies of mechanisms of antifungal action of Zp*E-*Ln*E

Morphological studies

Considering that changes in morphology of yeasts have often provided insight into the mechanism of antifungal action (Gunji et al., 1983; Fukushima et al., 1993), the morphological changes of fission yeast *S. pombe,* which is a cell model in cellular and

molecular biology, subjected to a sub-inhibitory concentration of *Zp*E-*Ln*E were observed by phase contrast and fluorescence microscopy.

The fluorochrome CW, used for staining *S. pombe* cells, binds to fibrillar β1,4 polysaccharides, like chitin or cellulose. *S. pombe* has no chitin, but CW binds specifically with high affinity to a special linear β1,3-D-glucan, which forms part of the primary septum and with much less intensity to growing poles (Cortés et al., 2007). Also, CW penetrates living cells that have perturbed plasma membrane integrity. Thus, fluorescence micrographs of CW stained cells allowed us to observe whether cytoplasmic membrane of fungal cells were intact in *S. pombe* cells treated with the *Zp*E-*Ln*E antifungal combination.

S. pombe cells treated with *Zp*E-*Ln*E at 1/2x MIC showed (Fig. 5, left) scorched and dead (b), and swollen, vacuolized and deformed (a) cells in phase contrast images compared to the control cells, likely due to a damage in the membrane and/or in the cell wall.

In CW-staining fluorescence images (Fig.5, right), a bright fluorescence was observed in septa and growing poles in control cells as expected, while the CW stain penetrated to the cytoplasm in some of the *Zp*E-*Ln*E treated cells (c and d), thus showing that the membrane could be heavily damaged losing its capacity to protect the cell as a permeability barrier against the environment and causing the cell death.

Interestingly, the malformations produced by *Zn*E-*Ln*E in *S. pombe* cells suggest that either the fungal cell-wall or the fungal membrane could be seriously damaged.

Assays related to the interaction with the fungal membrane

One important mode of action of antifungal drugs is related to the decrease of its ergosterol content (main sterol of the fungal membrane) either by binding it or by inhibiting some steps of its biosynthesis. Both mechanisms lead to fungal membrane disruption and cell death allowing the penetration of the CW into the fungal cell that is the effect observed in Fig. 5 right.

Binding of ZpE-LnE to ergosterol

To determine whether *Zp*E-*Ln*E binds fungal ergosterol, the MIC of this mixture was determined against *C. albicans* either with or without the addition of exogenous ergosterol. If the mode of action of the *Zp*E-*Ln*E combination is the binding ergosterol, the combination will bind the most attainable exogenous ergosterol and not the fungal membrane's ergosterol. As a consequence, a higher MIC will be obtained for the combination (Escalante and Zacchino, 2007).

Results showed (Table 2) that the MIC of *Zp*E-*Ln*E against *C. albicans* is enhanced in a dose-dependent manner in the presence of increasing concentrations (50, 100 and 200 μg/ml) of exogenous ergosterol, suggesting that the combination acts by binding to

ergosterol of the fungal membrane. AmpB that is used as a standard positive drug due to its known ergosterol binding properties, showed an expected enhanced MIC.

Ergosterol has many essential roles in fungal cell physiology, including functional regulation of membrane proteins, microdomain formation (sterol-rich domains or lipid rafts), endocytosis, vacuole fusion, cell division and cell signaling (Anderson et al., 2014). So, the binding of *Zp*E-*Ln*E to this sterol, concomitantly precludes its participation in multiple cellular functions leading to the death of fungal cells.

Ergosterol quantitation

To examine whether *Zp*E-*Ln*E interferes with any of the steps of the ergosterol biosynthesis, the total ergosterol content in *C. albicans* was quantified in the presence of *Zp*E-*Ln*E in comparison with the content in untreated strain. Itraconazole (Itra) was used as the positive control. A decrease in the ergosterol content would be a proof that the ergosterol biosynthesis was partially blocked on any of its synthesis steps and thus, the ergosterol was not formed in a normal amount.

Ergosterol and the late sterol intermediate 24(28) dehydroergosterol [24(28)DHE] show a characteristic four-peaked curve in an UV spectrum. Ergosterol and 24(28)DHE both absorb at 281.5 nm, whereas only 24(28)DHE shows an intense spectral absorption band at 230 nm. Therefore, the amount of ergosterol can be determined by calculating the total ergosterol plus 24(28)DHE and the only 24(28)DHE contents and then, subtracting this value of only 24(28)DHE from the amount of absorption due to total ergosterol plus 24(28)DHE as explained in Materials and methods.

Table 3 summarizes the ergosterol content in *C. albicans* in the presence of either *Zp*E-*Ln*E or Itra (Table 3 and Fig. 6).

Table 3 shows that cells grown with Itra showed a markedly reduction (from 0.036 to 0.009 %) in ergosterol content. Instead, the combination *Zp*E-*Ln*E did not show any reduction respective of the control. For the sake of clarity, the results were depicted in Fig. 6.

Assays related to the interaction of ZpE-LnE with the fungal cell wall

C. albicans cell wall is a rigid carbohydrate-containing structure that serves as a protective barrier to the extracellular environment. Cell wall provides osmotic integrity to the cell, maintains mechanical strength and defines cell shape (Cortés et al., 2016). Since fungal but not mammalian cells are encased in a rigid cell wall that is required for growth and viability of fungi, its inhibition represents an ideal mode of action of antifungal agents, due to the fact that agents acting by this mechanism of action would not be toxic to human cells.

Both cellular and enzymatic assays were performed in order to determine if the *Zp*E-*Ln*E could act through the inhibition of the synthesis or assembly of the main polymers of the fungal cell wall.

Cellular sorbitol assay

Many times, the effect of the antifungals that act by inhibiting the fungal cell wall is reversed in a medium containing an osmotic stabilizer of fungal protoplasts such as sorbitol (Frost et al., 1995) showing a much higher MIC than without sorbitol. Sorbitol also stabilizes strains with a weakened cell wall (Ribas et al., 1991; Muñoz et al., 2013). MIC determination for *Zp*E-*Ln*E with the method M27-A3 (CLSI, 2008) was conducted both with and without sorbitol. Results showed that when *C. albicans* was treated with *Zp*E-*Ln*E in the presence of sorbitol, the MICs values did not shift to a higher value compared to those without sorbitol, thus suggesting that the combination could not act directly through the inhibition of the cell wall synthesis or assembly (results not shown). In fact, the effect of binding the ergosterol and altering the plasma membrane would impede the sorbitol protection observed when the inhibition exclusively affects the cell wall. Therefore it is possible that the *Zp*E-*Ln*E combination had a combined effect on both membrane and cell wall synthesis and therefore, it could not be detected by sorbitol protection.

To further analyze whether *Zp*E-*Ln*E has an effect in the cell wall, the combination was tested *in vitro* for its capacity to inhibit *C. albicans* GS and ChS activities, enzymes that catalyze the synthesis of the major polymers of the fungal cell wall, β1,3-D-glucan and chitin, respectively.

For assessing GS inhibition, the incorporation of soluble UDP[14C]-glucose into insoluble β1,3-D-glucan (Ribas et al., 1991) was determined, while for the inhibition of ChS, the incorporation of soluble UDP-[U-^{14}C]GlcNAc into insoluble chitin was measured (Choi and Cabib, 1994). *Zp*E-*Ln*E was tested at three different concentrations: 1/2x MIC (= 32.98 µg/ml containing 9.42 µg/ml *Zp*E + 23.56 µg/ml *Ln*E), 1x MIC (= 65.96 µg/ml; 18.84 µg/ml *Zp*E + 47.12 µg/ml *Ln*E) and 2x MIC (= 131.92 µg/ml; 37.68 µg/ml *Zp*E + 94.24 µg/ml *Ln*E). Results showed a moderate dose-dependent decrease of residual *C. albicans* GS. At 1/2x, 1x and 2x MIC, the activity decreased to 87.47 %; 74.99 % and 52.07 % respectively (Fig. 7). In turn, ChS residual enzymatic activity (%) was reduced to 88.29 % (1/2x MIC), 65.63 % (1x MIC) and 23.89 % (2x MIC), indicating that at 2x MIC a good enzymatic inhibition was produced for both GS and ChS (Fig. 7).

From Fig. 7 it is clear that *Zp*E-*Ln*E combination showed a low and moderate capacity of inhibiting GS and ChS respectively with IC_{50} values of 131,92 µg/ml for GS and 90 µg/ml for ChS, while PapB and NikZ showed IC_{50} = 0.02 and 0.01 µg/ml respectively. However, it must be considered that whereas PapB and NikZ are pure compounds, *Zp*E-

*Ln*E is a combination of raw extracts and therefore the active inhibitors could be represented in a much lower amount. Additionally, these results show that the *Zp*E-*Ln*E present a dual fungicidal activity altering both the plasma membrane ergosterol and the cell wall synthesis.

Inhibition of Candida albicans virulence factors

Expression of *C. albicans* virulence factors like adherence to epithelial surfaces, germ tube formation, and the production of hydrolytic enzymes such as phospholipases, proteinases and haemolysins contributes to the pathogenesis of candidiasis (Sachin et al., 2012). To know the capacity of *Zp*E-*Ln*E to inhibit these virulence factors is an added value to its future development as a new bi-Herbal Medicinal Product.

Adherence to BECs

Adherence and colonization on an epithelial surface are the first steps by which a microorganism may initiate infection. It is important to note that there is a clear association between adherence and virulence. For example, species of *C. albicans* more commonly associated with disease tend to show greater adherence characteristics.

Results showed that adhesion of yeast cells to BECs decreased from 55.29 ± 29.12 in the control cells without compound to 23.69 ± 15.34 in the presence of 1x MIC *Zp*E-*Ln*E ($p < 0.05$) and to 19.20 ± 14.58 in the presence of 1x MIC AmpB ($p < 0.05$) (Fig. 8).

Fig. 9 shows light microscopy images of adhesion of *C. albicans* to BECs, either in absence (control) or in the presence of *Zp*E-*Ln*E or AmpB.

Fig. 9 A and B clearly show that the amount of adhered fungal cells to BEC in *Zp*E-*Ln*E- and AmpB- treated cells (B and C) were much lower than in the untreated cells (A), thus suggesting that there will be a degree of resistance to colonization of BECs by *C. albicans* in the presence of *Zp*E-*Ln*E combination similar to the effect of AmpB.

Germ tube formation

The transition of unicellular yeasts cells to the filamentous form is an attribute of *C. albicans* and is associated with virulence and pathogenicity although both forms may be involved in the progress of disease (Ishida et al., 2006).

In this study, the effect of different concentrations (1/8, 1/4, 1/2, 1, 2 and 4x MIC) of *Zp*E-*Ln*E on *C. albicans* germ tube formation was assessed. Results showed (Fig. 10) that in presence of the combination, the percentage of cells with germ tube formation was 77.00, 57.55 and 19.73 % at 1, 2 and 4x MIC respectively with respect to the percentage in control cells without the combination, while in the presence of AmpB (at MIC) the germ tube formation was 2 % that of control cells.

From Fig. 10 it is clear that *Zp*E-*Ln*E has not a significant effect on the germ tube formation since even at 4x MIC the inhibition still maintains a 18 % of germ tube

formation, which is a poor inhibition in comparison to the effect of complete inhibition of germ tube formation of the standard drug AmpB at its MIC.

Effect of ZpE-LnE on phospholipases secretion

It has been demonstrated in a murine model (Chen et al., 1997) that *C. albicans* strains that produce fewer amounts of phospholipases show lower virulence than strains producing higher amounts of them, thus suggesting that phospholipases may be virulence factors. Also a correlation was found between phospholipase activity, cellular adherence and pathogenicity of *C. albicans* (Barrett-Bee et al., 1985).

The combination *Zp*E-*Ln*E at 1x MIC was tested for its capacity of reducing the phospholipase production in six phospholipase-producing *C. albicans* strains, which possess Pz indices in the range 0.56-0.96 (Table 4).

Results showed that *Zp*E-*Ln*E completely inhibited the phospholipase production in the six strains, since the Pz index = 1 for the six strains.

In Fig. 11, a clear inhibition of phospholipase production can be observed in the six tested strains compared with the control strains growing in the absence of the combination.

Effect of ZpE-LnE on proteinases secretion

A positive correlation between proteinase production, adherence and pathogenicity of candidiasis was demonstrated by Ghannoum and Elteen (1986). However there are different reports that suggested not only a positive but also a negative relationship between proteinase production and virulence in *C. albicans* (Cutler, 1991).

In this work *Zp*E-*Ln*E (1x MIC) was tested for its capacity of inhibiting the proteinase secretion against three *C. albicans* strains. Results showed (Table 5) that in the presence of *Zp*E-*Ln*E, proteinases secretion was completely inhibited (Pz index = 1) in two *C. albicans* strains.

Effect of ZpE-LnE on haemolysins secretion

Pz indices obtained with *C. albicans* strains tested either without (control) or with *Zp*E-*Ln*E combination (at its 1x MIC) are shown in Table 6.

In control cells without *Zp*E-*Ln*E combination, the Pz index values vary between 0.83 to 0.92, indicating a weak haemolysins secretion of these strains. In the presence of *Zp*E-*Ln*E mixture, haemolysins secretion was not inhibited in none of the strains (Pz index between 0.77-0.90).

Cell viability assay

In order to evaluate the toxic effect of *Zp*E-*Ln*E and its component extracts *Zp*E and *Ln*E alone, human hepatoma (Huh7) cells were treated for 24 h with different

concentrations of each *Zp*E, *Ln*E and *Zp*E-*Ln*E (1x MIC, 1/2x MIC, 1/4x MIC and 1/8x MIC) and the cell viability was estimated by using the MTT assay. As shown in Table 7 and in Fig. 12, the viability of Huh7 cells was 60.13 % and 17.88 % in the presence of *Zp*E and *Ln*E respectively at their MICs (70.36 and 136.22 µg/ml respectively), while in the presence of *Zp*E-*Ln*E at its MIC (containing 18.84 µg/ml of *Zp*E and 47.12 µg/ml of *Ln*E), cell viability was much higher (74.12%), meaning that the combination with lower extracts' concentrations enhanced the cell viability. At lower concentrations (1/2 MIC, 1/4 MIC and 1/8 MIC) of extracts and combination, the cell viability increased, and this was greater in the presence of the combination.

These results showed that the combination produced lower toxicity than the extracts alone, thus giving the support for the development of a safe bi-Herbal Medicinal Product.

Conclusions

Our results suggest that *Zp*E-*Ln*E whose MIC has been previously reported, possess strong fungicidal properties and has the capacity of killing *C. albicans* cells in 8 h, a time lower than the time needed by AmpB, in both cases at their specific MFC.

In addition *Zp*E-*Ln*E produces at sublethal MIC concentrations different type of malformations of the fungal cells that suggest that it targets either the fungal membrane and/or the fungal cell wall. The exogenous ergosterol assay allowed determining that *Zp*E-*Ln*E binds to the ergosterol of the fungal membrane although it does not modify the ergosterol content of the fungal cells, thus suggesting that it does not act inhibiting any step of the ergosterol biosynthesis but altering the membrane structure.

Assays targeting the fungal cell wall, showed that the combination moderately inhibits the GS activity but it produces a higher inhibition of ChS activity, enzymes that catalyze the synthesis of β1,3-D-glucan and chitin, the two most important structural polymers of the fungal cell wall. In summary, these results together with absence of sorbitol protection of fungicidal activity suggest a dual fungicidal activity *Zp*E-*Ln*E combination altering both the plasma membrane ergosterol and the cell wall synthesis.

Regarding its effect on virulence factors, *Zp*E-*Ln*E significantly decreased the capacity of adhesion of fungal cells to eukaryote BECs, although it did not significantly inhibit the germ tube formation up to 4x MIC. In addition, *Zp*E-*Ln*E completely inhibited the secretion of phospholipases and two over three proteinases but not of haemolysins. The studies of toxicity of the *Zp*E-*Ln*E combination on Huh7 cells demonstrated that *Zp*E-*Ln*E possess a very low toxicity, much lower than that of the extracts alone.

The fungicidal properties of *Zp*E-*Ln*E against *C. albicans*, added to the evidences of the mode of action and its low toxicity, justify the continuation of the studies towards the development of this combination as a new antifungal bi-Herbal Medicinal Product

containing Argentinean medicinal plants. *Zp*E-*Ln*E would be a good candidate for replacing the toxic commercial antifungal agents and, due to the easy availability of both plant species, the combination could be widely used by the population, and mainly by those with lower economic resources.

Conflicts of interest

Authors declare that they do not have any conflict of interest.

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Legends for Fig.:

Fig. 1: Structures of selected markers for *Zuccagnia punctata* exudate (*Zp*E) of chalcones: galangin (**1**)**,** pinocembrin (**2**), 2,4-dihydroxychalcone (**3**) and 2,4-dihydroxy-3-methoxy chalcone (**4**); and for *Larrea nitida* exudate (*Ln*E) of lignans: nordihydroguiaretic acid (NDGA) (**5**), 3´-deoxy-nordihydroguiaretic acid (DNDGA) (**6**) and 3´-methyl-nordihydroguiaretic acid (MNDGA) (**7**).

Fig. 2: Composition of the synergistic combination of *Zuccagnia punctata* (*Zp*E) with *Larrea nitida* (*Ln*E) exudates (*Zp*E-*Ln*E) that showed a Loewe Index Lϕ = 0.62 at a affected fraction ϕ = 0.95 (95 % of fungal inhibition), and was suggested in the previous paper (Butassi et al., 2015) for a further development of a bi-herbal medicinal product with activity against *Candida albicans*. The proportion of markers **1**-**7** is indicated.

Fig. 3: Pz index = diameter of the colony (a)/total diameter of colony plus Pz (b) for phospholipases, proteinases or haemolysins test either in the absence or in the presence of inhibitor.

Fig. 4: Effect of *Zp*E-*Ln*E combination and AmpB (standard drug), both at their respective Minimum Fungicidal Concentration (MFC), on the $\log_{10}$ colony-forming units (CFU)/ml of *C. albicans* CCC 125, *versus* time (h).

Fig. 5. Left: Phase contrast images. Right: fluorescence micrographs [Calcofluor white (CW) stained] of *S. pombe* cells grown either in the absence (control) or in the presence of *Zp*E-*Ln*E (at 1/2x MIC) during 4.5 h. Bar: 5 µm. (a) swollen cells; (b) scorched and dead cells; in (c) and (d) the stain penetrated into the dead cells.

Fig. 6: UV spectrophotometric sterol analysis of *Candida albicans* CCC 125 either in absence (control) or in the presence of *Zp*E-*Ln*E at 1x MIC. Itraconazole (itra) (1x MIC) was used as standard positive drug.

Fig. 7: Residual enzymatic activity (%) of *C. albicans* β1,3-D-glucan synthase (GS) and chitin synthase (ChS) in the presence of *Zp*E-*Ln*E at different concentrations (µg/ml) 1/2x (32.98 µg/ml), 1x (65.96 µg/ml) and 2x (131.92 µg/ml) MIC. Papulacandin B (PapB) and Nikkomycin Z (NikZ) were used as standard positive drugs for GS and ChS, respectively. IC_{50} PapB= 0.02 µg/ml and IC_{50} NikZ= 0.01 µg/ml.

Fig. 8: Adherence values of *C. albicans* to human buccal epithelial cells (BECs) after incubation of the yeast cells in media containing either *Zp*E-*Ln*E or AmpB each at its MIC.

BECs adherence is expressed as number of yeasts/BEC (N=100). ***p*<0.05, Kruskall Wallis and Dunn tests show the significant difference between tests and control.

Fig. 9: Adhesion of *Candida albicans* (dark violet stain) to Buccal Epithelial Cells (BECs, pink stain). (a) Control: adherence of *C. albicans* to untreated BECs. (b) *C. albicans* and BECs treated with *Zp*E-*Ln*E combination. (c) *C. albicans* and BECs treated with Amphotericin B (AmpB). Light microscopy at 40x·magnification. Bar: 5 µm.

Fig. 10: *Candida albicans* germ tube formation (% of cells) either without (control) or with different concentrations of *Zp*E-*Ln*E: 1/8, 1/4, 1/2, 1, 2, and 4x MIC. Results are expressed as mean ± SD of three independent assays. *****Only this sample differed significantly ($p<0.05$) from the control cells, as determined by using the Kruskal-Wallis and Dunn's multiple comparison tests. Amphotericin B (AmpB) was used as standard drug at 1x MIC.

Fig. 11**:** Phospholipases secretion of *Candida albicans* strains (*Ca*): 1 (*Ca* CCC 125), 2 (*Ca* CCC 130-15), 3 (*Ca* CCC 132-15), 4 (*Ca* CCC 182-13), 5 (*Ca* CCC 193-13) and 6 (*Ca* CCC 131-15) either in absence (control) (A) or in the presence of *Zp*E-*Ln*E (B).

Fig. 12**:** Cellular viability (%) of human hepatoma Huh7 cells in the presence of different concentrations (1, 1/2, 1/4, and 1/8x MIC) of *Zp*E-*Ln*; *Zp*E alone and *Ln*E alone. Viability of treated cells was determined by MTT assay. Values are expressed as mean ± SD, determined in triplicate.

Figure 1

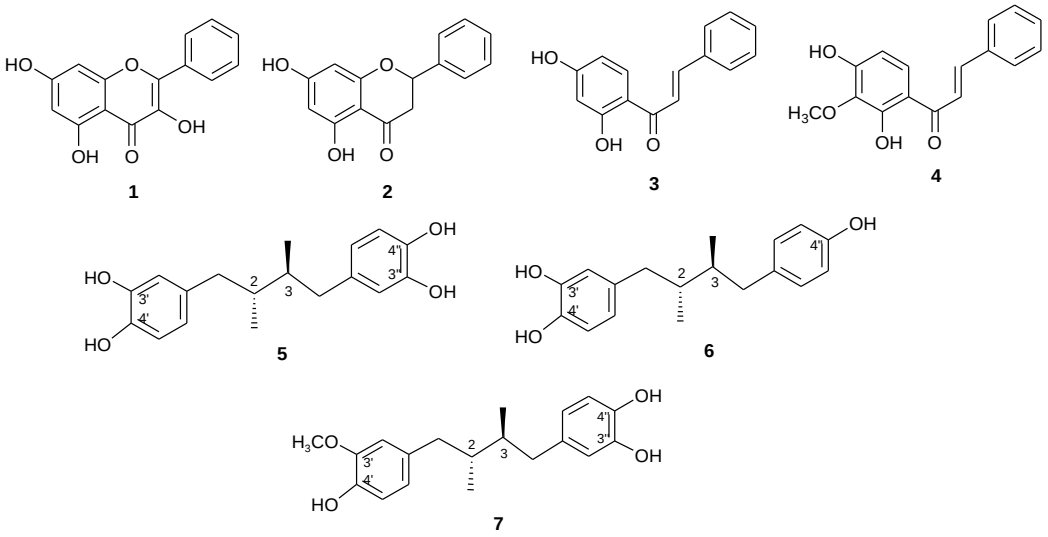

Fig. 1

Figure 2

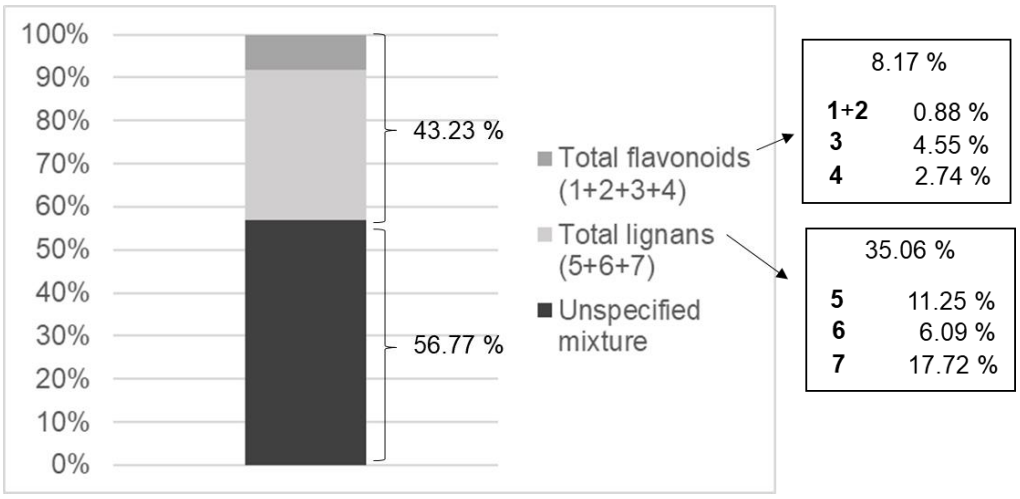

Fig. 2

Figure 3

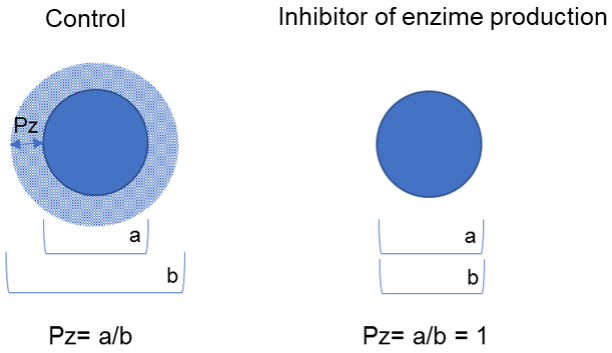

Fig. 3

Figure 4

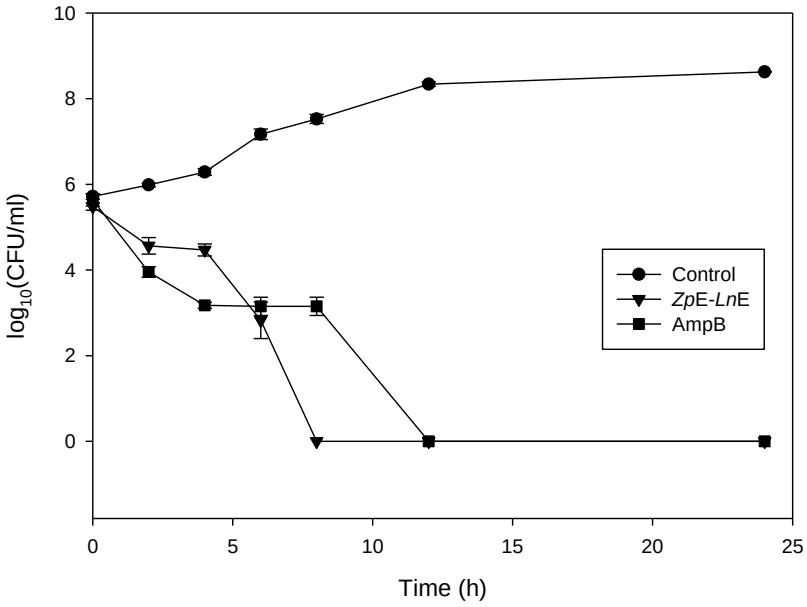

Fig. 4

Figure 5

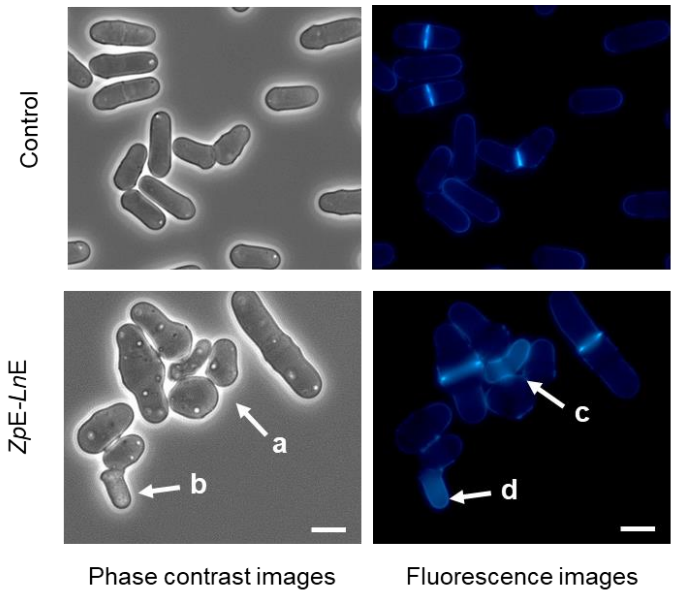

Fig. 5

Figure 6

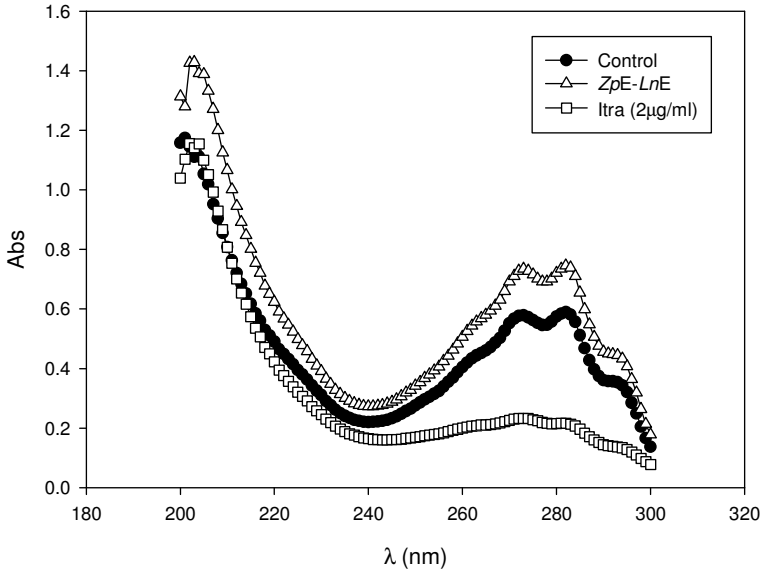

Fig. 6

Figure 7

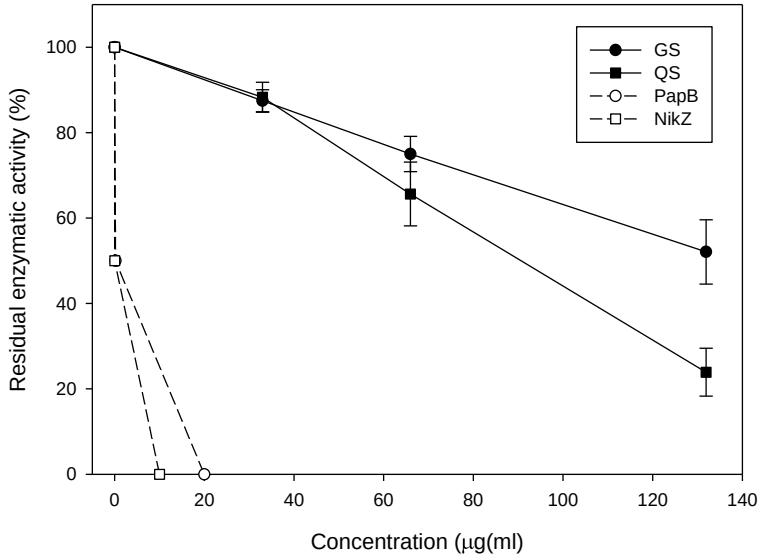

Fig. 7

Figure 8

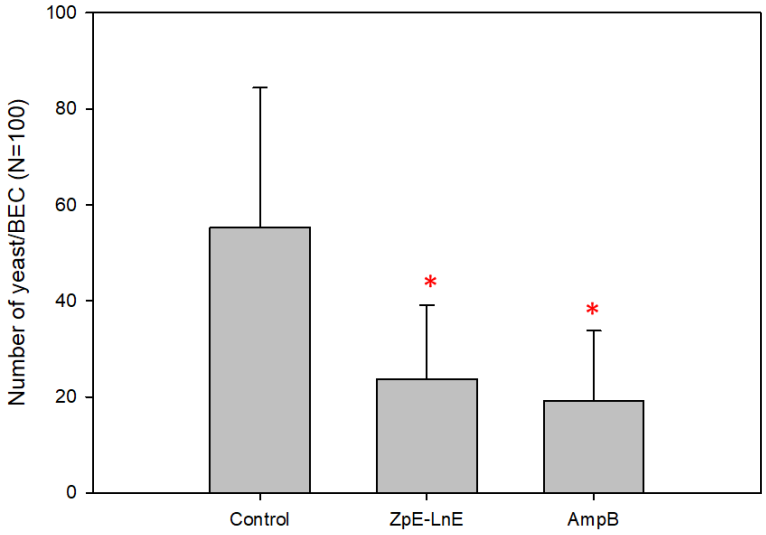

Fig. 8

Figure 9

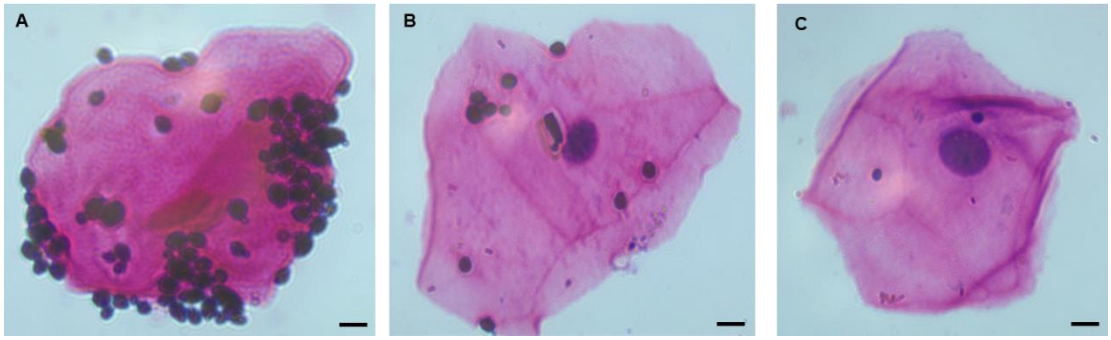

Fig. 9

Figure 10

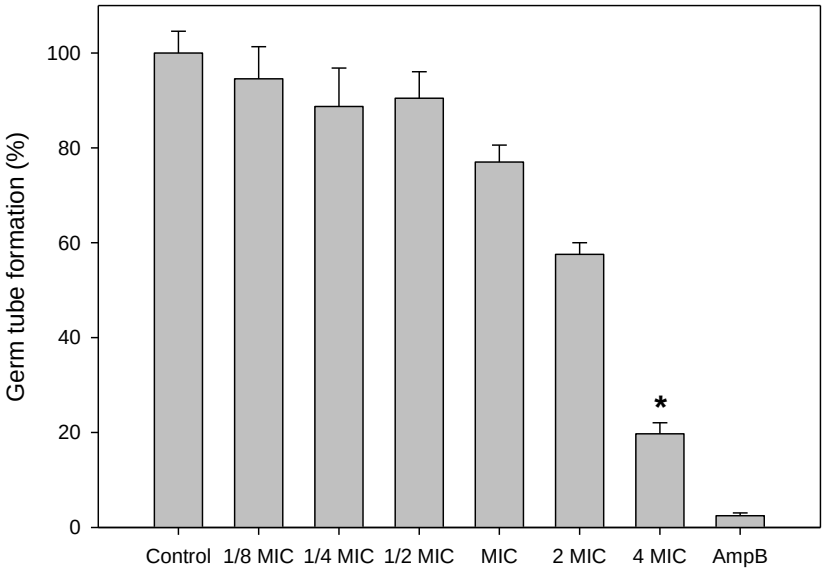

Fig. 10

Figure 11

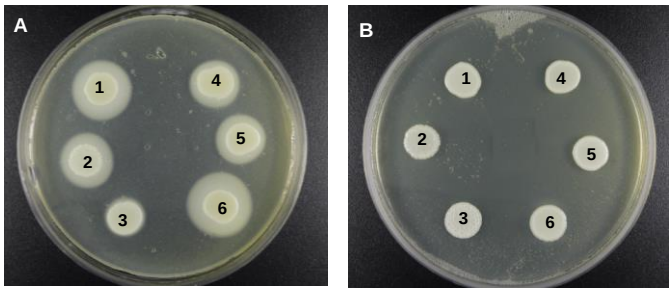

Fig. 11

Figure 12

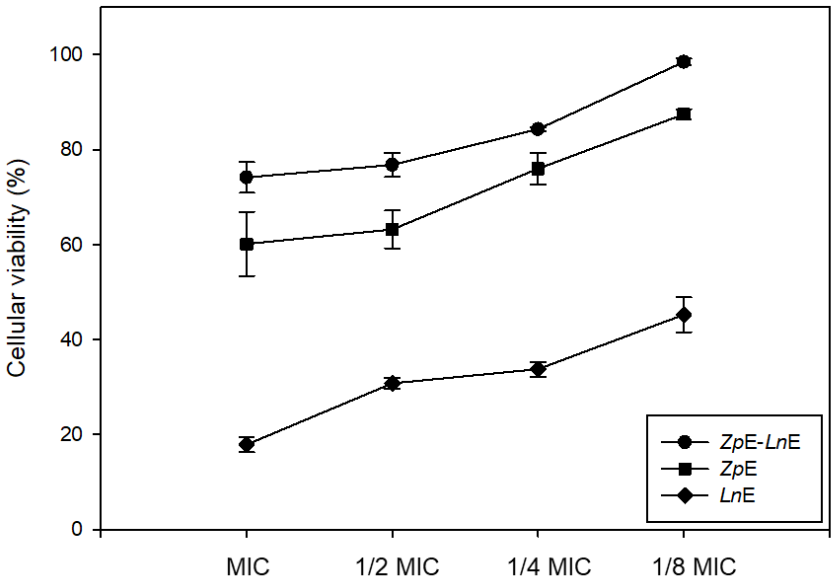

Fig. 12

Table 1

Table 1: MIC and MFC (µg/ml) of the combination *Zp*E-*Ln*E and each extract *Zp*E and *Ln*E alone, against *C. albicans* (*Ca*) CCC 125.

	*Zp*E-*Ln*E	*Zp*E in the combination (*Zp*Ec)	*Ln*E in the combination (*Ln*Ec)	*Zp*E alone (*Zp*E)	*Ln*E alone (*Ln*E)	DRI *Zp*E= *Zp*E/*Zp*Ec	DRI *Ln*E= *Ln*E/*Ln*Ec	AmpB
MIC	65.96	18.84	47.12	70.36	136.22	3.73	2.89	1.0
MFC	131.92	37.68	94.24	140.72	272.44	3.73	2.89	2.0

MIC= Minimum Inhibitory Concentration; MFC: Minimum Fungicide Concentration; *Zp*E: *Zuccagnia punctata* exudate; *Ln*E: *Larrea nitida* exudate; DRI= Dose Reduction Index; AmpB: Amphotericin B.

Table 2

Table 2: Exogenous ergosterol effect. MICs (μg/ml) of the most synergistic mixtures *Zp*E-*Ln*E against *Candida albicans* CCC 125, either in the absence (0 μg/ml) or in the presence (50, 100 or 200 μg/ml) of exogenous ergosterol. Amphotericin B (AmpB) was used as standard drug.

Ergosterol concentration	0 μg/ml	50 μg/ml	100 μg/ml	200 μg/ml
*Zp*E-*Ln*E	65.96 μg/ml (MIC)	131.92 μg/ml (2x MIC)	263.84 μg/ml (4x MIC)	527.68 μg/ml (8x MIC)
AmpB	0.25 (MIC)	4 (16x MIC)	>4 (>16x MIC)	>4 (>16x MIC)

Table 3

Table 3: Mean ergosterol content (expressed as a % of the wet weight of the cell ± SD) of *Candida albicans* cells grown either without (control) or with any of both, the mixture *Zp*E-*Ln*E or the positive control drug Itraconazole (at 1x MIC).

	(Ergosterol (A) +24(28)-dehydroergosterol (B)	24(28)-Dehydroergosterol (B)	Ergosterol (A)
Control	0.051 ± 0.08	0.015 ± 0.01	0.036 ± 0.01
*Zp*E-*Ln*E	0.222 ± 0.10	0.065 ±0.07	0.156 ± 0.06
Itraconazole	0.027 ± 0.08	0.017 ± 0.02	0.009 ± 0.01

Table 4

Table 4: Phospholipases secretion measured in terms of Pz (precipitation or halo zone) indices in six *Candida albicans* (*Ca*) colonies either without (control) or with *Zp*E-*Ln*E (at 1x MIC). Each value represents the mean ± SD of three different determinations. Pz index = 1: negative (no phospholipases secretion). The lower the Pz index, the higher phospholipases production.

C. albicans strains voucher number	Pz index control	Pz index *Zp*E-*Ln*E
CCC 125	0.60 ± 0.05	1.00 ± 0.00
CCC 130-15	0.67 ± 0.10	1.00 ± 0.00
CCC 132-15	0.96 ± 0.01	1.00 ± 0.00
CCC 182-13	0.69 ± 0.03	1.00 ± 0.00
CCC 193-13	0.69 ± 0.12	1.00 ± 0.00
CCC 131-15	0.56 ± 0.06	1.00 ± 0.00

Table 5

Table 5: Proteinases secretion measured in terms of Pz (precipitation or halo zone) indices in *Candida albicans* strains either without (control) or with *Zp*E-*Ln*E (at 1x MIC). Each value represents the mean ± SD of three different determinations. Pz index = 1: negative (no proteinases secretion). The lower the Pz index value, the higher proteinases production.

C. albicans strains voucher number	Pz index control	Pz index *Zp*E-*Ln*E
CCC 125	0.70 ± 0.05	1.00 ± 0.00
CCC 130-15	0.43 ± 0.02	0.42 ± 0.03
CCC 131-15	0.89 ± 0.03	1.00 ± 0.00

Table 6

Table 6: Haemolysins secretion measured in terms of Pz (precipitation or halo zone) indices in *Candida albicans* colonies either without (control) or with *Zp*E-*Ln*E (at 1x MIC). Each value represents the mean ± SD of three different determinations. Pz index = 1: negative (no haemolysins production). The lower the Pz index value, the higher haemolysins production.

C. albicans strains voucher number	Pz index control	Pz index *Zp*E-*Ln*E
CCC 115	0.83± 0.03	0.83 ±0.08
CCC 130-15	0.92 ± 0.06	0.90 ± 0.03
CCC 132-15	0.89 ± 0.09	0.83 ±0.05
CCC 131-15	0.90 ± 0.08	0.77± 0.10
CCC 129-15	0.91 ±0.06	0.80± 0.06

Table 7

Table 7: Cellular viability of Huh7 cells in the presence of *Zp*E, *Ln*E and *Zp*E-*Ln*E at different concentrations: 1, 1/2**,** 1/4 and 1/8x MIC. Viability of treated cells was determined by MTT assay. Values are expressed as mean ± SD, determined in triplicate.

	*Zp*E		*Ln*E		*Zp*E-*Ln*E	
	Conc. (µg/ml)	Cellular viability (%)	Conc. (µg/ml)	Cellular viability (%)	Conc. (µg/ml)	Cellular viability (%)
1x MIC	70.36	60.13 ± 6.75	136.22	17.88 ± 1.63	65.95 (18.84/47.12)	74.12 ± 3.25
1/2x MIC	35.18	63.16 ±3.97	68.11	30.78 ± 1.16	32.98 (9.42/23.56)	76.76 ± 2.52
1/4x MIC	17.59	75.96 ± 3.41	34.06	33.76 ± 1.54	16.49 (4.71/11.78)	84.29 ± 0.42
1/8x MIC	8.8	87.40 ± 1.09	17.03	45.23 ± 3.68	8.24 (2.36/5.88)	98.45 ± 0.67

Supplementary material Fig. S1

Approaches to the mechanism of antifungal activity of *Zuccagnia punctata*-*Larrea nitida* bi-herbal combination

Estefania Butassi[1], Laura A. Svetaz[1], Maximiliano A. Sortino[1,2], Ariel D. Quiroga[3,4], Vanessa S. D. Carvalho[5], Juan C. G. Cortés[5], Juan C. Ribas[5], Susana A. Zacchino[1,*]

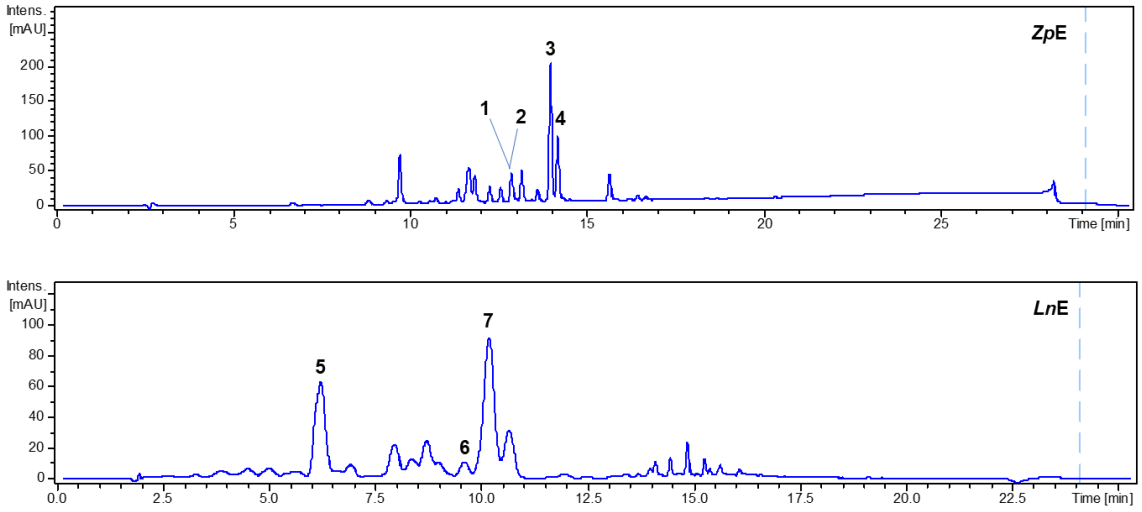

Fig. S1: HPLC-UV chromatograms of *Zuccagnia punctata* exudate (*Zp*E) (at 254 nm) and *Larrea nitida* exudate *Ln*E (at 280 nm).used in preparation of the *Zp*E-*Ln*E Marker compounds of *Zp*E and their retention times (Rt, min) are: galangin (**1**) 12.9 min; pinocembrin (**2**) 12.9 min; 2´,4´-dihydroxychalcone (**3**) 14.0 min; 2´,4´-dihydroxy-3´-methoxy chalcone (**4**) 14.9 min. Marker compounds of *Ln*E and their Rt are: NDGA (**5**) 6.2 min; DNDGA (**6**) 9.6 min; MNDGA (**7**) 10.1 min. More details in Butassi et al., 2015