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Synergistic mutual potentiation of antifungal activity of *Zuccagnia punctata* Cav. and *Larrea nitida* Cav. extracts in clinical isolates of *Candida albicans* and *Candida glabrata*

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ABSTRACT

Background: *Zuccagnia punctata* Cav. (Fabaceae) and *Larrea nitida* Cav. (Zygophyllaceae) are indistinctly or jointly used in traditional medicine for the treatment of fungal-related infections. Although their dichloromethane (DCM) extract have demonstrated moderate antifungal activities when tested on their own, antifungal properties of combinations of both plants have not been assessed previously.
Purpose: The aim of this study was to establish with statistical rigor whether *Z. punctata* (*Zp*E) and *L. nitida* DCM extract (*Ln*E) interact synergistically against the clinically important fungi *Candida albicans* and *Candida glabrata* and to characterize the most synergistic combinations.
Study design: For synergism assessment, the statistical-based Boik's design was applied. Eight *Zp*E–*Ln*E fixed-ratio mixtures were prepared from four different months of 1 year and tested against *Candida* strains. L_ϕ (Loewe index) of each mixture at different fractions affected (ϕ) allowed for the finding of the most synergistic combinations, which were characterized by HPLC fingerprint and by the quantitation of the selected marker compounds.
Methods: L_ϕ and confidence intervals were determined *in vitro* with the MixLow method, once the estimated parameters from the dose–response curves of independent extracts and mixtures, were obtained. Markers (four flavonoids for *Zp*E and three lignans for *Ln*E) were quantified in each extract and their combinations, with a valid HPLC–UV method. The 3D-HPLC profiles of the most synergistic mixtures were obtained by HPLC–DAD.
Results: Three over four IC_{50}*Zp*E/IC_{50}*Ln*E fixed-ratio mixtures displayed synergistic interactions at effect levels $\phi > 0.5$ against *C. albicans*. The dosis of the most synergistic ($L_\phi = 0.62$) mixture was 65.96 µg/ml (*Zp*E = 28%; *Ln*E = 72%) containing 8 and 36% of flavonoids and lignans respectively. On the other hand, one over four IC_{50}*Zp*E/IC_{50}*Ln*E mixtures displays synergistic interactions at $\phi > 0.5$ against *C. glabrata*. The dosis of the most synergistic ($L_\phi = 0.67$) mixture was 168.23 µg/ml (*Zp*E = 27%; *Ln*E = 73%) with 9.7 and 31.6% of flavonoids and lignans respectively.
Conclusions : Studies with the statistical-based MixLow method, allowed for the finding of the most *Zp*E–*Ln*E synergistic mixtures, giving support to a proper joint use of both antifungal herbs in traditional medicine.

Introduction

In 2012, the Ministry of Science, Technology and Productive Innovation of Argentina has launched a National Strategic Plan (2012–2015) for developing phytomedicines containing native or endemic plants based on the previous investigations conducted by research groups of this country.

Our group has already carried out a project devoted to the search for antifungal Latin American plants (Svetaz et al., 2010) and also to the isolation of their main antifungal components

Abbreviations: *Zp*E, *Zuccagnia punctata* dichloromethane extract; *Ln*E, *Larrea nitida* dichloromethane extract; L_ϕ, Loewe index; CI, combination index; DRI, dose reduction index; IC_x, inhibitory concentration to achieve *X*% effect; *Ca*, *Candida albicans*; *Cg*, *Candida glabrata*; NDGA, nordihydroguaiaretic acid; DNDGA, 3''-deoxy NDGA; MNDGA, 3′-*O*-methyl NDGA; MixLow, mixed-effects Loewe method; LOD, limit of detection; LOQ, limit of quantification; S, synergism; An, antagonism.

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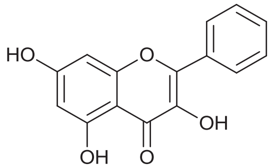
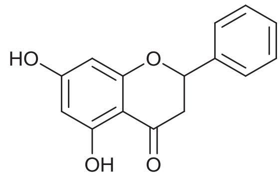
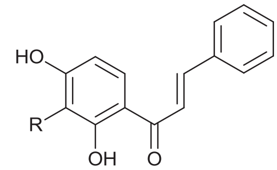


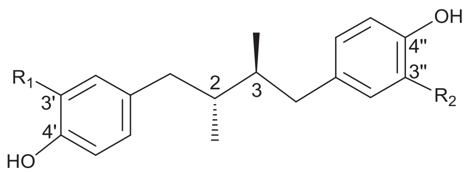
1
galangin



2
pinocembrin



3 R= H; 2',4'-dihydroxychalcone
4 R= OCH_3; 2',4'-dihydroxy-3'-methoxychalcone



5 R_1= OH R_2= OH; nordihydroguaiaretic acid
6 R_1= OH R_2= H; 4-[4-(4-hydroxyphenyl)-2,3-dimethylbutyl]-benzene-1,2-diol
7 R_1= OCH_3 R_2= OH; 3′-methyl nordihydroguaiaretic acid

Fig. 1. Isolated compounds from *Zp*E (**1–4**), and for *Ln*E (**5–7**).

(Escalante et al. 2008; Pacciaroni et al. 2008; Derita et al. 2009; Vila et al. 2010; López et al. 2011; Fernández et al. 2014; and others). Among the tested antifungal plants, two species, *Zuccagnia punctata* Cav. (Fabaceae) (Ulibarri, 1999, 2005) and *Larrea nitida* Cav. (Zygophyllaceae) showed moderate antifungal properties against clinical important fungi (Svetaz et al., 2007; Agüero et al., 2010, 2011; Alvarez et al., 2012; Nuño et al., 2014).

Z. punctata (common names "*jarilla macho*", "*jarilla de la puna*", "*laca*" or "*pus-pus*") is a monotypic species (Ulibarri, 2005) currently used in traditional medicine for bacterial and fungal infections. Moreover, this species can also be used for other pathologies like asthma, arthritis, rheumatism and tumors (Ratera and Ratera, 1980). During many years, *Z. punctata* has been described as growing in Chile and Argentina (Ulibarri, 1999). However, a thorough study based on its botanical and bibliographic collections, allowed to demonstrate that the shared distribution with Chile was erroneous and thus it was firmly established that *Z. punctata* is endemic of Argentina (Ulibarri, 2005).

Among the different extracts previously tested for antifungal properties, the dichloromethane (DCM) extract (*Zp*E) was the most active one (Svetaz et al., 2007; Agüero et al., 2010). This extract led to the isolation of several compounds of which 5,7-dihydroxi-3-flavonol (galangin, **1**), 5,7-dihydroxiflavanone (pinocembrin, **2**) and 2′,4′-dihydroxychalcone (**3**) showed antifungal properties while 2′,4′-dihydroxy-3′-methoxychalcone (**4**) (Fig. 1) was the most abundant (though inactive) compound. The other compounds did not show significant activity in concentrations up to 250 μg/ml.

In turn, the native plant *L. nitida* (common names "*jarilla de la sierra*" and "*jarilla fina*") (Del Vitto et al., 1997) is one of the four South American species of *Larrea* genus (Timmermann et al., 1979) that grows in Argentina and Chile (Hunziker, 2005). Antioxidant and antifungal activities were previously reported for this species (Torres et al., 2003; Agüero et al., 2011), being the DCM extract (*Ln*E) the most active one. Its bioassay-guided fractionation led to the isolation of five lignans of which only three, nordihydroguaiaretic acid (NDGA, **5**), 4-[4-(4-hydroxy-phenyl)-2,3-dimethyl-butyl]-benzene-1,2-diol (DNDGA, **6**) and 3′-*O*-methyl-nordihydroguaiaretic acid (MNDGA, **7**) (Fig. 1) showed moderate antifungal properties (Agüero et al. 2011).

Although *L. nitida* is less abundant and grows in more restricted regions, it is indistinctly or conjunctly used with *Z. punctata* due to their similar morphological characteristics and also to their common name (*jarilla*) (Del Vitto et al., 1997). However, until now, their combinations were used in an empirical basis, as no mixtures have been scientifically assessed by their type of interaction.

The use of bi-herbal mixtures 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). However, antagonistic or additive effects can be also found between the components of a plant combination (Williamson 2001; Odds 2003).

The study of antifungal interactions between two extracts is a complex task. The fungi target, the ratio between extracts in the mixture and the methodology for quantifying synergism must be chosen carefully in order to achieve trustworthy conclusions. In addition, since extracts are complex mixtures that usually present seasonal variations, several characterized batches must be combined in order to get quantified mixtures that display the highest synergism.

In this paper we report the study of the antifungal behavior of four bi-herbal *Zp*E–*Ln*E combinations, each of them prepared by mixing one sample of *Zp*E with one sample of *Ln*E collected in a same period of a year. This was made for the four periods in which the plants were collected.

Candida albicans and *Candida glabrata* were used as targets for the antifungal evaluation. The choice of *C. albicans* was due to this yeast is the most common cause of opportunistic fungal infections in immune compromised hosts (Pfaller and Diekema, 2007). In turn, the selection of *C. glabrata* was made because it has been identified as the second leading cause of adult candidemia particularly in patients with hematologic malignancies (Malani et al., 2005; Pfaller and Diekema, 2007).

Among the several mathematical methods to quantify synergism that have been proposed over the last few decades (Berembaum 1989; Merlin 1994; Greco et al. 1995; Tallarida 2001; Chou 2006; Boik et al. 2008), here we chose the mixed-effects Loewe (MixLow) method (Boik et al. 2008) to determine the Loewe index (L_ϕ) previously defined as combination index (CI) within the median-effect method (Chou 2006). Both the MixLow and the median-effect methods share the following characteristics: (i) assess the data from single ray fixed-ratio experiments; (ii) allow the identification of the optimal concentration (within the fixed-ratio) that will give the maximum synergy; (iii) present the results in a graphical form. However, MixLow method has the advantage over the median-effect that it allows for the statistically comparison of the combinations' effects by providing accurate dose–response curves' parameters and confidence