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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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Keywords:

Zuccagnia punctata
Larrea nitida
Bi-herbal combinations
Synergism
Antifungal
MixLow method

Abbreviations

*Zp*E	*Zuccagnia punctata* dichloromethane extract
*Ln*E	*Larrea nitida* dichloromethane extract
L_ϕ	Loewe Index
CI	Combination Index
DRI	Dose Reduction Index
ICx,	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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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 one 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 (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).

INSERT FIG. 1

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 for 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 intervals for L_ϕ that are vital to fully assess whether drugs in a mixture interact synergistically, antagonistically or additively.

As a result of this study, we could determine the L_ϕ of each mixture at different effects (ϕ), and the composition of the extracts in the two most synergistic *Zp*E-*Ln*E combinations against each fungus. The content of the selected markers in both combinations was determined by HPLC-UV following the International Conference on Harmonization guidelines (ICH 1996). The 3D-HPLC profiles of both synergistic combinations were provided too.

Materials and methods

Plant material

Aerial parts (AP) of *Z. punctata* and *L. nitida* (www.theplantlist.org) were collected over four periods of 2013 (February, May, September and November) at Las Flores and Bauchaceta towns, Agua Negra pathway, Iglesia district, San Juan province (Argentina), respectively. The plants were identified by Dr. Martin Hadad, Instituto de Biotecnología (IBT), Universidad Nacional de San Juan (UNSJ) and each batch was deposited at the herbarium of the IBT-UNSJ and identified as *Zp*LF AT-IBT 02 (abbreviated as *Zp*E Feb), *Zp*LF AT-IBT 05 (*Zp*E May), *Zp*LF AT-IBT 09 (*Zp*E Sept), and *Zp*LF AT-IBT 11 (*Zp*E Nov), *Ln*Bau AT-IBT 02 (*Ln*E Feb), *Ln*Bau AT-IBT 05 (*Ln*E May), *Ln*Bau AT-IBT 09 (*Ln*E Sept) and *Ln*Bau AT-IBT 11 (*Ln*E Nov).

Preparation of extracts

*Zp*Es and *Ln*Es were obtained by dipping fresh AP (500g) in cold DCM (3 x 1l) at room temperature for 40 s. The solutions were evaporated under vacuum to give semisolid yellow residues (*Zp*E Feb, 65 g, 13% w/w yield; *Zp*E May, 55 g, 11%; *Zp*E Sept, 60 g, 12%; *Zp*E Nov, 50 g, 10%. *Ln*E Feb, 50 g, 10%; *Ln*E May, 42.5 g, 8.5%; *Ln*E Sept, 45 g, 9% and *Ln*E Nov, 49 g, 9.8%).

Source of markers

*Zp*E's markers galangin (**1**) and pinocembrin (**2**) and *Ln*E's marker NDGA (**5**) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Instead, 2',4'-dihydroxychalcone (**3**), 2',4'-dihydroxy-3'-methoxychalcone (**4**) and MNDGA (**7**) were obtained in our laboratory from *Zp*E or *Ln*E as previously described (Svetaz et al. 2007; Agüero et al. 2010, 2011). The purities of reference compounds were ≥ 95% as determined by HPLC-DAD.

Preparation of extracts' and markers' solutions

Acetonitrile (ACN) solutions of *Zp*E and *Ln*E from Feb, May, Sept and Nov and of **1-7** were prepared at an appropriate concentration, filtered through a 30 mm, 0.45 µm Target Nylon Membrane Syringe Filter (Scientific Instrument Services, Ringoes, NJ) prior to injection into the HPLC system.

HPLC-ESI-MS/MS analyses

HPLC-ESI-MS/MS analyses of all batches of *Zp*E and *Ln*E were carried out by using a MicroTOFQ II apparatus (Bruker Daltonics, MA, USA), equipped with an ESI ion source with nitrogen as nebulizing gas (4 psi) and drying gas (8 l/min, 200 °C); capillary 4500 V and end plate offset at 500 V. Mass accuracy was verified by infusing a 10 mM solution of Na-formiate (Sigma-Aldrich) dissolved in MeOH:H_2O (50:50) and a C18-RP column Thermo Scientific (USA) Hypersil Gold (50 x 2.1 mm, 3 µm). Firstly, pure compounds were characterized by direct infusion to ESI using a syringe pump (Harvard Apparatus 11 Plus) recording both MS and MS/MS spectra. Because of the improved performance reached using direct infusion to ESI, we report only MS and MS/MS data obtained in the negative mode. Reference compounds and sample solutions were introduced in the HPLC (5 µl) using an autosampler (Agilent HiP-ALSSL+) at 25 °C. Flow rate was set to 0.20 ml/min and 0.25 ml/min for *Zp*E and *Ln*E samples and their own reference compounds, propelled by an Agilent 1200 series G1312B SL binary pump, using ultra-pure water or HPLC-grade ACN with 0.1% formic acid (solutions A and B respectively) following different methods: for *Zp*E extracts and reference compounds, the gradient started with 20% B, changing to 100% B within 10 min, then the composition was held 2 min, returning to 20% B in 3 min and keeping this condition for additional 5 min to achieve the column stabilization before the next run (total run time was 20 min). Eluted compounds were monitored at 254 nm. For *Ln*E extracts and reference compounds, the program started with 40% B, changing to 45% B within 10 min, and to 100% B the following 5 min, held by 4 min and returning to 40% B in 1 min (total run time 20 min). Eluted compounds were monitored at 280 nm. The HPLC flow was introduced into the Mass spectrometer *via* an ESI source. For the analyses of chromatograms and mass spectra, the Data Analysis 4.0 SP1 software (Bruker Daltonik GmgH, Germany) was used. Compounds **1-7** were identified by comparing their retention times (Rt), HRMS and MS/MS with those of reference compounds (Agüero et al. 2010, 2011). Markers´ content was quantified in the extracts of the different periods using the HPLC-UV chromatograms. Five calibration curves of pure compounds **1-5** were prepared with five appropriate dilutions of stock ACN solutions by triplicate. To monitor the samples, the wavelengths were chosen according to absorption maxima of markers: 267 nm (**1**), 289 nm (**2**), 341 nm (**3** and **4**) and 280 nm (**5**).

Method validation

Analytical method´s linearity, limit of detection and quantification (LOD and LOQ) and inter-day / intra-day precision were validated following the ICH guidelines (ICH 1996). Recovery was used to evaluate the accuracy of the method.

Strains used for the antifungal assessment of extracts and mixtures

For the antifungal evaluation of independent extracts and their combinations, clinical isolates of *C. albicans* (CCC 125) and *C. glabrata* (CCC 115) provided by Centro de Referencia en Micología (CEREMIC, CCC, Rosario, Argentina), were used. Strains were grown on Sabouraud-chloramphenicol agar slants for 48 h at 30 °C, maintained on Sabouraud-Dextrose Agar (SDA, Oxoid) and sub-cultured every 15 days to prevent pleomorphic transformations. Inocula were adjusted to 1-5 x 10^3 cells with colony forming units (CFU)/ml according to the Clinical and Laboratory Standards Institute (CLSI 2008).

Synergism studies design

The nature of the interaction between *Zp*E and *Ln*E in the presence of either *C. albicans* or *C. glabrata* inoculum, was analyzed by the L_ϕ (CI) defined by Eq. (1).

$$L\phi = \frac{\text{ICx } Zp\text{E in mixture}}{\text{ICx } Zp\text{E alone}} + \frac{\text{ICx } Ln\text{E in mixture}}{\text{ICx } Ln\text{E alone}} \quad (1)$$

Where ICx is the inhibitory concentration used to achieve X% effect (measured as diminution of fungal growth) of each extract alone and in the mixture. The estimation of L_ϕ (Boik et al. 2008) was a three-step (a-c) process (Scheme 1): (a) preparation of dose-response curves for each extract alone and for their combinations at a wide range of concentrations. Estimation of parameters that define the shape of each dose-response curve by using a non-linear regression model (outlined in Scheme 1, a_1, a_2); (b) use of the estimated parameters for calculating L_ϕ; (c) generation of confidence intervals for L_ϕ.

INSERT SCHEME 1

Dose-response curves of the independent extracts against Candida strains

Broth microdilution techniques were performed in 96-well microplates according to the M27-A3 document of CLSI for yeasts (CLSI 2008). Each plate was designed as follows (Boik et al. 2008): 10 different concentrations of each extract (wells 3-12, Fig. 2), by sextuplicate (wells A-F, Fig. 2) were prepared [T wells (= 60), Fig. 2] from DMSO (maximum concentration ≤ 1%) 0stock solutions of each extract, diluted with RPMI-1640. In addition, 36 control wells [12 treatment control growth wells (TC) containing culture medium plus inoculum; 4 medium control wells (MC) (culture medium plus water) and 20 blanks-by-tray

control wells (BT) (extracts plus water)] were included in the plate (Fig. 2). Each assay was repeated thrice. So, 18 replicates of each concentration (i.e. (3A-3F) x3; (4A-4F) x3, and so on) were prepared.

INSERT FIG. 2

In each plate, *Zp*E and *Ln*E concentrations ranges were selected in order to cover growth percentages within 95-5% range. To better distribute the points in *x*-axis, and therefore obtain a better adjustment of the sigmoidal curve, equi-spaced concentrations in the logarithmic scale (ln) were prepared (Table S1 of Supplementary Material). An inoculum suspension (100 µl) was added to each well (final well volume = 200 µl) and plates were incubated 24 h at 28-30 ºC in a moist dark chamber. After incubation, the wells' absorbance values were recorded at 405 nm with a VERSA Max microplate reader (Molecular Devices, Sunnyvale, CA, USA) and introduced in the MixLow package of R software (Boik and Narasimhan 2010). Dose-response curves modeled as a sigmoidal function of responses $Y_{d,t,w}$ (*y*-axis) and extracts' concentrations (*c*) (*x*-axis) (Eq. 2 and 3) were generated.

$$Y_{d,t,w} = \exp(\mu + b_t)\left(1 - \phi_{d,w,t}\right) + \epsilon_{d,t,w} \quad (2)$$

where

$$\phi_{d,t,w} = 1 - \frac{1}{1 + \left(\frac{\exp(c_{d,t,w})}{\exp(\psi_{d,0.5})}\right)^{\gamma_d}} \quad (3)$$

ϕ = E[*Fa*], where *Fa* (fraction affected) represents the fraction of fungi affected by an extract at a concentration *c*, and E[•] is the expected value; $c_{d,t,w}$ refers to the log of the extract's concentration for the *d* (d^{th} extract), *t* (t^{th} tray), *w* (w^{th} well) that is a known value. Each sigmoidal curve is parameterized by three constants: $\psi_{d,0.5}$, γ_d and μ (Scheme 1, a_1). The parameter $\psi_{d,0.5}$ is the log concentration of each extract that produce a $\phi = 0.50$ (50% inhibition relative to controls). By convention, $\exp(\psi_{d,0.5})$ is called the IC_{50}; γ_d is a shape parameter and µ is the mean of control wells from all trays (Boik et al. 2008).

Dose-response curves of mixtures against Candida strains

Mixtures of *Zp*E and *Ln*E were prepared by mixing the mass that produces an equipotent effect of each partner, in the fixed ratio IC_{50}*Zp*E/IC_{50}*Ln*E and submitted to antifungal evaluation in 96-wells microplates with the same experimental design of independent extracts. In brief, within each plate, 10 different concentrations of each fixed-ratio were prepared (Table S2 of Supplementary material). Each sigmoidal curve is parameterized by three constants: $\psi_{m,0.5}$, γ_m and μ (Scheme 1, a_2).

Quantification of synergism

Once obtained the estimated parameters from the dose-response curves of the independent extracts ($\psi_{d,0.5}$ and γ_d) and mixtures ($\psi_{m,0.5}$ and.γ_m), the MixLow package can calculate the L_ϕ at different ϕ effects using Eq. (4).

$$L_\phi = \sum_{d=1}^{n} \tau_d \exp\left(\log\left(\left(\frac{\phi}{1-\phi}\right)^{1/\gamma_m}\right) + \psi_{m,0.5} - \log\left(\left(\frac{\phi}{1-\phi}\right)^{1/\gamma_d}\right) - \psi_{d,0.5}\right) \quad (4)$$

where τ_d is the fraction of the mixture that is composed of extract *d*.

The confidence intervals for the L_ϕ at different ϕ are calculated with Eq. (5).

$$\exp(\log(L_\phi) \pm t_{df,1-\frac{\alpha}{2}} \mathrm{SE}(\log L_\phi)) \quad (5)$$

Dose Reduction Index (DRI)

The DRI is a measure of how many times the dose of each drug in a synergistic combination is reduced at a given effect level compared with the doses of each independent drug. The DRI value for each drug is calculated using Eq. 6 (Chou 2006). A greater DRI indicates a greater dose reduction for a given effect level.

$$\mathrm{DRI} = \frac{\text{ICx extract alone}}{\text{ICx extract in mixture}} \quad (6)$$

3D-HPLC profile

The most synergistic mixtures were dissolved in ACN, filtered by a membrane filter and subjected to HPLC analysis in a Hewlett Packard 1050 (Palo Alto, CA), coupled to a DAD detector (HP/Agilent series 1050 DAD), with a quaternary pump and autosampler (HP/Agilent series 1050) and Luna C18-RP column (Phenomenex) of 25 cm x 4.6 mm, 5 µm of particle size. The isocratic solvent phase was composed of ultra-pure water supplemented with formic acid 0.1% (40%) and HPLC-grade ACN (60%). The flow rate was 0.5 ml/min and the injection volume, 5 µl. Peaks in the extracts, monitored at λ = 254 nm, were assigned based on the Rt of reference compounds.

Statistical analysis

Statistical analysis was performed with GraphPad Prism™ 4.0 (GraphPad software Inc., La Jolla, CA). The data were analyzed using both, Kruskal-Wallis test (non-parametric ANOVA) and Dunn test to verify the difference between treatments; *p* values < 0.05 were considered significant.

Results and discussion

Markers for HPLC analyses

The choice of markers **1-7** (Fig. 1) was based on our previous reports. Flavonoids **1-3** and lignans **5-7** were considered active markers (EMEA 2007) due to they previously showed to be the main anti-*Candida* constituents of ZpE and *Ln*E respectively (Svetaz et al. 2007; Agüero et al. 2010, 2011) and **4** was considered an analytical marker (EMEA 2007) since, although inactive, it was present in high amounts in *Zp*E (Agüero et al. 2010).

HPLC fingerprints of ZpE and LnE batches

HPLC fingerprints of *Zp*E and *Ln*E batches (Feb, May, Sept and Nov), are shown in Fig. 3; **1-4** and **5-7** were unequivocally identified (Rt, HRMS and MS/MS data) (Table 1) in each batch of *Zp*E, and *Ln*E respectively (Agüero et al. 2011).

INSERT FIG. 3 and TABLE 1

Quantitative assessment of markers in ZpE and LnE batches

The quantification of **1-4** and **5-7** was made by HPLC-UV in each batch of *Zp*E or *Ln*E respectively. HPLC method was first validated for linearity, LOD and LOQ, precision and accuracy following ICH guidelines (ICH 1996), with compounds **1-5**. Compounds **6** and **7** of *Ln*E were quantified by the relative response factors (Gao et al. 2009) based on the calibration curve of **5**.

Linearity and calibration curves: Linearity of pure **1-5** calibration curves was established by calculating the slope, intercepts and R^2 coefficient. The regression equation and R^2 (0.99-1) showed good linearity response in the ranges detailed in Table 2. LOD and LOQ were calculated as 3.3 σ/S and 10 σ/S respectively, being σ the response standard deviation, and S the slope of each marker.

Precision: Intra- and inter- day variability test was determined for three times within 1 day and 3 separated days at three different concentrations, respectively. Variations were expressed by the relative standard deviations (RSD) (Table 2), confirming the precision of the proposed method.

Accuracy: Three concentrations of pre-analyzed sample solutions were spiked with known quantities of the standards and injected in triplicate to perform recovery studies. The percentage recovery for **1-5** were between 90.6-106.6% (RSD < 4%, n = 3), confirming the accuracy of the proposed method.

INSERT TABLE 2

Results of quantitative analyses of markers in the eight extracts are shown in Table 3.

INSERT TABLE 3

The non-parametric Kruskall-Wallis test found significant differences between the medians of markers content (data not shown) for all the periods ($p < 0.05$). The Dunn test showed statistically significant differences ($p < 0.05$) in: (a) the content of **1**, **2** and **4** between *Zp*E Feb and *Zp*E May;

(b) the content of **3** between *Zp*E Sept and *Zp*E Nov; (c) the content of **5** and **7** between *Ln*E Feb and *Ln*E Nov and (d) the content of **6** between *Ln*E May and *Ln*E Nov. This variation in markers' composition among batches justified the preparation of mixtures with each of them, since the interactions would be different.

Statistically supported synergism assessment by using fixed-ratio mixtures

Each *Zp*E or *Ln*E batch was tested against the yeasts *C. albicans* and *C. glabrata* in 96-wells microplates prepared as explained in Materials and Methods (Fig. 2).

With the absorbance values obtained from 18 replicates of each concentration, a sigmoidal dose-response curve for each *Zp*E or *Ln*E batch was constructed and analyzed with the MixLow method, which gave the estimated parameters $\psi_{d,0.5}$ [and thus $\exp(\psi_{d,0.5}) = IC_{50}$], γ_d and μ, which are shown in Table S3. Table 4 (columns 3 and 4) shows the IC_{50} values of each extract against each fungus.

INSERT TABLE 4

Ten different concentrations of each IC_{50}*Zp*E/IC_{50}*Ln*E fixed-ratio mixture were prepared according to Materials and Methods (Table 4, column 5). Each fixed-ratio concentrations' set can be regarded as a ray, in a so-called "ray design". All sets were tested against either *C. albicans* or *C. glabrata* in 96-wells plates following the same design used for the independent extracts. After incubation, the absorbance values were introduced in the MixLow package of R software generating sigmoidal dose-response curves and the corresponding parameters $\psi_{m,0.5}$, γ_m and μ for all combinations (Table S3). Fig. 4 and 5 (A,B,C,D) show the dose-response curves obtained for *Zp*E and *Ln*E on their own and in fixed-ratio combinations against *C. albicans* and *C. glabrata* respectively in the four periods of the year. In Fig. 4 and 5 (A'B'C'D') the affected fraction of yeasts' population at a giving drug concentration ϕ was plotted *vs* the Loewe Index L_ϕ. In these graphs, full lines indicate the L_ϕ at different ϕ for a given mixture and dotted lines indicate the ninety-five percent confidence intervals of the index. $L_\phi = 1$ indicates additivism (Ad) or no interaction; $L_\phi > 1$ indicates antagonism (An) and $L_\phi < 1$ indicates synergism (S) (Boik et al. 2008; Liu et al. 2013). Within values of $L_\phi < 1$, the lower L_ϕ, the greater synergism (Chou 2006). As previously suggested (Chou 2006), L_ϕ = 0.91-1.09 represents near (n)Ad; L_ϕ = 0.85-0.90, slight (sl)S; 0.70-0.84, moderate (m)S; 0.30-0.69, medium (med)S; 0.10-0.29 strong (s)S and < 0.1, very strong (vs)S; 1.10-1.20 slight (sl)An; 1.21-1.45 moderate (m)An; 1.46-3.30 medium (med)An; 3.40-10, strong (s)An and >10 very strong (vs)An.

INSERT FIG. 4 AND 5

In Fig. 4 (A'-D'), it is clear that, when acting against *C. albicans*, all *Zp*E-*Ln*E mixtures showed some degree of synergism in the following ϕ ranges: $0.02 \leq \phi \leq 0.74$ (Feb); $0.02 \leq \phi \leq 0.94$ (May); $0.50 \leq \phi \leq 0.94$ (Sept.) and $0.06 \leq \phi \leq 0.94$ (Nov.). In turn, when acting against *C. glabrata* (Fig. 5, A´-D'), *Zp*E-*Ln*E Feb and May mixtures showed synergism in the ϕ ranges: $0.40 \leq \phi \leq 0.94$ and $0.02 \leq \phi \leq 0.49$ respectively, while *Zp*E-*Ln*E Sept and Nov mixtures showed antagonism ($L_\phi > 1$) at all ϕ. For the sake of clarity, the L_ϕ values and confidence intervals for ϕ = 0.50, 0.80, 0.90 and 0.95 extracted from Fig. 4 and 5 (A'-D') are recorded in Table 5.

INSERT TABLE 5

Table 5 clearly shows that the *Zp*E-*Ln*E May mixture was the most synergistic against *C. albicans*, displaying the lowest L_ϕ values = 0.62, 0.65 and 0.68 [(med)S] at ϕ = 0.80-0.95. It also showed (m)S with L_ϕ = 0.73 at ϕ = 0.50 against this clinically important fungus.

Instead, *C. glabrata* was less sensitive to the tested mixtures. The only combination that showed synergism was that of Feb, which achieved a L_ϕ= 0.67 [(med)S] when ϕ = 0.95, and L_ϕ= 0.71 or 0.77 [(m)S] at ϕ = 0.90 or 0.80. Interesting enough, all mixtures of May, Sept and Nov showed (n)Ad or (sl) or (m)An, but none of them showed S.

Composition of synergistic mixtures at different effects levels and DRI values

For the most synergistic mixtures (*Zp*E-*Ln*E May for *Ca* and *Zp*E-*Ln*E Feb for *Cg*), the concentrations of each extract in each mixture were calculated using Eq. (2) and (3) (see Materials and Methods) at ϕ = 0.50, 0.80, 0.90 and 0.95. Table 6 shows that for achieving an effect level of ϕ= 0.95 against *C. albicans*, the sum of *Zp*E and *Ln*E in the mixtures should be 65.96 µg/ml (column III), composed by 18.84 µg/ml of *Zp*E (column IV) and 47.12 µg/ml of *Ln*E (column V). Instead, for achieving lower ϕ (0.9, 0.8 and 0.5), the sum of *Zp*E and *Ln*E doses were 57.70 µg/ml (*Zp*E: 16.48 and *Ln*E: 41.22 µg/ml); 49.91 (*Zp*E 14.26 and *Ln*E 35.65 µg/ml) and 38.94 µg/ml (*Zp*E 11.80 and *Ln*E 27.14 µg/ml) respectively. Instead, for achieving an effect level of ϕ = 0.95 against *C. glabrata*, the sum of *Zp*E and *Ln*E concentrations in the mixtures should be 168.23 µg/ml (column III) composed by 45.47 µg/ml of *Zp*E (column IV) and 122.76 µg/ml of *Ln*E (column V). In addition, for lower ϕ (0.90, 0.80 and 0.50), their *Zp*E and *Ln*E doses are also higher (158.56, 148.69 and 133.22 µg/ml) than those needed for inhibiting *C. albicans.*

INSERT TABLE 6

DRI values were calculated for different ϕ from Eq. 6. As shown in Table 6 (columns VII and VIII), *Zp*E in combination with *Ln*E improved 2.66 and 1.29-fold (= DRI values) the capability of inhibition of 50% or 80% of *C. albicans* growth compared to when acting on their own (IC_{50} and IC_{80} decreased from 31.40 to 11.80 µg/ml and from 45.91 to 14.26 µg/ml respectively, columns I

and IV). Interestingly, IC_{90} and IC_{95} diminished 3.48 and 3.73-fold (IC_{90}, from 57.33 to 16.48 µg/ml and IC_{95} from 70.36 to 18.84 µg/ml), indicating that a significant reduction of the doses of *Zp*E in the combination respective to the independent extract, is required for a complete inhibition of the fungus. From the point of view of *Ln*E, IC_{50}, IC_{80}, IC_{90} and IC_{95} against *C. albicans* decreased 2.71, 2.76, 2.83 and 2.89-fold respectively.

Against *C. glabrata*, *Zp*E Feb within the combination, improved 1.85 and 1.86-fold the capability of 90 or 95% growth inhibition, compared to when acting on its own (IC_{90} and IC_{95} decreased from 79.43 to 42.86 µg/ml and from 84.69 to 45.47 µg/ml respectively). Additionally, *Ln*E Feb improved 6.02 and 7.83-fold the capability of inhibition of 90 or 95% of *C. glabrata* growth in combination with *Zp*E Feb, compared to when acting on its own (IC_{90} and IC_{95} of *Ln*E decreased from 697.03 µg/ml and 960.28 µg/ml to 115.70 and 122.76 µg/ml respectively).

Markers content in most synergistic mixtures

Table 7 shows the concentrations of each marker in May and Feb mixtures at different effect levels (ϕ) against *C. albicans* and *C. glabrata*, respectively.

INSERT TABLE 7

Against *C. albicans*, the most synergistic *Zp*E-*Ln*E mixture contains 8% of flavonoids **1**-**4** and 36% of lignans **5**-**7**. Within *Zp* markers, a predominance of 2,4-dihydroxychalcone **3** (4.5%) followed by 2,4-dihydroxy-3-methoxy chalcone **4** (2.6%) and pinocembrin **1** + galangin **2** (0.9%) was observed. Within *Ln* markers, a prevalence of MNDGA **7** (18%) followed by NDGA **5** (12%) and then DNDGA **6** (6%) was observed. However, although the proportion of markers are the same for all ϕ, the doses of the whole mixtures are different for achieving the different effects ϕ = 0.50, 0.80, 0.90 and 0.95 (38.94, 49.91, 57.70 and 65.96 µg/ml respectively) (Fig. 6). Obviously, the dosis of 65.96 µg/ml for achieving a ϕ = 0.95 (95% inhibition of *C. albicans*) (with markers proportion described above) should be chosen for preparing a phytomedicine with activity against *C. albicans* infections.

Against *C. glabrata*, *Zp*E-*Ln*E the most synergistic mixture contains 9.7% of flavonoids **1**-**4** and 31.6% of lignans **5**-**7**. Within *Zp* markers, a predominance of **3** (4.7%) was observed, closely followed by **4** (4.4%) and with lower concentrations of **1** + **2** (0.90%). Within *Ln* markers, a prevalence of **7** (17%) followed by **5** (8%) and then **6** (7%) was observed. The whole combinations' doses for achieving ϕ = 0.50, 0.80, 0.90 and 0.95 were 133.22, 148.69, 158.56 and 168.23 µg/ml, much higher that the doses needed for inhibiting *C. albicans* (Fig. 6). The dosis of 168.23 µg/ml (with the proportion of markers described above) for ϕ = 0.95 should be chosen when preparing a phytomedicine that is able to control *C. glabrata* infections. Fig. 6 comparatively shows the doses needed for inhibiting each fungus for all ϕ = 0.50, 0.80, 0.90 and 0.95.

INSERT FIG. 6

It is clear that the dosis of the fixed ratio mixture of *Zp*E+*Ln*E necessary to achieve the higher synergism at the best effect level $\phi = 0.95$ against *C. glabrata* (168.23 µg/ml) is 2.55 times higher than the dosis required to inhibit *C. albicans* (65.96 µg/ml). The ratios between the amount of flavonoids markers **1-4**, and lignans markers **5-7** will be 0.22 (5.39 µg/ml/23.63 µg/ml) against *C. albicans* and 0.30 (16.35 µg/ml/53.24 µg/ml) against *C. glabrata*, clearly showing that lignans are required in higher proportions than flavonoids against both fungi, although this difference is less pronounced when acting against *C. glabrata*.

Declaration of herbal extracts and their most effective synergistic combination characteristics

Considering that the most synergistic combinations are those that have an effect level $\phi = 0.95$ (95% fungal growth inhibition or higher), it can be declared that, in accordance to EMA guidelines (EMA 2010), each dosis (1 ml) of the most synergistic mixture against *C. albicans,* should contain 65.96 µg of the whole mixture, composed of 18.84 µg of *Zp*E dry extract from *Z. punctata* Cav. aerial parts (9:1) and 47.12 µg of *Ln*E dry extract from *L. nitida* Cav. aerial parts (12:1), corresponding to 5.39 µg of *Zp*E flavonoids markers and 23.63 µg of *Ln*E lignans markers. In turn, each dosis (1 ml) of the most synergistic mixtures against *C. glabrata* should contain 168.23 µg of the whole mixture, composed of 45.47 µg of ZpE dry extract from *Z. punctata* Cav. aerial parts (8:1) and 122.76 µg of *Ln*E dry extract from *L. nitida* Cav. aerial parts (10:1), corresponding to 16.35 µg of *Zp*E flavonoids markers and 53.24 µg of *Ln*E lignans markers.

3D HPLC chromatograms

The 3D HPLC profiles of the most synergistic mixtures against *C. albicans* (*Zp*E-*Ln*E May) and *C. glabrata* (*Zp*E-*Ln*E Feb) are shown in Fig. 7A and 7B respectively. This chromatogram was monitored at 254 nm, which allows to visualize both groups of compounds, lignans (λ_{max} 280 nm) and flavonoids (λ_{max} 267, 289 and 341 nm).

INSERT FIG. 7

Conclusions

Synergism studies with the statistical-based MixLow method, allowed us to give support to the jointly use of *Zp*E and *Ln*E in traditional medicine. As the results of this study, we could determine that 3 over 4 fixed-ratio mixtures, whose composition were estimated by a valid method, showed synergism against *C. albicans* while only one showed synergism against *C. glabrata* (at $\phi > 0.5$).

Of them, one herbal-quantified *Zp*E-*Ln*E preparation acting against *C. albicans* and one acting against *C. glabrata* are both of great interest for the development of an antifungal phytomedicine.

Conflict of interest

The authors declare they have no conflict of interest.

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Graphical Abstract

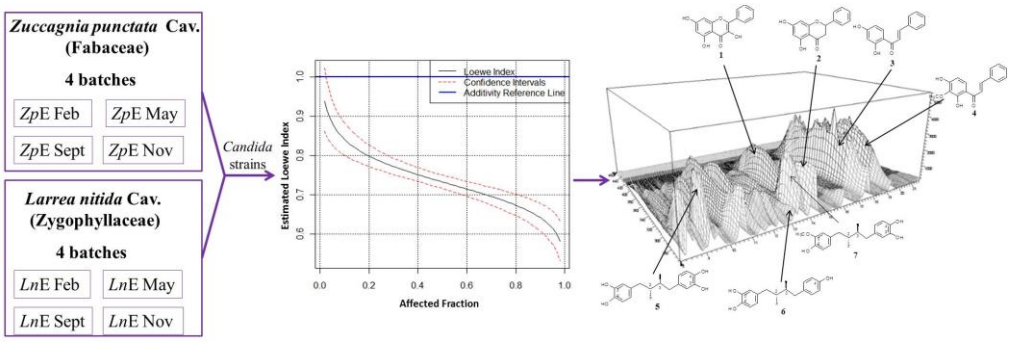


Table 1

Identification data of markers **1-4** (*Zp*E) and **5-7** (*Ln*E): Retention time (Rt), HRMS values and MS/MS fragments. Molecular Formulas (MF) and Molecular Weights (MW) are included.

Markers	Rt (min)	MF	MW	HRMS (error in ppm)				MS/MS fragments
				Feb	May	Sept	Nov	
*Zp*E								
1	12.9	$C_{15}H_{10}O_5$	270.05	269.0451 (1.8)	269.0457 (-0.5)	269.0446 (3.7)	269.0446 (3.6)	269.0451, 169.0653, 171.0443, 195.0431, 143.0496
2	12.9	$C_{15}H_{12}O_4$	256.07	255.0655 (3.1)	255.0656 (2.7)	255.0650 (4.9)	255.0649 (4.9)	255.0655, 227.0704, 151.0033, 123.0083,107.0145
3	14.0	$C_{15}H_{12}O_3$	240.08	239.0704 (3.9)	239.0709 (1.9)	239.0696 (4.8)	239.0703 (4.7)	239.0704, 197.0625, 169.0606, 148.0176,135.0100
4	14.2	$C_{16}H_{14}O_4$	270.09	269.0813 (2.2)	269.0814 (1.9)	269.0807 (4.4)	269.0812 (2.6)	269.0813, 254.0580, 150.9983, 106.0055, 94.0056
*Ln*E								
5	6.2	$C_{18}H_{22}O_4$	302.15	337.1207 (1.6)	337.1204 (2.5)	337.1213 (-0.3)	337.1201 (3.3)	302.1470, 273.1486, 122.0366
6	9.6	$C_{18}H_{22}O_3$	286.16	321.1256 (2.0)	321.1249 (4.3)	321.1253 (3.1)	321.1253 (3.0)	123.0407, 122.0366, 108.0211
7	10.1	$C_{19}H_{24}O_4$	316.17	351.1366 (0.8)	351.1358 (3.1)	351.1362 (1.9)	351.1363 (1.5)	300.1357, 149.0596, 135.0440, 122.0367

The Rt was determined by three individual analysis (n=3). The detected compounds had the greatest responses under the negative mode and so, the $[M-H]^-$ was used as the precursor ion for **1-4**, and $[M+Cl]^-$ for **5-8**.

Table 2

Linear regression data, precision, LOD and LOQ of compounds **1**-**5**.

Comp.	λ (nm)	Linear regression data			Precision, RSD (%)			LOD (mg/ml)	LOQ (mg/ml)
		Regressive equation	R^2	Linear range (mg/ml)	Conc. (mg/ml)	Intra-day (n=3)	Inter-day (n=3)		
1	267	y=39148.63 x -2097.40	0.99	0.10-0.50	0.10	0.15	0.99	0.03	0.09
					0.30	0.24	1.00		
					0.50	0.98	1.20		
2	289	y=33843.54 x -104.24	1.00	0.05-0.60	0.05	0.22	0.83	0.01	0.04
					0.20	0.19	0.95		
					0.60	0.28	0.99		
3	341	y=9045.88 x -89.18	0.99	0.50-2.00	0.50	0.24	0.90	0.15	0.46
					1.00	0.78	1.16		
					2.00	0.58	1.53		
4	341	y=10600.88 x -162.71	1.00	0.10-1.00	0.10	0.57	0.82	0.01	0.03
					0.50	0.48	0.93		
					1.00	0.30	1.20		
5	280	y=4149.35 x -304.55	1.00	0.30-2.00	0.30	1.87	1.90	0.10	0.30
					0.50	0.61	0.80		
					2.00	0.68	0.99		

Table 3

Quantitative assessment (g of compound /100 g extract) of the seven markers **1-7** in the four batches of each *Zp*E and *Ln*E (of February, May, September and November of one year) by HPLC-UV method. Values are the mean ± Standard Deviation calculated from three replicates.

Markers	**Feb**	**May**	**Sept**	**Nov**
*Zp*E				
1	03.36 ± 0.01	03.10 ± 0.01	03.11 ± 0.01	03.26 ± 0.03
2	03.36 ± 0.01	03.10 ± 0.01	03.11 ± 0.01	03.26 ± 0.03
3	16.39 ± 0.05	15.93 ± 0.07	15.51 ± 0.09	16.54 ± 0.10
4	16.22 ± 0.09	09.64 ± 0.06	15.15 ± 0.01	10.37 ± 0.21
*Ln*E				
5	10.39 ± 0.01	16.80 ± 0.05	12.48 ± 0.11	19.09 ± 0.08
6	09.14 ± 0.02	08.53 ± 1.03	09.66 ± 0.02	10.24 ± 0.01
7	23.84 ± 0.07	24.81 ± 0.10	27.37 ± 0.09	28.46 ± 0.02

Table 4

IC_{50} values (µg/ml) of each extract alone obtained from the dose-response curves against *C. albicans* (*Ca*) and *C. glabrata* (*Cg*). For synergism studies, the ratio of concentrations tested (IC_{50} *Zp*E/IC_{50} *Ln*E) is recorded. Each mixture was prepared from extract of the same period.

Fungal strain	Period	IC_{50} *Zp*E (µg/ml).	IC_{50} *Ln*E (µg/ml).	Ratio tested (IC_{50} *Zp*E/IC_{50} *Ln*E)
Ca	Feb	27.33 ± 0.01	53.14 ± 0.02	0.51
	May	31.40 ± 0.02	73.50 ± 0.01	0.43
	Sept	31.90 ± 0.03	57.20 ± 0.01	0.56
	Nov	28.63 ± 0.01	36.88 ± 0.01	0.78
Cg	Feb	65.79 ± 0.01	271.70 ± 0.03	0.24
	May	66.47 ± 0.01	164.72 ± 0.01	0.40
	Sept	39.56 ± 0.01	152.16 ± 0.01	0.26
	Nov	58.73 ± 0.01	154.31 ± 0.01	0.38

Amphotericin B was used as standard drug: IC_{50} against *C. albicans* = 0.25 µg/ml; against *C. glabrata* = 0.25 µg/ml.

Table 5

L_ϕ values (combination indexes) and confidence intervals and type of interaction of the mixtures *Zp*E-*Ln*E from different months of a year, against *C. albicans* (*Ca*) and *C. glabrata* (*Cg*) at fractions affected (ϕ) = 0.50, 0.80, 0.90 and 0.95 (inhibitory percentages 50, 80, 90 and 95 %).

Fungal strain	Combination	L_ϕ values at ϕ (inhibition percentage)							
		0.50 (50%)	**Int (clasif)**	**0.80 (80%)**	**Int (clasif)**	**0.90 (90%)**	**Int (clasif)**	**0.95 (95%)**	**Int (clasif)**
Ca	*Zp*E-*Ln*E Feb	0.92 ± 0.02	Ad (n)	0.96 ± 0.02	Ad (n)	0.99 ± 0.03	Ad (n)	1.01 ± 0.04	Ad (n)
	*Zp*E-*Ln*E May	0.73 ± 0.01	S (m)	0.68 ± 0.02	S (med)	0.65 ± 0.03	S (med)	0.62 ± 0.04	S (med)
	*Zp*E-*Ln*E Sept	0.95 ± 0.02	Ad (n)	0.77 ± 0.02	S (m)	0.71 ± 0.03	S (m)	0.67 ± 0.04	S (med)
	*Zp*E-*Ln*E Nov	0.89 ± 0.01	S (sl)	0.88 ± 0.01	S (sl)	0.87 ± 0.02	S (sl)	0.87 ± 0.02	S (sl)
Cg	*Zp*E-*Ln*E Feb	0.91 ± 0.01	Ad (n)	0.77 ± 0.02	S (m)	0.71 ± 0.02	S (m)	0.67 ± 0.02	S (med)
	*Zp*E-*Ln*E May	0.98 ± 0.01	Ad (n)	1.00± 0.01	Ad (n)	1.03 ± 0.02	Ad (n)	1.05 ± 0.02	Ad (n)
	*Zp*E-*Ln*E Sept	1.21 ± 0.01	An (m)	1.13 ± 0.01	An (sl)	1.09 ± 0.02	Ad (n)	1.06 ± 0.02	Ad (n)
	*Zp*E-*Ln*E Nov	1.20 ± 0.01	An (m)	1.15 ± 0.01	An (sl)	1.12 ± 0.02	An (sl)	1.09 ± 0.02	Ad (n)

Int: type of interaction; Clasif: classification of Synergism (S), Antagonism (An) and Additivism (Ad) in: slight (sl), moderate (m), medium (med), (n): near. *Ca*: *C. albicans* CCC 125; *Cg*: *C. glabrata* CCC 115.

Table 6

IC_x (Inhibitory Concentration to achieve X% of effect) of *Zp*E and *Ln*E alone and in the May or February combination against *C. albicans* (*Ca*) and *C. glabrata* (*Cg*) respectively, and DRI values, at ϕ = 0.5, 0.8, 0.90 and 0.95 (inhibitory percentages 50, 80, 90 and 95%).

		I	II	III (= IV+V)	IV	V	VI	VII (I/IV)	VIII (II/V)
Fungal strain	**ϕ (X% effect)**	**IC_x *Zp*E alone (µg/ml)**	**IC_x *Ln*E alone (µg/ml)**	**IC_x *Zp*E + IC_x *Ln*E in the combination (µg/ml)**	**IC_x *Zp*E in the combination (µg/ml)**	**IC_x *Ln*E in the combination (µg/ml)**	**L_ϕ = CI**	**DRI *Zp*E**	**DRI *Ln*E**
Ca	0.50 (50%)	31.40	73.50	38.94	11.80	27.14	0.73±0.01	2.66	2.71
	0.80 (80%)	45.91	98.28	49.91	14.26	35.65	0.68±0.02	1.29	2.76
	0.90 (90%)	57.33	116.48	57.70	16.48	41.22	0.65±0.03	3.48	2.83
	0.95 (95%)	70.36	136.22	65.96	18.84	47.12	0.62±0.03	3.73	2.89
Cg	0.50 (50%)	65.79	271.70	133.22	26.12	107.09	0.91±0.01	2.52	2.54
	0.80 (80%)	74.09	492.32	148.69	40.19	108.50	0.77±0.02	1.84	4.54
	0.90 (90%)	79.43	697.03	158.56	42.86	115.70	0.71±0.02	1.85	6.02
	0.95 (95%)	84.69	960.28	168.23	45.47	122.76	0.67±0.24	1.86	7.83

Table 7

Concentrations (μg/ml) of markers **1-7** in the most synergistic *Zp*E-*Ln*E mixtures against *C. albicans* and *C. glabrata* respectively, at different ϕ.

ϕ	**1** + **2**	**3**	**4**	**Total *Zp*E flavonoids**	**5**	**6**	**7**	**Total *Ln*E lignans**	**Whole mixture**
*Zp*E-*Ln*E best synergistic mixture (May) against *C. albicans*									
0.50	0.34 ± 0.03	1.77 ± 0.02	1.07 ± 0.01	3.18 (8%)	4.67 ± 0.01	2.37 ± 0.04	6.90 ± 0.03	13.94 (36 %)	38.94
0.80	0.44 ± 0.02	2.27 ± 0.01	1.37 ± 0.01	4.08 (8%)	5.99 ± 0.02	3.04 ± 0.04	8.85 ± 0.04	17.88 (36%)	49.91
0.90	0.51 ± 0.02	2.63 ± 0.01	1.58 ± 0.05	4.72 (8%)	6.92 ± 0.02	3.52 ± 0.03	10.23 ± 0.04	20.67 (36 %)	57.70
0.95	0.58 ± 0.01	3.00 ± 0.04	1.81 ± 0.02	5.39 (8%)	7.92 ± 0.03	4.02 ± 0.05	11.69 ± 0.05	23.63 (36 %)	65.96
*Zp*E-*Ln*E best synergistic mixture (Feb) against *C. glabrata*									
0.50	1.28 ± 0.02	5.84 ± 0.03	5.80 ± 0.03	12.92 (9.7 %)	10.12 ± 0.01	8.92 ± 0.02	23.04 ± 0.08	42.15 (31.6 %)	133.22
0.80	1.35 ± 0.02	6.57 ± 0.03	6.52 ± 0.02	14.44 (9.7 %)	11.27 ± 0.02	9.92 ± 0.02	25.86 ± 0.10	47.05 (31.6 %)	148.69
0.90	1.44 ± 0.01	7.01 ± 0.01	6.96 ± 0.04	15.41 (9.7%)	12.02 ± 0.01	10.58 ± 0.01	27.58 ± 0.10	50.18 (31.6 %)	158.56
0.95	1.53 ± 0.01	7.44 ± 0.02	7.38 ± 0.04	16.35 (9.7%)	12.75 ± 0.03	11.23 ± 0.01	29.26 ± 0.09	53.24 (31.6 %)	168.23

Markers of *Zp*E are: **1** (galangin), **2** (pinocembrin), **3** (2´,4´-dihydroxychalcone) and **4** (2´,4´-dihydroxy-3´-methoxychalcone). Markers of *Ln*E are: **5** (NDGA), **6** (DNDGA) and **7** (MNDGA). Compounds **1** and **2** had same Rt, so they could not quantify separately.

Scheme 1. Whole process of MixLow method: (**a**) Preparation of three dose-response curves: (**a_1**) one curve for each extract alone, confectioned with the data of each extract (*Zp*E and *Ln*E). Estimation of parameter values; (**a_2**) Preparation of a dose-response curve for a fixed-ratio mixture of both extracts. Estimation of parameter values. (**b**) Use estimated parameters in calculating the Loewe Index ($L_{\square}$). (**c**) Generation of confidence intervals for $L_{\square}$, and obtaining an Affected fraction vs $L_{\square}$ graph.

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

Fig. 2. Design of a 96-well microplate used for analysis of antifungal activity of the extracts alone and mixtures.

Fig. 3. HPLC-UV chromatograms of four batches of each *Zuccagnia punctata* extract (*Zp*E) (left) at 254 nm and *Larrea nitida* extract (*Ln*E) (right) at 280 nm. Marker compounds of *Zp*E and their Retention times (Rt, min) are: 5,7-dihydroxy-3-flavonol (galangin **1**) 12.9 min; 5,7-dihydroxy flavanone (pinocembrin **2**) 12.9 min; 2',4'-dihydroxychalcone (**3**) 14.0 min; 2',4'-dihydroxy-3'-methoxy chalcone (**4**) 14.2 min. Marker compounds of *Ln*E and their Rt are: nordihydroguairetic acid (NDGA **5**) 6.2 min; 4-[4-(4-hydroxyphenyl)-2,3-dimethyl-butyl]-benzene-1,2-diol (DNDGA **6**) 9.6 min; 3'-methyl nordihydroguiaretic acid (MNDGA **7**) 10.1 min.

Fig. 4. (A-D): Dose-response curves of single and combined *Zp*E and *Ln*E, prepared with plants collected in four periods of one year against *C. albicans*. Mixtures *Zp*E-*Ln*E were tested at the fixed ratio IC_{50}*Zp*E/IC_{50}*Ln*E at 10 equi-spaced concentrations in the ln scale. (A'-D'): Affected fraction (ϕ) *vs* Estimated Loewe Index (L_ϕ) (full line) with 95% confidence interval (dotted lines). Full line at L_ϕ = 1 represents additivity line. Lines below or above the additivity show synergism or antagonism, respectively.

Fig. 5. (A-D): Dose-response curves of single and combined *Zp*E and *Ln*E, prepared with plants collected in four periods of one year against *C. glabrata.* Mixtures *Zp*E-*Ln*E were tested at the fixed

ratio IC_{50}*Zp*E/IC_{50}*Ln*E at 10 equi-spaced concentrations in the ln scale. (A'-D'): Affected fraction (ϕ) *vs* Estimated Loewe Index (L_ϕ) curves (full line) with 95% confidence interval (dotted lines). Full line at L_ϕ = 1 represents additivity. Lines below or above L_ϕ = 1 denote synergism or antagonism, respectively.

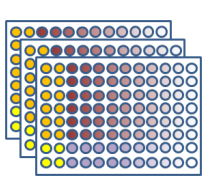
Fig. 6. Doses of the whole synergistic *Zp*E-*Ln*E combinations and their content in marker compounds for achieving an effect level □ = 0.50, 0.80, 0.90 and 0.95 against *C. albicans* and *C. glabrata.*

Fig. 7. 3D-HPLC profile of the two-herbal combination formed by *Z. punctata* and *L. nitida* in the concentration that produces 95% of growth inhibition. The representative peaks of the markers of both herbs were indicated. Peaks number **1-4** belong to *Z. punctata* and peaks number **5-7** belong to *L. nitida*. (A) *Zp*E-*Ln*E mixture of May. (B) *Zp*E-*Ln*E mixture of February. Detection was at 254 nm.

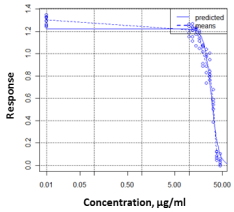
Scheme 1

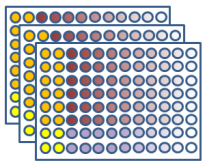
a

a_1

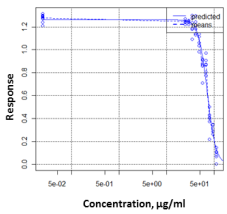


ZpE





LnE



$$Y_{ZpE,t,w} = \exp(\mu + b_t)\left(1 - \phi_{ZpE,w,t}\right) + \epsilon_{ZpE,t,w}$$

$$\phi_{ZpE,t,w} = 1 - \frac{1}{1 + \left(\frac{\exp(c_{ZpE,t,w})}{\exp(\psi_{ZpE,0.5})}\right)^{\gamma_{ZpE}}}$$

$$\gamma_{ZpE}; \psi_{ZpE,0.5};\ \mu$$

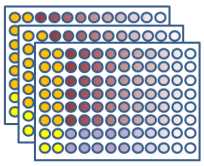
$$Y_{LnE,t,w} = \exp(\mu + b_t)\left(1 - \phi_{LnE,w,t}\right) + \epsilon_{LnE,t,w}$$

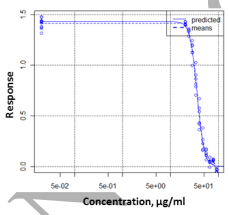
$$\phi_{LnE,t,w} = 1 - \frac{1}{1 + \left(\frac{\exp(c_{LnE,t,w})}{\exp(\psi_{LnE,0.5})}\right)^{\gamma_{LnE}}}$$

$$\gamma_{LnE}; \psi_{LnE,0.5};\ \mu$$

a_2

Fixed ratio at different doses
(Ic_{50} ZpE/ Ic_{50} LnE)





$$Y_{m,t,w} = \exp(\mu + b_t)\left(1 - \phi_{m,w,t}\right) + \epsilon_{m,t,w}$$

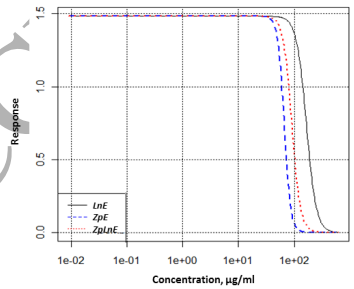
$$\phi_{m,t,w} = 1 - \frac{1}{1 + \left(\frac{\exp(c_{m,t,w})}{\exp(\psi_{m,0.5})}\right)^{\gamma_m}}$$

$$\gamma_m; \psi_{m,0.5};\ \mu$$

b

$$L_\phi = \sum_{d=1}^{n} \tau_d \exp\left(\log\left(\left(\frac{\phi}{1-\phi}\right)^{1/\gamma_m}\right) + \psi_{m,0.5} - \log\left(\left(\frac{\phi}{1-\phi}\right)^{1/\gamma_d}\right) - \psi_{d,0.5}\right)$$

c



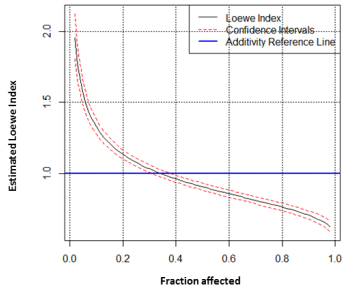
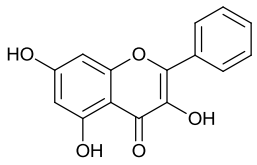
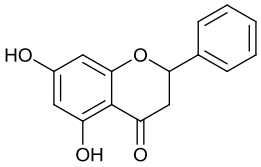


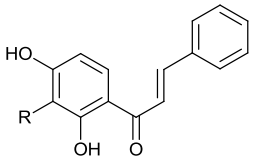
Fig. 1



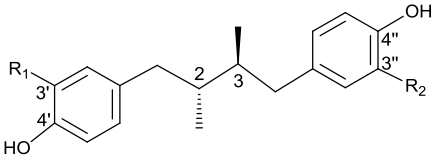
1
galangin



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. 2

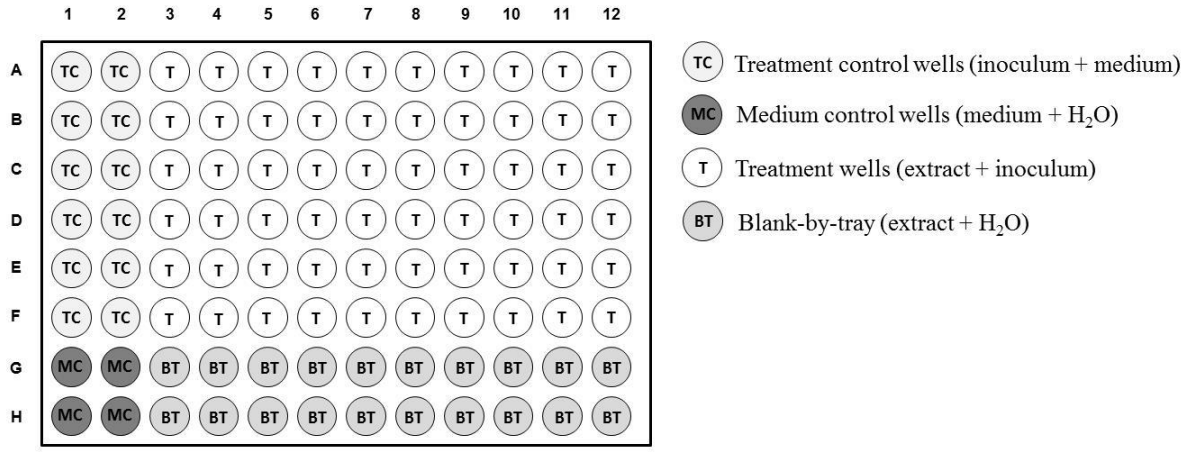


Fig. 3

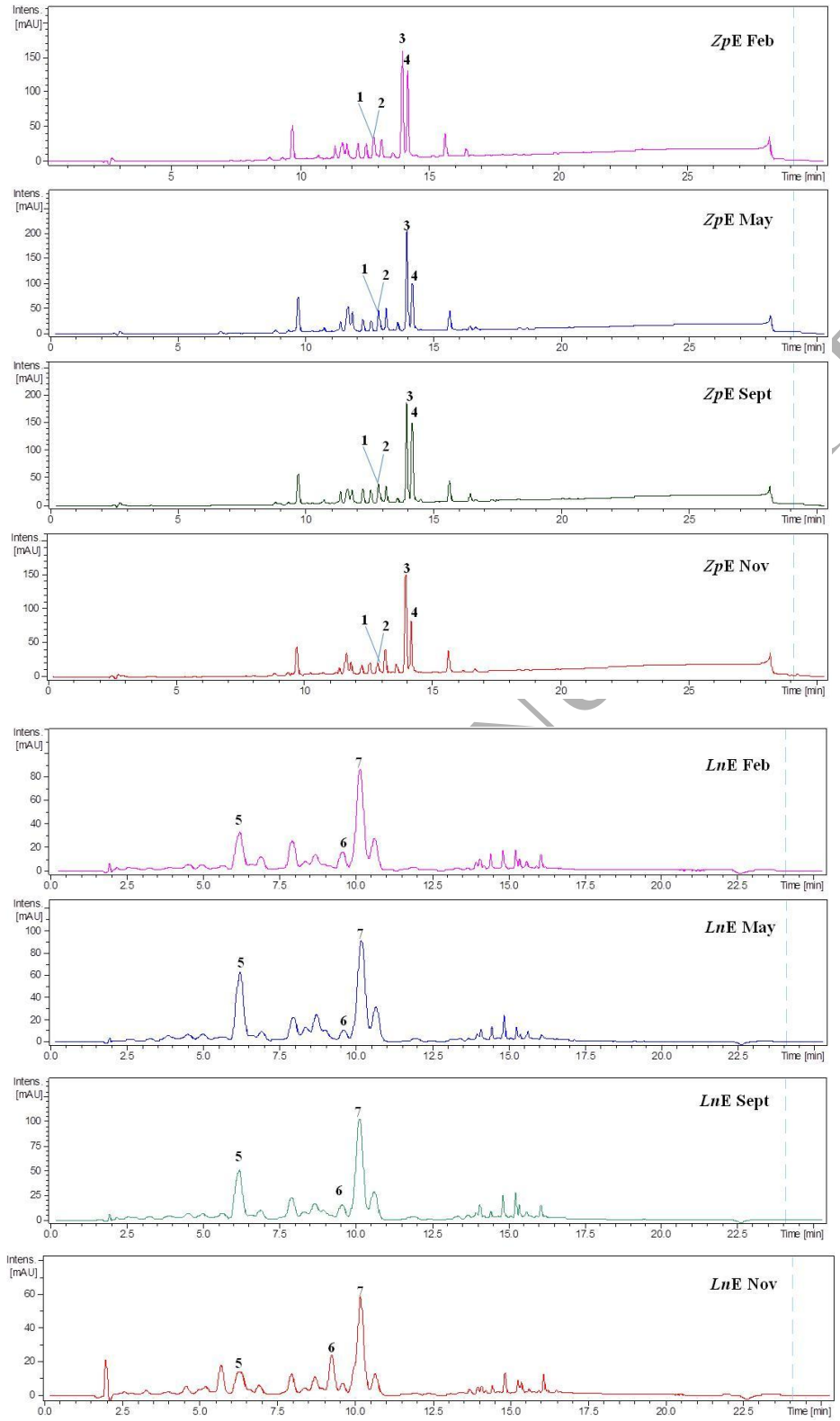

ZpE Feb
ZpE May
ZpE Sept
ZpE Nov
LnE Feb
LnE May
LnE Sept
LnE Nov
Intens. [mAU]
Time [min]

Fig. 4

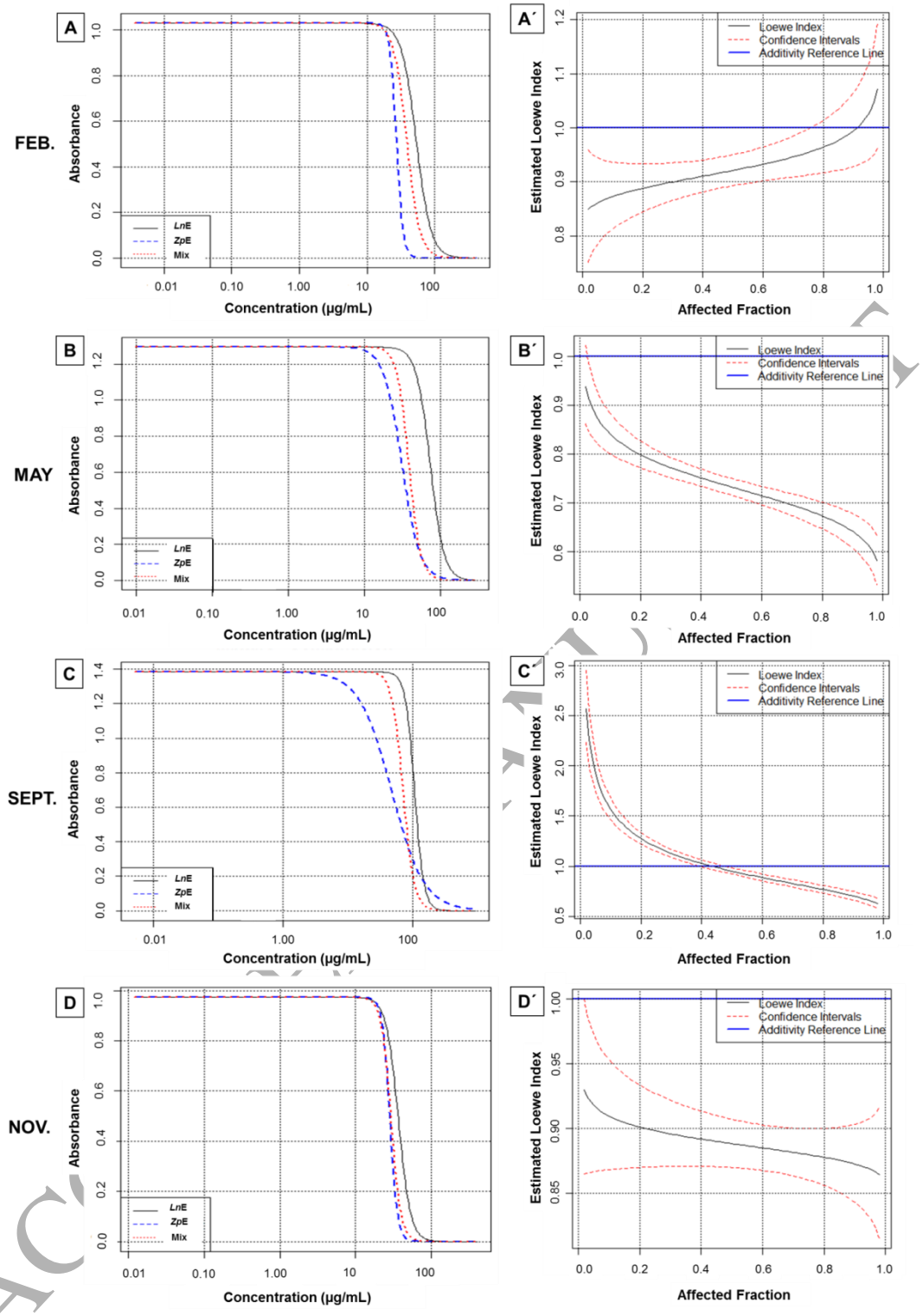

A
A´
FEB.
B
B´
MAY
C
C´
SEPT.
D
D´
NOV.
Absorbance
Concentration (µg/mL)
LnE
ZpE
Mix
Estimated Loewe Index
Affected Fraction
Loewe Index
Confidence Intervals
Additivity Reference Line

Fig. 5

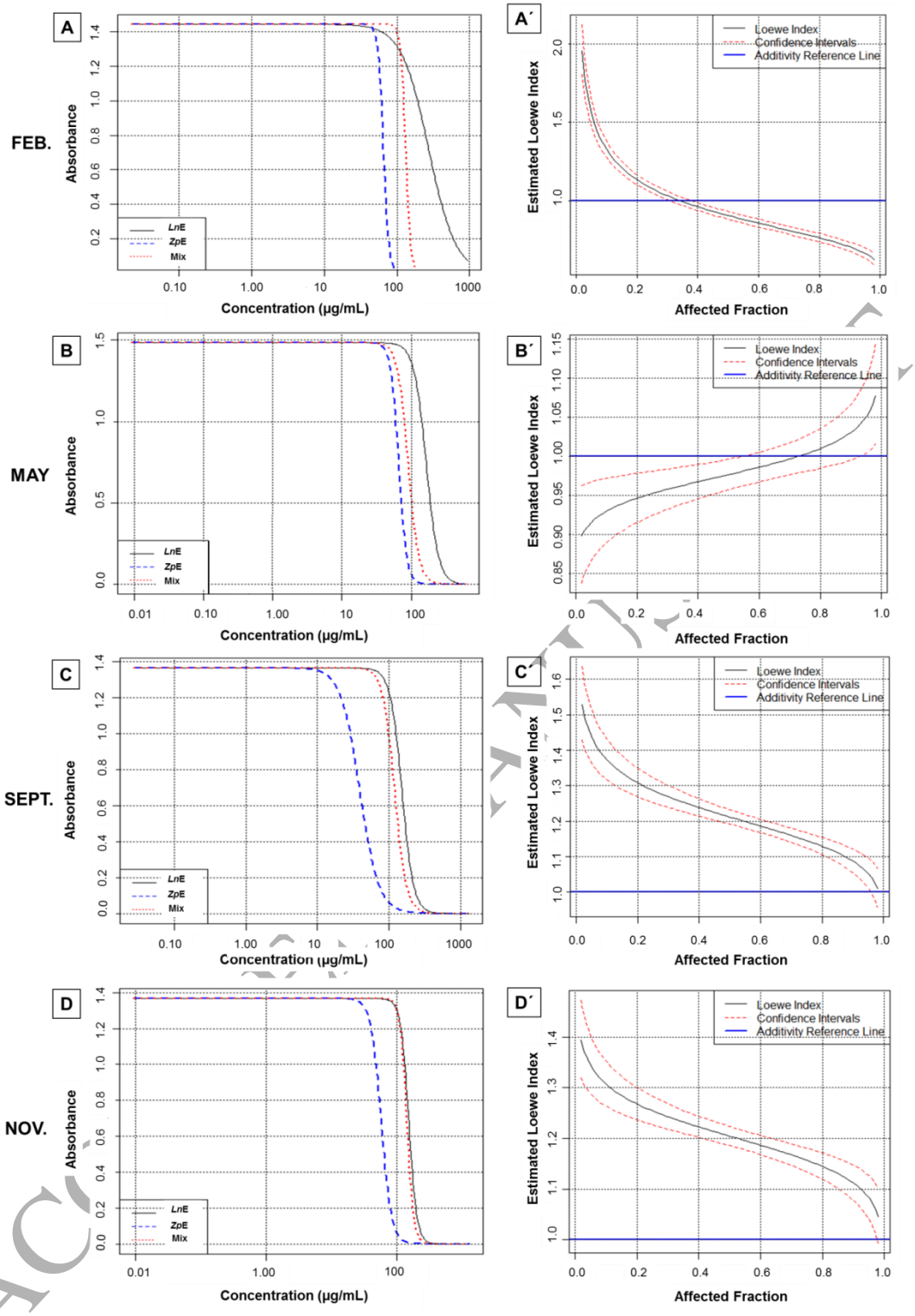

A
FEB.
Absorbance
Concentration (µg/mL)
LnE
ZpE
Mix
A´
Estimated Loewe Index
Affected Fraction
Loewe Index
Confidence Intervals
Additivity Reference Line
B
MAY
B´
C
SEPT.
C´
D
NOV.
D´

Fig. 6

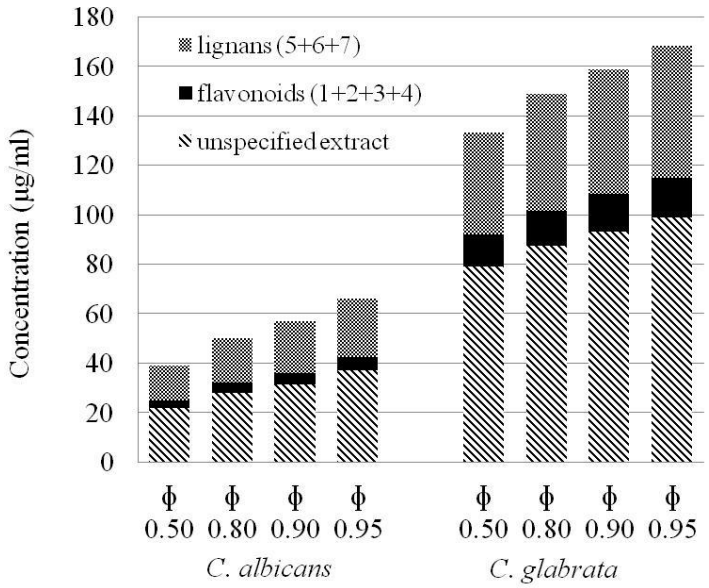


Fig. 7