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Antifungal activity and cytotoxicity of extracts and triterpenoid saponins obtained from the aerial parts of *Anagallis arvensis* L.

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ABSTRACT

Ethnopharmacological relevance: *Anagallis arvensis* L. (Primulaceae) is used in argentinean northwestern traditional medicine to treat fungal infections. We are reporting the isolation and identification of compounds with antifungal activity against human pathogenic yeast *Candida albicans*, and toxicity evaluation.
Aim of the study: to study the antifungal activity of extracts and purified compounds obtained form *A. arvensis* aerial parts, alone and in combinations with fluconazole (FLU), and to study the toxicity of the active compounds.
Materials and methods: Disk diffusion assays were used to perform an activity-guided isolation of antifungal compounds from the aerial parts of *A. arvensis*. Broth dilution checkerboard and viable cell count assays were employed to determine the effects of samples and combinations of FLU + samples against *Candida albicans*. The chemical structures of active compounds were elucidated by spectroscopic analysis. Genotoxic and haemolytic effects of the isolated compounds were determined.
Results: Four triterpenoid saponins (**1–4**) were identified. Anagallisin C (AnC), exerted the highest inhibitory activity among the assayed compounds against *C. albicans* reference strain (ATCC 10231), with MIC-0 =1 μg/mL. The Fractional Inhibitory Concentration Index (FICI=0.129) indicated a synergistic effect between AnC (0.125 μg/mL) and FLU (0.031 μg/mL) against *C. albicans* ATCC 10231. AnC inhibited *C. albicans* 12–99 FLU resistant strain (MIC-0 =1 μg/mL), and the FICI=0.188 indicated a synergistic effect between AnC (0.125 μg/mL) and fluconazole (16 μg/mL). The combination AnC+ FLU exerted fungicidal activity against both *C. albicans* strains. AnC exerted inhibitory activity against *C. albicans* ATCC 10231 sessile cells (MIC_50=0.5 μg/mL and MIC_{80}=1 μg/mL) and against *C. albicans* 12–99 sessile cells (MIC_50=0.75 μg/mL and MIC_{80}=1.25 μg/mL). AnC exerted haemolytic effect against human red blood cells at 15 μg/mL and did not exerted genotoxic effect on *Bacillus subtilis* rec strains.
Conclusions: The antifungal activity and lack of genotoxic effects of AnC give support to the traditional use of *A. arvensis* as antifungal and makes AnC a compound of interest to expand the available antifungal drugs.

1. Introduction

C. albicans is an opportunistic fungal pathogen, and a common cause of invasive fungal infections in humans, producing infections that can involve any organ (Soberón et al., 2015). There is a limited number of therapeutic antifungal agents, and a decrease in the activity, even for new drugs (e.g. echinocandins), due to an increase of resistance mechanisms, enhanced by the ability of some strains to form biofilms (Favre-Godal et al., 2015). Biofilms are structured microbial communities with a complex three-dimensional architecture characterized by a network of adherent cells connected by water channels and encapsulated within an extracellular matrix (Bachmann et al., 2002). Most candidiasis are related with the formation of biofilms, which show resistance to antifungal compounds, thus increasing the concentration of antifungals, which may become toxic (Bachmann et al., 2002). Fluconazole (FLU) is the main therapeutic antifungal drug employed in

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developing countries (Flynn et al., 2009). The fungistatic action of azoles is an aspect that triggers the development of drug resistance (Sanglard et al., 2003), an effect that could be avoided through association of drugs. Drug associations could be described as indifferent (i.e. no interaction), antagonistic or synergistic (Soberón et al., 2015). An advantage of using drugs combinations is the possibility to obtain a synergistic effect, which could lead to a fungicidal mix, highly desirable to increase efficacy and reduce resistance development (Fiori and Van Dijck, 2012). These facts indicate the need for the discovery of compounds with antifungal activities against both planktonic and biofilm cells (Denning and Perlin, 2011), or compounds that could be combined with commercial fungistatic drugs to yield a fungicidal association (Soberón et al., 2015).

Higher plants are interesting sources of antimicrobial agents (Soberón et al., 2014). *Anagallis arvensis* L. (Primulaceae) is a small annual weed spread all over the world, used to treat fungal infections in Argentinean northwestern traditional medicine (Rondina et al., 2010), and also in other counties, such as India (Mitscher, 1975) or Palestine (Ali-Shtayeh et al., 1998). Aerial parts are used to prepare an ointment for the treatment of external infections (López et al., 2011). The leaves are consumed raw by humans and other mammals, as sheeps (Middleditch, 2012). There are few reports on the antifungal activity of *A. arvensis* extracts: Al-Abed et al. (1993) evaluated the antifungal activity against phytopathogenic fungi, Ali-Shtayeh and Abu Ghdeib (1999) proved the antifungal activity against dermatophytes, and López et al. (2011) proved the antifungal activity of an ethanolic extract against *C. albicans*. All these reports deal with raw extracts. This article describes the antifungal activity study of *A. arvensis* ethanolic extracts, activity guided purification, structural elucidation and the antifungal activity analysis of compounds obtained from *A. arvensis* ethanolic extract, alone and combined with FLU against planktonic *C. albicans* cells. The isolated compounds were also evaluated on their haemolytic and genotoxic effects. The most active compound isolated was also evaluated on its ability to inhibit *C. albicans* sessile cells.

2. Material and methods

2.1. Chemicals

Analytical and HPLC grade solvents were from Sintorgan Labs (Buenos Aires, Argentina). FLU, menadione, and 2H-tetrazolium-2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl] hydroxide salt (XTT) were from Sigma-Aldrich (MO, USA). Sodium phosphate, KCl and NaCl were from Biopack (Buenos Aires, Argentina). Sabouraud dextrose (SD) medium and agar were from Britania Labs (Buenos Aires, Argentina). RPMI 1640 medium was from Microvet Labs (Buenos Aires, Argentina). CHROMagar® Candida was from CHROMagar Microbiology, France.

2.2. Strains and media

Two *C. albicans* strains were employed: one reference strain was from *American Type Culture Collection* (ATCC 10231), and the other was *C. albicans* 12–99 clinical isolate strain highly resistant to FLU (overexpressing *ERG11*, *CDR1*, *CDR2* and *MDR1*) (White et al., 2002). Strains were regularly refreshed from frozen permanents (CLSI, 2008). Cells cultures purity and viability were frequently assessed by plating yeast colonies on CHROMagar® Candida. FLU was dissolved in DMSO.

2.3. Plant material

Aerial parts of *A. arvensis* L. [Primulaceae] were used. Plants were collected in the province of Tucumán, in Argentinean northwest, on September 2013 (latitude −26.79°, longitude −65.32°). Plants were taxonomically identified and a voucher specimen was deposited in the herbarium of the Instituto de Estudios Farmacológicos for future references (voucher specimen number: IEF-2013-09-007-JRS). Plant materials were dried in the shade in a well ventilated chamber, ground to a coarse powder and stored into closed flasks at −20 °C in the dark until they were used.

2.4. Extract preparation

Ethanolic extract (EE) was prepared by adding plant coarse powder with 96% ethanol (10 g/100 mL) in glass flasks under stirring (40 cycles per minute); the flask were sealed and leaved at 37 °C for 7 days. Finally the extract was filtered through Whatman No.1 filter paper. The filtered extract was dried under reduced pressure using rotary vacuum evaporator at 30 °C. The dried material obtained represented the extracted material (EM).

2.5. Activity-guided fractionation of A. arvensis ethanolic extract

An antifungal activity-guided fractionation of *A.arvensis* ethanolic extract was conducted as follows: 10 g of dried EE was suspended in water: ethanol (3:2 v:v) and successively extracted with hexane, chloroform and n-butanol in an order of increasing solvent polarity, which yielded hexane (HX), chloroform (CH), n-butanol (nBu) and aqueous residual (Aq) fractions. Extracts were evaporated under reduced pressure (at 45 °C) and finally dried by lyophilization. The residues were dissolved in methanol for further assays. Subsequent purification steps were conducted with nBu. An aliquot of nBu containing 1 g of EM was chromatographed on Sílica Gel 60 (0.040–0.063 mm, 230–400 mesh, 120 mL bed volume) employing chloroform:MeOH mix (in increasing polarity order 9:1, 4:1, 3:2 and 1:1 v:v) as mobile phase. The 17 aliquots (50 mL each one) resulting from column elution were analyzed by TLC, joined according to their chemical composition into six groups (G1-G6) and evaporated under reduced pressure (at 45 °C) to yield EM residues which were dissolved in methanol for further experiments. Aliquots were taken from G1-G6 groups for disk diffusion and TLC experiments.

2.6. TLC experiments

Samples from EE, Hx, CH, nBu, Aq, and G1-G6 were analyzed by TLC on silica gel 60 F254 plates. The mobile phases were ethyl acetate: formic acid: acetic acid: water (100: 11: 11: 27, v:v:v:v). The eluted dried plates were visualized under visible and UV light (254 and 366 nm, UV Lamp Model UV 5L-58 Mineralight Lamp) before and after staining with either 1% methanolic 2-aminoethyl diphenylborate reagent (flavonoids detection), *p*-anisaldehyde/sulfuric acid reagent (terpenes detection) and Liebermann Burchard reagent (steroids and saponins detection), according to Wagner and Bladt (1996).

2.7. HPLC experiments

G5 group was selected for HPLC analysis. The first HPLC run was performed in analytical conditions to adjust the conditions on a Gilson HPLC (Villiers Le Bel, Val d'Oise, France) using an IB-SIL 5 C18 column (5 µm, 250×4.6 mm ID) from Phenomenex (Torrance, CA, USA), a 132 RI detector from Gilson and a Rheodyne injector fitted with a 50 µL loop. An isocratic elution was performed with MeOH: water (80:20 v:v) for 30 min. Compounds were detected at a flow rate of 0.9 mL/min. The retention times (Rts) were registered. The semi-preparative HPLC experiments employed an LUNA PFP column (5 µm, 250×10 mm ID) from Phenomenex, a Rheodyne injector fitted with a 500 µL loop and the same mobile phase an detector employed on analytical conditions were used at flow rate of 2.7 mL/min for 30 min. The detected compounds were collected, dried by lyophilization and dissolved in methanol for further experiments. Multiple injections in semipreparative conditions (5 mg of EM at a time) were carried out to obtain adequate quantity of material. Compounds collected were

analyzed by TLC, and their purities were verified by analytical HPLC experiments.

2.8. Electrospray ionization (ESI) - High-resolution mass spectra (HRMS) analysis

High-resolution mass spectra were recorded on a MicroTOF II spectrometer (Bruker Daltonics, Bremen, Germany) with sample introduction performed by a syringe pump (KD Scientific, KDS 100) and an ESI source operating in positive or negative modes. General conditions were as follows: dyring gas temperature of 180 °C, 4 L/min drying gas flow, capillary voltage of 4.5 kV and nebulizing gas pressure 0.4 bar. Mass spectra were acquired by scanning along the m/z 300–3000 range. G5 was diluted in acetonitrile to 50 mg/mL without any additives and loaded into a 500 mL volume Hamilton gastight 1700 series syringe operating at a flow rate of 3 mL/min. The accurate mass data of the molecular ions were processed through the software DataAnalysis 4.0 (Bruker Daltonics).

2.9. NMR spectra

Fourteen milligrams a compound purified from G5 were analyzed by one-dimensional NMR techniques (^{1}H NMR, ^{13}C NMR and ^{13}C DEPT) and bidimensional NMR techniques (^{1}H–^{1}H COSY, ^{1}H–^{13}C HSQC, ^{1}H–^{13}C HMBC and ^{1}H-NOE). NMR spectra were recorded on a Bruker Avance instrument (300 MHz for ^{1}H NMR and 75.14 MHz for ^{13}C NMR). Chemical shifts were recorded in δ (ppm) values relative to the nondeuterated impurities of the used solvents as internal standard (pyridine-d5) using standard Bruker Topspin software.

2.10. Polarimetric assays

Optical rotations were determined for purified compounds dissolved in MeOH, on a Horiba Sepa-300 polarimeter (Horiba Ltd., Kyoto, Japan) at 589 nm, in a 0.1 dm cell at 25 °C. $[\alpha]_D^{25}$ values were calculated and compared with literature data (Mahato et al., 1991).

2.11. Hydrolysis and derivatization

A compound purified from G5 was hydrolyzed by standard procedures (Soberón et al., 2010a), yielding an aqueous extract containing sugar moieties, which were separately analyzed by GC-MS after silylation procedure (Leblanc and Ball, 1978). 1 µL of sample was injected into a MicroTOF II spectrometer equipped with FID detector at 250 °C, using a Zebron MultiResidue-1 column (30 m×0,25 mm×0,25 µm - Phenomenex, Torrance, California, USA). General conditions were: dyring gas temperature of 200 °C and 2 mL/min drying gas flow. Mass spectra were acquired by scanning along the m/z 100–500 range. Sugars were identified according to the retention times, compared with those generated by commercial standards.

2.12. Disk diffusion assay

The assay was employed as inexpensive reliable fast method to assess the antifungal activity of samples (i.e. extract, fractions and purified compounds) alone and in combination with FLU (synergy testing) against *C. albicans* cells. The assay was performed as described elsewhere (Endo et al., 2010) with *C. albicans* ATCC 10231 (FLU sensitive strain) and *C. albicans* 12–99 (FLU resistant strain). Briefly, yeast cells were obtained from a 24 h culture on SD agar (i.e. exponential growing cells). The final inoculum was adjusted to 10^3 CFU/mL in sterile distilled water and then spread uniformly onto SD agar plates. Plates used for synergy tests were previously added with 6 µg/mL of FLU (Endo et al., 2010). 5 mm paper disks impregnated with 50 µg of samples were placed onto agar plates. The diameters (in mm) of growth inhibition zones were measured after incubation at 35 °C for 24 h. Experiments were repeated at least three times.

2.13. Broth microdilution and cell viability assays

The experiments were performed in sterile multiwell microdilution plates according to CLSI reference procedure (CLSI, 2008) using RPMI 1640 medium. Experiments were performed with isolated compounds and FLU. The isolated compounds concentrations ranged from 0.125 to 8 µg/mL, while FLU concentrations ranged from 0.015 to 4 µg/mL for experiments with ATCC 10231 strain, and from 0.5 to 128 µg/mL for experiments with 12–99 strain. The inoculum was obtained as previously described, and adjusted to10^3 UFC/mL in each well. Plates were incubated at 35 °C and read after 48 h for ATCC 10231 strain, and 24 h for 12–99 strain. Minimum inhibitory concentration (MIC) was the minimum concentration of FLU which produced 50% decrease in optical density (MIC-2) or 100% decrease in optical density for isolated compounds (MIC-0 or optically clear wells). Microdilution experiments were repeated for isolated compounds using a more narrow concentration range in order to more clearly identify the MICs (Vidovic et al., 2015); the concentrations employed were: 0.50, 0.75, 1.00, 1.25, 1.50, 1.75, 2.00 and 2.25 µg/mL for compounds 1 and 2, and 3.00, 3.25, 3.50, 3.75, 4.00, 4.25 and 4.50 µg/mL for compounds 3 and 4. Optical densities were recorded at 550 nm using a Bio-Rad 550 microplate reader (Bio-Rad Laboratories, CA, USA). Cell viability assays were carried out as follows: 25 µL aliquots taken from each well were washed, serially diluted, plated on SD agar plates and incubated at 35 °C for 48 h. Colony forming units (CFU) were registered. Experiments were repeated at least three times.

2.14. Checkerboard assays

The experiments were performed to assess antifungal interactions between FLU and isolated compounds as described elsewhere (Martinez-Irujo et al., 1996). Briefly, the top row wells (A) of a flat bottomed 96-well microtiter plate were added with FLU at the same final concentrations described in Section 2.13 (columns 3–11). Column 12 contained the isolated compound at the same final concentrations described in Section 2.13 (rows B-H). Column 1 was the growth control; column 2 was the sterility control. The remaining wells contained a unique combination of FLU and the compound under assay. All the wells (except sterility controls) were added with exponential growing cells (suspended in RPMI 1640 medium) of either *C. albicans* ATCC 10231 or 12–99 till reach 10^3 CFU/mL. The final volume in each well was 200 µL. The plates were covered and incubated at 35 °C for 48 h (Iten et al., 2009). CFU measurements were obtained as described in Section 2.13. The 2-log units criteria (i.e. a reduction in CFU/mL ≥2 log units exerted by the combination over the most active agent alone) was employed for determining the synergism of a combination (Pfaller et al., 2004). Fractional Inhibitory Concentration (FIC) of each evaluated substance was calculated as the ratio between MIC of the substance in the combined experiments and the MIC of the same substance alone. The interactions between FLU and isolated compounds were also assessed through Fractional Inhibitory Concentration Index (FICI), i.e. the sum of FICs (Odds, 2003). FICI data interpretations were: “synergy” (FICI ≤0.5), “antagonism” (FICI > 4.0) and “no interaction” (FICI > 0.5–4.0), as suggested by Odds (2003). The MIC values for FLU (alone) which were > 4 µg/mL experimentally (against *C. albicans* ATCC 10231) and > 128 µg/mL experimentally (against *C. albicans* 12–99), were considered to be 8 µg/mL and 256 µg/mL respectively (referred to as MIC-0_{FLU}) for calculation purposes, a criterion reported by other authors (Fiori and Van Dijck, 2012). Experiments were repeated at least three times.

2.15. Fungicidal activity assays

Fungicidal activity was assessed through viable count determinations after checkerboard assay performed with 10^4 CFU/mL initial inocula (Cantón et al., 2003). This inocula allowed to determine the > 99.9% viability lost of initial inoculums or 3 log unit decrease in CFU/mL compared to starting inoculum, above the limit of detection (i.e. 10 CFU/mL). The minimal fungicidal concentration (MFC) was the minimal concentration of a compound under assay which caused > 99.9% growth inhibition. *C. albicans* ATCC 10231 and 12–99 strains were employed. Plates were incubated for 48 h at 35 °C. Experiments were repeated at least three times.

2.16. Antifungal activity on C. albicans biofilm

The antifungal activity of AnC against *C. albicans* ATCC 10231 and *C. albicans* 12–99 sessile cells was performed through a 96-well plate method based on the ability of metabolically active sessile cells to reduce XTT to water-soluble orange formazan compounds, the intensity of which can then be determined using a microtiter-plate reader (Pierce et al., 2008). Briefly, 100 µL of a 10^6 cells/mL inoculum prepared in RPMI medium were placed in a 96-well plate and incubated at 37 °C for 48 h. The obtained biofilms were washed three times with phosphate-buffered saline (PBS –10 mM phosphate buffer, 2.7 mM KCl, 137 mM NaCl, pH 7.4). Increasing quantities of sterile AnC aqueous solutions were added to the washed biofilms till reach final concentrations ranging from 5 to 1280 µg/mL. After 48 h incubation at 37 °C, biofilms were washed three times with PBS and then added with 100 µL of XTT solution (sterile solution of 0.5 µg/mL XTT added with 1 µM menadione). After 2 h incubation at 37 °C in the dark, absorbances were recorded at 490 nm using a Bio-Rad 550 microplate reader. The sessile minimum inhibitory concentrations (SMICs), $SMIC_{50}$ and $SMIC_{80}$ were the AnC concentrations which produced a 50% or 80% decrease in absorbance as compared with the control biofilms (i.e. in the absence of AnC). Experiments were repeated at least three times.

2.17. Toxicity against human red blood cells

The toxicity of isolated compounds against human red blood cells was assessed through quantification of the haemolytic effect, as described elsewhere (Ahmad et al., 2010). The compounds were assayed between 1–200 µg/mL. The results were expressed as the 100% haemolytic dose (HD_{100}), which represented the sample concentration inducing the 100% haemolysis after 1 h incubation at 25 °C. Triton X-100 was used as positive control drug (i.e. 100% haemolysis).

2.18. Genotoxic activity

Genotoxic response was assessed by using *Bacillus subtilis* rec assay followed by quantification and Probit transformation of the differential growth curves of *B. subtilis* rec strains exposed to samples, as described elsewhere (Matsui, 1988). Isolated compounds were assayed between 0.03–20 µg/mL. The results, referred to as S-Probit were compared with reference values previously described (Soberón et al., 2010b). $K_2Cr_2O_7$ and FLU were used as genotoxic and non-genotoxic reference drugs respectively.

2.19. Statistic analysis

Data were analyzed by either one-way ANOVA or Student's *t*-test. Probability levels below 0.05 ($p < 0.05$) were considered as statistically significant.

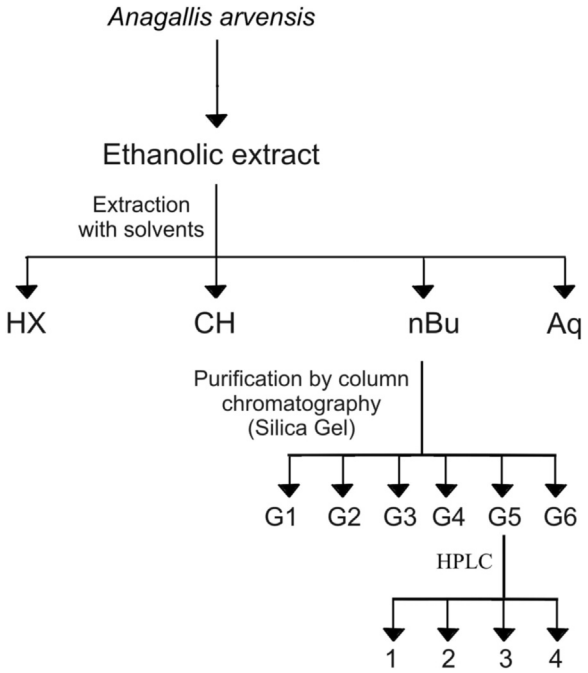


Fig. 1. : Flow chart of the purification procedure.

3. Results and discussion

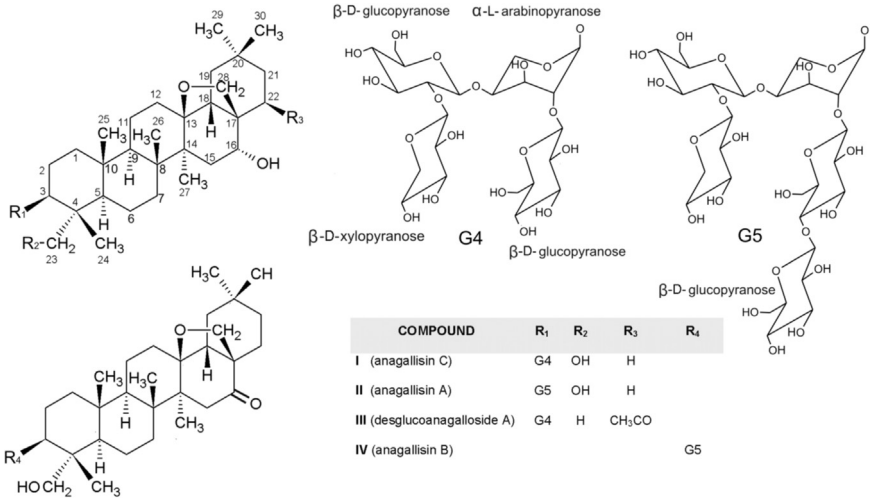
3.1. Activity-guided fractionation of A. arvensis ethanolic extract

The extraction yield obtained from EE was 30.3 ± 3.2 g of EM per 100 g of dry plant material. A detailed flow chart of the purification procedure is shown in Fig. 1. The EM obtained in HX, CH, nBu and Aq was 0.94 mg, 2.70 g, 5.93 g and 0.30 g, respectively. Neither of HX, CH and Aq exhibited growth inhibition against the *C. albicans* ATCC10231 strain (i.e. no inhibition halo observed), while nBu showed inhibitory activity against this strain, with inhibition halo diameters of 8.0 ± 0.2 cm (experiments without FLU) and 9.0 ± 0.2 cm (experiments with FLU). The inhibition halo produced by nBu was clear in both experiments suggesting a fungicidal activity (Onyewu et al., 2003). nBu was selected for further purification steps.

Column chromatography of nBu yielded group G5 with antibacterial activity against *C. albicans* ATCC10231 with and without FLU, determined by disk diffusion assay.

3.2. Identification of purified compounds

HPLC experiments performed with G5 yielded four triterpenoid monodesmosidic saponins (i.e. sugar moiety attached al C3 hydroxyl group), **compound 1:** Anagallisin C (AnC) (also named Desglucoanagalloside B), **compound 2** Anagallisin A (also named Anagallosaponin I), **compound 3:** Anagallisin B and **compound 4:** Desglucoanagalloside A (also named Anagallosaponin VIII). The chemical structures were determined through ESI-HRMS experiments and polarimetric measures (Mahato et al., 1991; Shoji et al., 1994a and Shoji et al., 1994b). The chemical structure of compound 1 was also confirmed through NMR experiments. The sugars in the glycon portion of compound 1 were confirmed in the aqueous fraction after acid hydrolysis and derivatization by GC-MS. The results could be found as Supplementary material (Tables S1, S2 and S3; Figs. S1, S2 and S3). The chemical structures of compounds are showed in Fig. 2. These compounds belong to a rare group of oleanane saponins that possess a completely saturated pentacyclic skeleton with a 13β,28-epoxy bridge, and were reported exclusively in the Myrsinaceae and Primulaceae families (Foubert et al., 2008).



COMPOUND	R_1	R_2	R_3	R_4
I (anagallisin C)	G4	OH	H	
II (anagallisin A)	G5	OH	H	
III (desglucoanagalloside A)	G4	H	CH_3CO	
IV (anagallisin B)				G5

Fig. 2. : Chemical structures of isolated compounds.

3.3. Disk diffusion assay

The antifungal activities of compounds 1–4 were preliminary evaluated through disk diffusion assay with and without FLU. All of the compounds showed antifungal activity against both *C. albicans* strains assayed, though the inhibition halos obtained with AnC were significant higher ($p < 0.05$) than those generated by the other compounds, against both assayed strains with and without FLU (data not shown). Preliminary studies using the agar disk diffusion method found that AnC (from methanolic extract of *Lysimachia candida* Lindl.) was the main antifungal compound against *Aspergillus flavu* (filamentous fungi) (Xia et al., 2013). Through disk diffusion experiments we found similar results against human pathogenic yeasts. The inhibition growth zones diameters generated by AnC against *C. albicans* ATCC 10231 with and without FLU were 9.5 ± 0.2 mm and 8.0 ± 0.1 mm respectively ($p > 0.05$), while the growth inhibition zones diameters for AnC against *C. albicans* 12–99 with and without FLU were 8.6 ± 0.1 mm and 7.8 ± 0.2 mm respectively ($p > 0.05$). These results suggested that AnC antifungal activity against FLU sensitive and FLU resistant *C. albicans* strains could be increased by FLU presence.

3.4. Broth microdilution and cell viability assays

Compounds 1–4 were quantitatively evaluated on their antifungal activities against both strains, alone and combined with FLU by broth microdilution and checkerboard assay. Table 1 summarizes the MICs obtained.

AnC was the most active compound against both strains. The viable cell count experiments performed with 0.125 μg/mL of AnC after checkerboard assays (initial inoculum of 10^3 CFU/mL) showed a decrease on the viability of *C. albicans* ATCC 10231 from 7.02 log units (cells exposed to AnC alone) to 3.91 for those cells exposed to the combination of AnC (0.125 μg/mL) + FLU (0.031 μg/mL), i.e.≥2 log units decrease by the combination over AnC alone (line with squares in Fig. 3A), which suggested a synergistic combination between AnC and FLU (FICI =0.129). For experiments conducted with *C. albicans* 12–99, there was a decrease from 7.21 log units (cells exposed to AnC alone at 0.125 μg/mL) to 4.71 for cells exposed to the combination of AnC (0.125 μg/mL) + FLU (16 μg/mL), i.e.≥2 log units decrease (line with squares in Fig. 3B), suggesting a synergistic combination between AnC and FLU against this strain (FICI =0.188). There were no differences between the MICs generated by AnC against both *C. albicans* strains, which suggests that the mechanisms responsible of *C. albicans 12–99* resistance to FLU, i.e. alterations in the gene encoding the enzyme *ERG11,* overexpression of *CDR1*, *CDR2*, and *MDR1* efflux pump genes (White et al., 2002), did not affected the sensibility of this yeast to AnC. As some authors reported, antimicrobial compounds which act through different modes of action could have a synergistic effect on target cells (Cuenca-Estrella et al., 2005; Endo et al., 2010). Despite saponins and FLU cause alterations in the fungal cell membrane, they act over different target, which may contribute to the synergistic effect observed in our experiments.

Table 1
MIC values for evaluated compounds against *C. albicans* ATCC 10231 and 12–99.

Strain	MIC-2 (μg/mL)	MIC-0 (μg/mL)				
	FLU	FLU	1 (AnC)	2	3	4
ATCC 10231	1	8[a]	1.0	2.0	4.0	4.0
12–99	> 128	256[a]	1.0	2.0	4.0	4.0

[a] Criterion described by Fiori and Van Dijck (2012). See Section 2.14 for detailed explanation. Numbers 1 – 4 refer to isolated compounds.

3.5. Fungicidal activity assays

Fungicidal activity and subsequent cell viability assays were performed for AnC alone and combined with FLU, using 10^4 CFU/mL inocula, against both strains. No viable *C. albicans* ATCC 10231 cells were recovered from wells containing 4.0 μg/mL of AnC alone, thus the MFC_{AnC}=4.0 μg/mL. No cell growth was obtained for those experiments containing AnC (1.0 μg/mL) + FLU (≥0.25 μg/mL) (Fig. 4A) against *C. albicans* ATCC 10231, suggestive of > 99.9% viability lost of initial inoculum. Identical experiments performed against *C. albicans* 12–99 showed MFC_{AnC}=4.0 μg/mL, and no cell growth was obtained for experiments containing AnC (1.0 μg/mL) + FLU (> 2 μg/mL) (Fig. 4B). These evidences suggest that AnC + FLU combination exert fungicidal action against both *C. albicans* strains. Though the assayed saponins are well known compounds, this is the first detailed report on their antifungal activity against *C. albicans*. An antimicrobial activity is referred as “very interesting” for a pure compound with an inhibitory concentration below 10 μg/mL (Rios and Recio, 2005). We found that AnC at 0.125 μg/mL and at 1 μg/mL were sufficient to produce respectively a synergistic and fungicidal combination with FLU. López et al. (2011) found that an alcoholic extract from *A. arvensis* was strongly active against *C. albicans*, and proposed that saponins might be involved in antimicrobial properties. Our results confirm these statements.

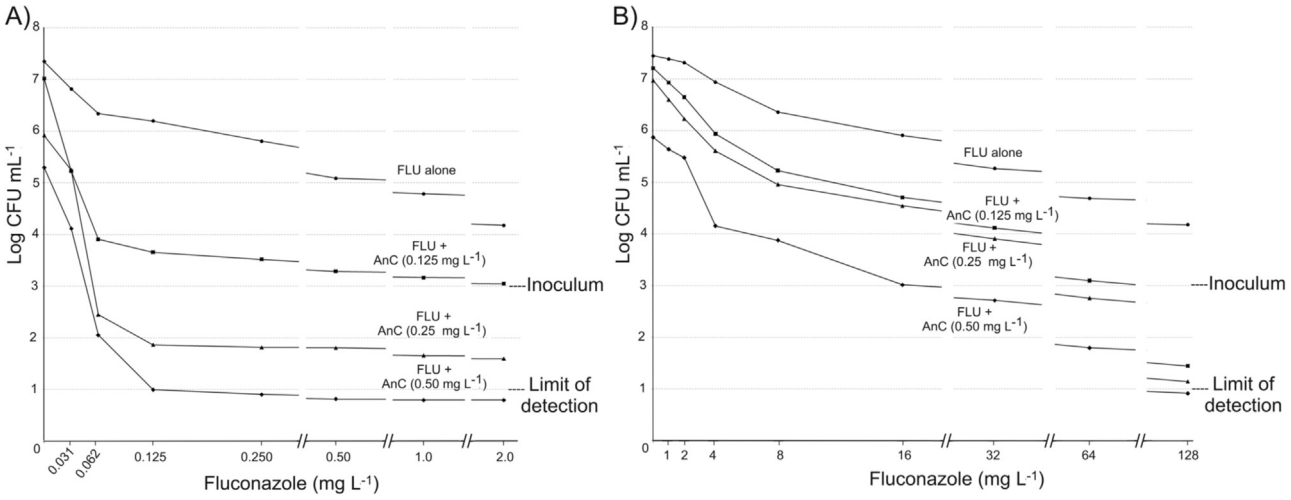


Fig. 3. : Viable cell count assays with 10^3 CFU/mL initial inoculums. A) Experiments with *C. albicans* ATCC 10231. B) Experiments with *C. albicans* 12–99.

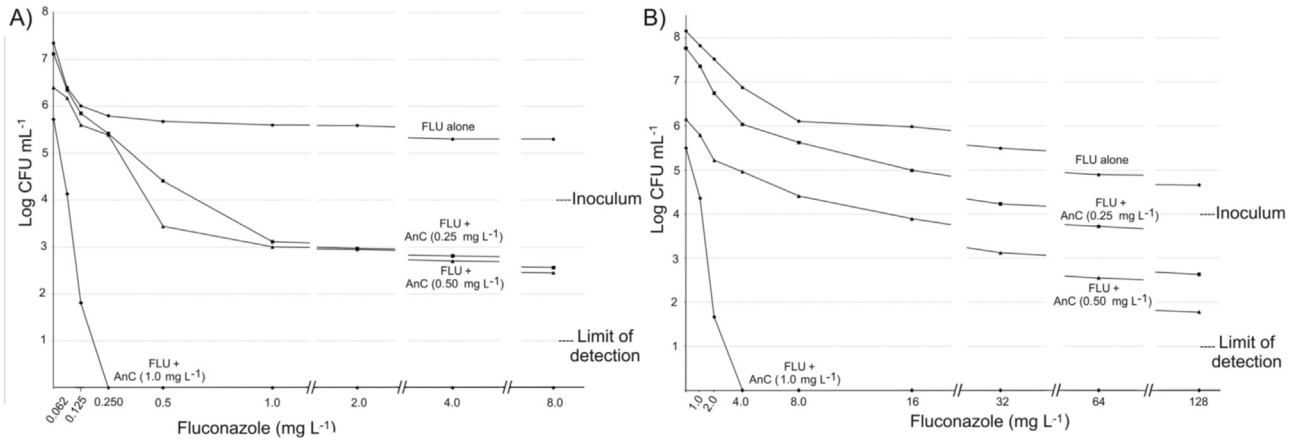


Fig. 4. : Viable cell count assays with 10^4 CFU/mL initial inoculums. A) Experiments with *C. albicans* ATCC 10231. B) Experiments with *C. albicans* 12–99.

Table 2
Haemolytic activity generated by purified compounds against sheep red blood cells.

Compound	HD_{100} (μg/mL)
1(AnC)	15.0
2	20.0
3	20.0
4	17.5

Table 3
Genotoxic activity of isolated compounds and reference substances.

Sample	S-Probit	Result
1(AnC)	0.183	Non-genotoxic (–)
2	0.151	Non-genotoxic (–)
3	0.140	Non-genotoxic (–)
4	0.178	Non-genotoxic (–)
$K_2Cr_7O_7$	2.95	Strong genotoxic (++)
FLU	0.117	Non-genotoxic (–)

3.6. Antifungal activity on C. albicans biofilm

Since AnC exerted the highest inhibitory activity against *C. albicans* planktonic cells, we assessed the antifungal activity exerted by AnC against *C. albicans* ATCC 10231 and *C. albicans* 12–99 sessile cells. AnC inhibited mature *C. albicans* ATCC 10231 sessile cells with $SMIC_{50}$ and $SMIC_{80}$ values of 0.5 μg/mL and 1.0 μg/mL respectively. *C. albicans* 12–99 sessile cells have been reported to be resistant to FLU, as well as planktonic cells do (Peralta et al., 2015). Experiments carried out with *C. albicans* 12–99 sessile cells generated $SMIC_{50}$ and $SMIC_{80}$ values of 0.75 μg/mL and 1.25 μg/mL respectively. Saponins' antifungal activity is attributed to an interaction between these compounds and membrane sterols, which leads to an increase in the ion's permeability (Sparg et al., 2004). The $SMIC_{50}$s obtained in our experiments were smaller than MICs obtained through microdilution assay against planktonic cells. Coleman et al. (2010), who evaluated antifungal activity of some triterpenoid saponins against planktonic and sessile *C. albicans* cells, found similar results in their experiments, and suggested that they could be due an increase of hyphae cell membrane permeability in sessile cells compared to that of planktonic cells. These results are significant because the commercial antifungals (except echinocandins) do not cause significant inhibition on *C. albicans* biofilms (Coleman et al., 2010).

3.7. Toxicity against human red blood cells

Saponins are well known to cause haemolysis (Glauert et al., 1962). Actually, blood reagent is used to detect haemolytically active saponins on TLC plates (Wagner and Bladt, 1996). Abe et al. (1978) found that the bridged 13β,28- epoxyoleanene system, the axial C_{16}-OH, the C_{23}-OH and the number of sugar moieties groups in saikosaponins (triterpenoid saponins structurally close to anagallosaponins) were significant for haemolysis. Moreover, monodesmosidic triterpenoid saponins with protoprimulagenin A ring (as the anagallosaponins evaluated in the present study) were described as haemolytic against

sheep red blood cells, yielding a HD100=15 μg/mL (Voutquenne et al., 2002). These authors also stated that monodesmosidic saponins with four sugar units reach the maximum haemolytic activity. Our results match with these statements: we found that compounds 1–4 caused 100% haemolysis at concentrations ≥15 μg/mL (Table 2), and AnC, a saponin that includes four sugar units and all of the structural requirements stated by Abe et al. (1978), exerted the highest haemolytic activity among the isolated compounds, though the concentration needed for this effect was almost 60 (sixty) times higher than the MIC in combinatory experiments against both *C. albicans* strains. López et al. (2011) affirmed that *A. arvensis* is employed to prepare an infusion to threat internal or systemic infections, though they are popularly known to be toxic during long-term consumption. The haemolytic activity exerted by *A. arvensis* saponins could be responsible for the toxic effect referred by these authors.

3.8. Genotoxic activity

Safety evaluation is important in determining the potential application of a given agent in therapy, especially in the case of saponins because of several reports of the genotoxicity (Koczurkiewicz et al., 2016). S-Probit values and interpretations obtained through *B. subtilis* rec assay are shown in Table 3.

Compounds **1–4** and FLU were not genotoxic on *B. subtilis* rec strains (−0.123 > S-Probit > 0.199), while $K_2Cr_2O_7$ exerted a strong genotoxic effect (S-Probit > 0.593). These results are in agreement with those obtained by Koczurkiewicz et al. (2016), who found that AnC (named as CIL-1) did not exhibit genotoxic potential through *Vibrio harveyi* assay.

4. Conclusion

The bioassay-guided fractionation from aerial parts of *A. arvensis* allowed the detection of AnC as the compound with the highest antifungal activity against *C. albicans* FLU sensitive and FLU resistant strains. AnC combinated with FLU yielded a synergistic mix with fungicidal activity against both strains. This monodesmosidic triterpenoid saponin exerted inhibitory activity against *C. albicans* sessile cells, and the toxicity on red blood cells was almost 60 times higher than MICs obtained on combinatory experiments. We found no genotoxic response of the assayed saponins up to 10 μg/mL (higher than MICs in combinatory assays). To the best of our knowledge, this is the first report on anti-candida activity of compounds purified from *A. arvensis* aereal parts. The absence of cytotoxic and genetic effects observed at the antifungal concentrations of isolated compounds give additional support to the ethnopharmacological use of *Anagallis arvensis* as antifungal agent in traditional medicine. The evaluated saponins could represent another group of compound with antifungal activity, in order to expand the current classes of available antifungal drugs.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jep.2017.03.056.

References

Abe, H., Sakaguchi, M., Konishi, H., Tani, T., Arichi, S., 1978. The effects of saikosaponins on biological membranes. Planta Med. 34, 160–166.
Ahmad, A., Khan, A., Manzoor, N., Khan, L.A., 2010. Evolution of ergosterol biosynthesis inhibitors as fungicidal against *Candida*. Microb. Pathog. 48 (1), 35–41.
Al-Abed, A.S., Qasem, J.R., Abu-Blan, H.A., 1993. Antifungal effects of some common wild plant species on certain plant pathogenic fungi. Dirasat. Ser. B Pure Appl. Sci. 20 (3), 149–158.
Ali-Shtayeh, M.S., Yaghmour, R.M.R., Faidi, Y.R., Salem, K., Al-Nuri, M.A., 1998. Antimicrobial activity of 20 plants used in folkloric medicine in the Palestinian area. J. Ethnopharmacol. 60 (3), 265–271.
Ali-Shtayeh, M.S., Abu Ghdeib, S.I., 1999. Antifungal activity of plant extracts against dermatophytes. Mycoses 42 (11-12), 665–672.
Bachmann, S.P., VandeWalle, K., Ramage, G., Patterson, T.F., Wickes, B.L., Graybill, J.R., López-Ribot, J.L., 2002. In vitro activity of caspofungin against *Candida albicans* biofilms. Antimicrob. Agents Chemother. 46 (11), 3591–3596.
Cantón, E., Pemán, J., Viudes, A., Quindós, G., Gobernado, M., Espinel-Ingroff, A., 2003. Minimum fungicidal concentrations of amphotericin B for bloodstream *Candida* species. Diag Microbiol Infect. Dis. 45, 203–206.
Clinical Laboratory Standards Institute (CLSI), 2008. protocols. Reference method for broth dilution antifungal susceptibility testing of yeasts. Approved standard – 3rd Edition, M27-A3. Wayne, PA, USA.
Coleman, J.J., Okoli, I., Tegos, G.P., Holson, E.B., Wagner, F.F., Hamblin, M.R., Mylonakis, E., 2010b. Characterization of plant-derived saponin natural products against *Candida albicans*. ACS Chem. Biol. 5 (3), 321–332.
Cuenca-Estrella, M., Gomez-Lopez, A., Garcia-Effron, G., Alcazar-Fuoli, L., Mellado, E., Buitrago, M.J., Rodriguez-Tudela, J.L., 2005. Combined activity in vitro of caspofungin, amphotericin B, and azole agents against itraconazole-resistant clinical isolates of *Aspergillus fumigatus*. Antimicrob. Agents Chemother. 49, 1232–1235.
Denning, D.W., Perlin, D.S., 2011. Azole resistance in *Aspergillus*: a growing public health menace. Future Microbiol. 6 (11), 1229–1232.
Endo, E.H., Cortez, D.A.G., Ueda-Nakamura, T., Nakamura, C.V., Dias Filho, B.P., 2010. Potent antifungal activity of extracts and pure compound isolated from pomegranate peels and synergism with fluconazole against Candida albicans. Res. Microbiol. 161, 534–540.
Favre-Godal, Q., Dorsaz, S., Queiroz, E.F., Marcourt, L., Ebrahimi, S.N., Allard, P.M., Sanglard, D., 2015. Anti-Candida Cassane-Type Diterpenoids from the Root Bark of *Swartzia simplex*. J. Nat. Prod. 78 (12), 2994–3004.
Fiori, A., Van Dijck, P., 2012. Potent synergistic effect of doxycycline with fluconazole against *Candida albicans* is mediated by interference with iron homeostasis. Antimicrob. Agents Chemother. 56 (7), 3785–3796.
Flynn, S., Hollis, A., Palmedo, M., 2009. An economic justification for open access to essential medicine patents in developing countries. J. Law Med. Ethics 37 (2), 184–208.
Foubert, K., Theunis, M., Apers, S., Vlietinck, A.J., Pieters, L., 2008. Chemistry, distribution and biological activities of 13, 28-epoxy-oleanane saponins from the plant families Myrsinaceae and Primulaceae. Curr. Org. Chem. 12 (8), 629–642.
Glauert, A.M., Dingle, J.T., Lucy, J.A., 1962. Action of saponin on biological cell membranes. Nature 196, 952–955.
Iten, F., Saller, R., Abel, G., Reichling, J., 2009. Additive antimicrobial effects of the active components of the essential oil of *Thymus vulgaris*–chemotype carvacrol. Planta Med. 75, PJ100.
Koczurkiewicz, P., Kowolik, E., Podolak, I., Wnuk, D., Piska, K., Łabędź-Masłowska, A., Michalik, M., 2016. Synergistic cytotoxic and anti-invasive effects of mitoxantrone and triterpene saponins from *Lysimachia ciliata* on human prostate cancer cells. Planta Med.. http://dx.doi.org/10.1055/s-0042-117537.
Leblanc, D.J., Ball, A.J.S., 1978. A fast one-step method for the silylation of sugars and sugar phosphates. Anal. Biochem. 84 (2), 574–578.
López, V., Jäger, A.K., Akerreta, S., Cavero, R.Y., Calvo, M.I., 2011. Pharmacological properties of Anagallis arvensis L.("scarlet pimpernel") and Anagallis foemina Mill. ("blue pimpernel") traditionally used as wound healing remedies in Navarra (Spain). J. Ethnopharmacol. 134 (3), 1014–1017.
Mahato, S.B., Sahu, N.P., Roy, S.K., Sen, S., 1991. Structure elucidation of four new triterpenoid oligoglycosides from Anagallis arvensis. Tetrahedron 47 (28), 5215–5230.
Martinez-Irujo, J.J., Villahermosa, M.L., Alberdi, E., Santiago, E., 1996. A checkerboard method to evaluate interactions between drugs. Biochem. Pharmacol. 51, 635–644.
Matsui, S., 1988. The *Bacillus subtilis*/microsome rec-assay for the detection of DNA-damaging substances in waters of a night soil treatment plant. Environ. Toxicol. 3, 173–193.
Middleditch, B.S., 2012. Kuwaiti Plants: Distribution, Traditional Medicine, Pytochemistry, Pharmacology and Economic Value 2. Elsevier.
Mitscher, L.A., 1975. Antimicrobial Agents from Higher Plants. In: *Recent advances in phytochemistry* - volume 9. Runeckles, V.C. Ed. Plenum Press. New York and London. Pp. 250.
Odds, F.C., 2003. Synergy, antagonism, and what the chequerboard puts between them. J. Antimicrob. Chemother. 52, 1.
Onyewu, C., Blankenship, J.R., Del Poeta, M., Heitman, J., 2003. Ergosterol biosynthesis inhibitors become fungicidal when combined with calcineurin inhibitors against *Candida albicans*, *Candida glabrata*, and *Candida krusei*. Antimicrob. Agents Chemother. 47 (3), 956–964.
Peralta, M.A., da Silva, M.A., Ortega, M.G., Cabrera, J.L., Paraje, M.G., 2015. Antifungal activity of a prenylated flavonoid from Dalea elegans against Candida albicans biofilms. Phytomedicine 22, 975–980.

Pfaller, M.A., Sheehan, D.J., Rex, J.H., 2004. Determination of fungicidal activities against yeasts and molds: lessons learned from bactericidal testing and the need for standardization. Clin. Microbiol. Rev. 17, 268–280.
Pierce, C.G., Uppuluri, P., Tristan, A.R., Wormley, F.L., Mowat, E., Ramage, G., Lopez-Ribot, J.L., 2008. A simple and reproducible 96-well plate-based method for the formation of fungal biofilms and its application to antifungal susceptibility testing. Nat. Protoc. 3 (9), 1494–1500.
Rios, J.L., Recio, M.C., 2005. Medicinal plants and antimicrobial activity. J. Ethnopharmacol. 100 (1), 80–84.
Rondina, R.V., Bandoni, A.L., Coussio, J.D., 2010. Argentine medicinal plants with potential antifungal activity. Dominguezia 26 (1), 31–39.
Sanglard, D., Ischer, F., Marchetti, O., Entenza, J., Bille, J., 2003. Calcineurin A of *Candida albicans*: involvement in antifungal tolerance, cell morphogenesis and virulence. Mol. Microbiol. 48 (4), 959–976.
Shoji, N., Umeyama, A., Yoshikawa, K., Arihara, S., 1994a. Structures of Anagallosaponins 1-V and their companion substances from *Anagallis arvensis* L. Chem. Pharm. Bull. 42 (9), 1750–1755.
Shoji, N., Umeyama, A., Yoshikawa, K., Arihara, S., 1994b. Triterpenoid glycosides from Anagallis arvensis. Phytochemistry 37 (5), 1397–1402.
Soberón, J.R., Sgariglia, M.A., Sampietro, D.A., Quiroga, E.N., Sierra, M.G., Vattuone, M.A., 2010a. Purification and identification of antibacterial phenolics from *Tripodanthus acutifolius* leaves. J. Appl. Microbiol. 108 (5), 1757–1768.
Soberón, J.R., Sgariglia, M.A., Sampietro, D.A., Quiroga, E.N., Vattuone, M.A., 2010b. Free radical scavenging activities and inhibition of inflammatory enzymes of phenolics isolated from *Tripodanthus acutifolius*. J. Ethnopharmacol. 130 (2), 329–333.
Soberón, J.R., Sgariglia, M.A., Maderuelo, M.R.D., Andina, M.L., Sampietro, D.A., Vattuone, M.A., 2014. Antibacterial activities of *Ligaria cuneifolia* and *Jodina rhombifolia* leaf extracts against phytopathogenic and clinical bacteria. J. Biosci. Bioeng. 118 (5), 599–605.
Soberón, J.R., Lizarraga, E.F., Sgariglia, M.A., Juárez, M.B.C., Sampietro, D.A., Ben Altabef, A., Vattuone, M.A., 2015. Antifungal activity of 4-hydroxy-3-(3-methyl-2-butenyl) acetophenone against *Candida albicans*: evidence for the antifungal mode of action. Antonie Van. Leeuwenhoek 108 (5), 1047–1057.
Sparg, S., Light, M.E., Van Staden, J., 2004. Biological activities and distribution of plant saponins. J. Ethnopharmacol. 94 (2), 219–243.
Vidovic, S., Elder, J., Medihala, P., Lawrence, J.R., Predicala, B., Zhang, H., Korber, D.R., 2015. ZnO nanoparticles impose a panmetabolic toxic effect along with strong necrosis, inducing activation of the envelope stress response in *Salmonella enterica* serovar *enteritidis*. Antimicrob. Agents Chemother. 59, 3317–3328.
Voutquenne, L., Lavaud, C., Massiot, G., Men-Olivier, L.L., 2002. Structure-activity relationships of haemolytic saponins. Pharm. Biol. 40 (4), 253–262.
Wagner, H., Bladt, S., 1996. Plant Drug Analysis. A Thin Layer Chromatography Atlas 2[a] ed. Springer-Verlag, Berlin, Heidelberg, Germany.
White, T.C., Holleman, S., Dy, F., Mirels, L.F., Stevens, D.A., 2002. Resistance mechanisms in clinical isolates of *Candida albicans*. Antimicrob. Agents Chemother. 46 (6), 1704–1713.
Xia, X., Wei, X., Lin, L., 2013. Triterpenoid saponins from *Lysimachia candida* Lindl. Pharmacogn. J. 5, 119–122.