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Effects of the binding of a *Helianthus annuus* lectin to *Candida albicans* cell wall on biofilm development and adhesion to host cells.

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Abstract

Background: In our previous study, we isolated and characterized a lectin called Helja from *Helianthus annuus* (sunflower) and then, in a further study, demonstrated its antifungal activity against *Candida* spp. Since *Candida* infections are a major health concern due to the increasing emergence of antifungal resistant strains, the search for new antifungal agents offers a promising opportunity for improving the treatment strategies against candidiasis.

Purpose: The aim of this work was to get insights about the mechanism of action of Helja, an antifungal lectin of *H. annuus*, and to explore its ability to inhibit *Candida albicans* biofilm development and adherence to buccal epithelial cells (BEC).

Study Design / Methods: Yeast viability was evaluated by Evans Blue uptake and counting of colony forming units (CFU). The yeast cell integrity was assessed using Calcofluor White (CFW) as a cell wall perturbing agent and sorbitol as osmotic protectant. The induction of oxidative stress was evaluated using 3,3'-diaminobenzidine (DAB) for detection of hydrogen peroxide. The adherence was determined by counting the yeast cells attached to BEC after methylene blue staining. The biofilms were developed on polystyrene microplates, visualized by confocal laser scanning microscopy and the viable biomass was quantified by CFU counting. The binding lectin-*Candida* was assessed using Helja conjugated to fluorescein isothiocyanate (Helja-FITC) and simultaneous staining with CFW. The cellular surface hydrophobicity (CSH) was determined using a microbial adhesion to hydrocarbons method.

Results: *C. albicans* cells treated with 0.1 μg/μl of Helja showed a drastic decrease in yeast survival. The lectin affected the fungal cell integrity, induced the production of hydrogen peroxide and inhibited the morphological transition from yeast to filamentous forms. Helja caused a significant reduction of adherent cells and a decrease in biofilm biomass and coverage area. The treatment with the protein also reduced the surface hydrophobicity of fungal cells. We show the binding of Helja-FITC to yeast cells distributed as a thin outer layer to the CFW signal, and this interaction was displaced by mannose and Concanavalin A.

Conclusion: The results demonstrate the interaction of Helja with the mannoproteins of *Candida albicans* cell wall, the disruption of the cell integrity, the induction of oxidative stress, the inhibition of the morphological transition from yeast to filamentous forms and the fungal cell viability loss. The binding Helja-*Candida* also provides a

possible explanation of the lectin effect on cell adherence, biofilm development and CSH, relevant features related to virulence of the pathogen.

Keywords

Adherence, Antifungal lectin, Biofilm, *Candida*, Cell wall, Jacalin.

Abbreviations

BEC, Buccal epithelial cells; CFU, Colony forming units; CFW, Calcofluor White; CLSM, Confocal laser scanning microscopy; ConA, Concanavalin A; CSH, Cellular surface hydrophobicity; DAB, 3,3'-diaminobenzidine; EF, Extracellular fluid; FITC, Fluorescein isothiocyanate; FM4-64, N-(3-triethylammoniumpropyl)-4-(6-(4-diethylaminophenylhexatrienyl) pyridinium dibromide; Helja, *Helianthus annuus* jacalin; PBS, Phosphate-buffered saline; ROS, reactive oxygen species.

Introduction

Candida species are opportunistic pathogens that usually live on the mucosal surfaces of healthy individuals. However, when host defense mechanisms are impaired they can cause systemic infections in the human host, which are associated to high mortality rates (Pappas et al., 2018). Thus, *Candida* infections are a major health concern for immunocompromised individuals, due to the limited repertoire of drugs for its efficient treatment and the increased drug resistant in clinical isolates. The identification of antifungal agents with new active principles offers a promising opportunity for the development of successful therapeutic approaches against these infections (Cavalheiro and Teixeira, 2018).

The fungal cell wall plays a crucial role for the pathogenicity of *Candida albicans*. It is composed of chitin, glucans and mannans, organized as two layers: an inner skeletal layer of chitin and β1,3-linked glucan and an outer layer of β1,6-glucan and highly glycosylated cell wall proteins (Hall and Gow, 2013). These latter include enzymes involved in cell wall biogenesis as well as proteins essential for adhesion and biofilm formation, all of which influence the pathogenicity of the organism (Hall and Gow, 2013). The ability of *Candida* cells to adhere to the mucosal surfaces of various host organs is essential for the recognition of the target cell and constitutes a critical first step in the infectious process (Zhu and Filler, 2010). In addition, biofilm formation is considered as an important factor for C. *albicans* virulence, showing high resistance to antifungal drugs and to the host defense mechanisms (Olsen, 2015). Biofilms are defined as a microbial community in which the cells are connected to a substrate, or to each other, embedded in an extracellular matrix of polymeric substances (produced by themselves) and exhibit an altered growth rate and gene transcription (Ganguly and Mitchell, 2011). Accordingly, the inhibition of both, the adherence and the ability to develop biofilms would contribute to find alternative strategies to prevent infections caused by this organism (Cavalheiro and Teixeira, 2018).

The lectins are a group of proteins that bind specifically to free and cell surface attached carbohydrates and, although they were originally discovered in plant seeds, it is now known to be widely distributed in nature (Dang and Van Damme, 2015). Despite the large number of lectins that have been purified and characterized, only a few of them displayed antifungal activity (Regente et al., 2014; Neto et al., 2017; da Silva et al., 2018). Although the mechanism by which lectins display their antifungal action remains

elusive, effects produced by the binding to the carbohydrates on the surface of the fungal cell wall have been suggested (Dang and Van Damme, 2015). We have previously identified a mannose-binding lectin by a proteomic study of seedlings extracellular fluids of *Helianthus annuus* L. (Asteraceae) (common name sunflower), and subsequently it was isolated by D-mannose affinity chromatography (Pinedo et al., 2012). The protein was called Helja (*H. annuus* jacalin) since the proteomic analysis showed that it belonged to the jacalin family. As Supporting material (Fig. S1), we inform the deduced amino acid sequence from the full-length Helja cDNA, although the details of its molecular characterization can be found in Pinedo et al. (2015). Helja showed to possess antifungal activity against several spp. of the *Candida* genus (Regente et al., 2014). In fact, 0.2 μg/μl of Helja produced growth inhibition of *C. albicans*, *C. parapsilosis* and *C. tropicalis*. By fluorescence microscopy using SYTOX-Green it was determined that Helja alters the membrane permeability of *C. albicans* and *C. tropicalis*. In addition, Helja induces the production of reactive oxygen species in *C. tropicalis* cells. Since *C. albicans* is the major species responsible for invasive candidiasis, the aim of this work was to get insights about the antifungal mechanism of Helja and to study the lectin ability to inhibit *C. albicans* biofilm formation and adherence to buccal epithelial cells (BEC).

Materials and methods

Helja purification

Dehulled *H. annuus* seeds (Sunny Pampas line provided by Andreoli S.A., Chivilcoy, Buenos Aires, Argentina) were used for Helja isolation. The seeds were subjected to the extraction of the extracellular fluid (EF) by a standard infiltration-centrifugation procedure (Regente et al., 2014) and the isolation of Helja was carried out according to Pinedo et al. (2012), with some modifications. The EF was loaded on a 1 ml D-mannose-agarose resin (Sigma-Aldrich, St Louis, MO, USA) equilibrated with 50 mM HCl-Tris pH 7.5, 100 mM NaCl (buffer A). Non-bounded proteins were washed with buffer A, and subjected to elution of retained proteins with 0.2 M mannose in the same buffer. The eluted fraction was exhaustively dialyzed against distilled water to allow the release of mannose from the protein fraction.

C. albicans culture conditions

C. albicans strain NGY152 (Brand et al., 2004) was used in this study. *C. albicans* cultures were maintained on Sabouraud agar (1% peptone, 2% glucose and 1.7% agar-agar) at 30ºC. For the preparation of the suspensions, an inoculum of yeast was plated on Sabouraud agar, allowed to grow at 30ºC for 48 h and transferred to sterile Sabouraud broth. The yeast cells were quantified in a Neubauer chamber or by optical density at 600 nm for further calculation of appropriate dilutions.

Viability assays

To monitor the effect of Helja on the yeast viability, the Evans Blue uptake method was used. Yeast cells were incubated in the absence or presence of Helja (0.1 μg/μl) for 48 h, stained with Evans Blue 0.05% and observed by optical microscopy. Cell viability was also evaluated by counting of colonies forming units (CFU) after Helja treatment. A cell suspension (10^4 cells/ml) in Sabouraud broth culture medium was incubated with protein sample (0.1 μg/μl) or water as control during 48 h at 30ºC. Subsequently, the cells were washed once and diluted 1000-fold in Sabouraud broth culture medium, and an aliquot of 100 μl from this dilution was spread over the surface of a Sabouraud agar medium with a Drigalski loop and grown at 30 °C for 48 h. At the end of this period, CFU were determined, and the Petri dishes were photographed. The experiments were carried out in triplicate. The percentage of cell viability in the presence of Helja was

calculated relative to the control in the absence of the lectin, which was considered as 100%.

Yeast growth inhibition assays

The yeast growth inhibition assays were performed in 96-well flat microplates incubating *C. albicans* (10^4 cells/ml) with protein sample (0.1 μg/μl) or water as control in Sabouraud broth. The fungal growth was monitored by 630 nm optical reading at the indicated times during 48 h of incubation at 30ºC (Regente et al., 2014). The effect of Helja on yeast cell integrity was assessed adding 20 μg/ml Calcofluor White (Sigma-Aldrich, St Louis, MO, USA) as cell wall perturbing agent (Ansari et al., 2016) or 0.8 M sorbitol (Sigma-Aldrich, St Louis, MO, USA) as osmotic support (Frost et al., 1995). Fluconazole (Sigma-Aldrich, St Louis, MO, USA) was used as a positive control of growth inhibition at the indicated concentrations. The percentage of growth inhibition was calculated relative to the control in the presence of water, which was considered as 100 % growth. Yeast growth tests were performed by triplicate and repeated at least twice. Following the yeast growth assays the samples were mounted on glass slides, stained with CFW (0,02 %), and visualized by confocal laser scanning microscopy (CLSM). CFW was excited at 355 nm and detected at 300-440 nm. Acquisition and processing of images were performed as indicated below.

Detection of hydrogen peroxide

Detection of hydrogen peroxide in *C. albicans* cells was performed by a staining procedure using 3,3'-diaminobenzidine (DAB) (Del Rio et al., 2018). Briefly, the yeast cells (1 x 10^5 cells/ml) were incubated for 2 h on microslides with water (negative control), the protein sample (0.1 μg/μl) or 300 mM hydrogen peroxide (positive control) in the presence of 0.5 mg/ml DAB. The slides were microscopically evaluated for detection of hydrogen peroxide as a brown pellet. To determine the percentage of cells showing the presence of peroxides a count of cells brown-stained related to the total cells was performed for the samples treated with Helja and the controls from at least 10 images of three independent experimental replicates.

C. albicans morphological switching assay

Cells were grown as described above and transferred to YPD broth (peptone 2%, yeast extract 1% and glucose 2%). Then, a cell suspension (10^5 cells/ml) was incubated with

protein sample (0.1 μg/μl) or water as control during 5 h or 24 h protected from light at 37ºC, and then observed by optical microscopy (Nadeem et al., 2013). The assays were performed by triplicate and repeated at least twice. To quantify the filamentation a count of the filamentous forms related to the total cells (yeast +filamentous forms) was performed for the samples treated with Helja and the controls from at least 10 images of two independent experimental replicates. The percentage of filamentation was calculated relative to the control in the presence of water, which was considered as 100 %.

Adherence assays

BEC were collected from healthy adult volunteers after information of the study was disclosed and the written informed consent was signed. This study was approved on August 1, 2018, by the Bioethics Committee from Universidad Nacional de Mar del Plata, registered in the Registro Provincial de Comités de Ética en Investigación, dependent on the Comité de Ética Central en Investigación, Ministerio de Salud de la Provincia de Buenos Aires, Argentina, under Nº 061/2016, Folio 124, Book 2. BEC were obtained via soft scraping of the cheek mucous membrane with sterile cotton swabs. BEC or yeast cells were re-suspended in 0.02 M phosphate-buffered saline (PBS) pH 7.2, washed twice by centrifugation (2500 *g*, 5 min each) with PBS and finally quantified in a Neubauer chamber. Equal volumes (100 μl) of BEC (10^5 cells/ml) and yeast (10^7 cells/ml) suspensions were incubated with PBS (control) or Helja (0.1 μg/μl) at 37ºC under gently stirring for 2 h. After incubation, the mix was washed with 4 volumes of PBS through paper filter (Whatman Nº 12) and the contents of the filter were transferred to microscope slides. The samples were stained with 1 % methylene blue and examined using light microscopy to count the number of yeast cells attached to 100 BEC. The experiments were carried out in triplicate.

Antibiofilm assays

The effect of Helja on *C. albicans* biofilm formation was assessed in polystyrene 96-well flat microplates containing a suspension of yeasts in Sabouraud broth (10^8 cells/ml) in a final volume of 100 μl. The plates were incubated for 48 h at 37ºC to allow biofilm development. The protein sample (0.1 μg/μl) or PBS (control) were added at the indicated times. At the end of incubation the wells were washed with PBS to remove the planktonic cells. The viable biomass of biofilm was quantified by CFU count. The cells of the biofilm from each well were scraped and suspended in 10 ml of sterile PBS. After

serial dilutions, 100 µl were plated on Sabouraud agar, incubated for 48 h at 30ºC and the CFU count was performed. The inhibition percentage of biofilm development in the presence of Helja was calculated relative to the control in the absence of the lectin, which was considered as 100% development. Antibiofilm tests were performed by quintupled and repeated at least twice.

Confocal laser scanning microscopy analysis

Fluorescein isothiocyanate (FITC) (Sigma-Aldrich, St Louis, MO, USA) was covalently coupled to Helja according to the methodology previously described (Del Rio et al., 2018). The yeast cells were treated with 0.05 µg/µl of Helja-FITC for 1 h or 24 h. When indicated, 500 mM D-mannose (Sigma-Aldrich, St Louis, MO, USA) or 0.05 µg/µl Concanavalin A (Sigma-Aldrich, St Louis, MO, USA) was added to the incubation. After the treatments, the materials were mounted on glass slides and visualized by CLSM to analyze the binding of Helja to fungal cells. The screening along the z axis of yeast cells was performed taking optical sections of 1 µm thickness. When indicated, a staining with fluorescent probe 0.02 % CFW was performed. FITC was excited at 450-490 nm and detected at 500 nm. CFW was excited at 355 nm and detected at 300-440 nm. Microscopic analysis was performed using a Nikon C1 confocal laser scanning microscope (Nikon Instruments Inc., Melville, NY, USA). Images were acquired with Super Fluor 40.0x/1.30/0.22 oil-immersion lens. The post-processing of images was performed with the aid of EZ-C1 FreeViewer version 3.2 software.

To determine the biofilm coverage on polystyrene surface, after washing planktonic cells, the microplate was air dried at room tempeture for 24 h. Then, the plates were mounted on microscope platen for analysis with fluorescent probes 0.02 % CFW and N-(3-triethylammoniumpropyl)-4-(6-(4-diethylaminophenylhexatrienyl) pyridinium dibromide (FM4-64) (Sigma-Aldrich, St Louis, MO, USA) (2 µg/ml) for labeling of yeast cell wall and the cell membrane system respectively. FM4-64 was excited at 488 nm and detected at 650-750 nm. Images were acquired with Plan Apochromat 20.0x/0.75/1.00 Dry spring-loaded. The post-processing of images was performed as described above. Quantification of biofilm coverage was performed by image processing program ImagJ from CLSM microscopy photographs.

Cellular surface hydrophobicity determination

The cellular surface hydrophobicity (CSH) was determined using a microbial adhesion to hydrocarbons method according to Blanco et al. (2008). Yeast suspensions were prepared in PBS to 0.4 optical density at 600 nm. After incubation of 300 µl of cell suspension in the absence (control) or presence of Helja (0.1 µg/µl) for 2 h, 100 µl xylene was added, it was vortexed for 1 min, allowed to settle for 5 min, then optical density at 600 nm was measured again. The CSH index was calculated as: % CSH = [(ODInitial - ODFinal) / ODInitial] × 100. The experiments were carried out in triplicate.

Statistical analysis

Numerical data were presented as means +/- standard deviation (SD). Number of repetitions individual assays was different and was given in the description of each of them. Statistical differences between sets of data were evaluated using Tukey's 2-tailed t-test, assuming unequal variance. *P*-values of 0.05 were considered significant.

Results

Effect of Helja on the viability of C. albicans.

To deepen the study of the anti-*Candida* activity displayed by Helja, different experimental approaches were performed using concentrations of the lectin included in the range in which it shows antifungal activity (Fig. S2) (Regente et al., 2014). Fig. S3 shows the percentage of *C. albicans* growth inhibition displayed by 0.1 μg/μl Helja compared to different doses of Fluconazole, a known antifungal drug among the most used for the treatment of fungal infections. To assess the effect of Helja on yeast viability, Evans Blue staining was performed, which is based on penetration of the dye into dead cells. A yeast suspension was treated with Helja (0.1 μg/μl) and after 48 h of incubation, part of the cells appeared blue-stained, revealing the presence of non-viable cells (Fig. 1A). To quantify this effect, the cells were plated on solid media and subjected to the count of colony forming units (CFU). Fig. 1B shows the drastic decrease in survival of *C. albicans* cultures treated with Helja (0.1 μg/μl) compared to controls, corresponding to a viability loss of 82 % (Fig. 1C). Taken together, the results of Fig. 1 highlight that the inhibitory ability of Helja on *C. albicans* is based on a fungicidal action.

Effect of Helja on the cell integrity and oxidative status of C. albicans.

It was previously determined that Helja alters the membrane permeability of *C. albicans* through the penetration of SYTOX-Green fluorophore into yeast cells. To further investigate the effect of Helja on fungal cell integrity, we performed yeast growth inhibition assays in presence of the cell wall perturbing agent Calcofluor White (CFW), which binds to chitin emitting a blue fluorescence (Ansari et al., 2016). Yeast cultures treated with Helja (0.1 μg/μl) in the absence or presence of CFW showed a significant reduction of fungal growth relative to controls. However, no significant differences were seen in the fungal growth between the Helja treated culture and the one treated with the lectin and CFW simultaneously (Fig. 2A). In contrast, the microscopic observation of the cultures revealed changes in the cell morphology of the yeasts treated with Helja and CFW simultaneously with respect to the controls and even in relation to the cells treated only with the lectin (Fig. 2B). Thus, the images show that control cells and those treated only with CFW or only with Helja, presented a regular oval shape and a homogeneous size of about 4.5 μm in diameter. However, morphological alterations,

such as loss of the typical oval shape of the yeasts and irregular sizes that quadruple the diameter of the cells, were observed in the cultures treated with the lectin and CFW simultaneously (Fig. 2B). Then, to explore the influence of Helja on the integrity of the fungal cell wall, a sorbitol protection assay was conducted (Frost et al., 1995). If a compound negatively interferes with the fungal cell wall, the dose required to generate a growth inhibition will be greater in the presence of osmotic support. The percentages of growth inhibition of *C. albicans* in liquid medium for Helja 0.1 µg/µl in the presence and absence of sorbitol were determined. Helja generated a growth inhibition of 60 % +/- 17 in relation to the controls. However, the lectin in the presence of sorbitol showed no inhibitory effect, since the cultures showed the same levels of fungal growth as the controls. These results indicate that Helja affects the cellular integrity of *C. albicans* and its mechanism of action would include a perturbation of the cell wall structure.
Since a disturbance in the oxidative status can result in damage to the cellular components, we asses if the cytotoxic effect of Helja on fungal cells involves the induction of reactive oxygen species (ROS). The oxidation of 3, 3-diaminobenzidine (DAB) in the presence of peroxides is observed as a dark-brown signal indicative of the local formation of an insoluble precipitate at the reaction site (Del Rio et al., 2018). The yeast cells were incubated with 0.1 µg/ µl Helja for 2 h in the presence of DAB and observed by optical microscopy. Several cells treated with the lectin developed a strong brown pellet while cells incubated with water appeared mostly unstained (Fig. 3). In fact, the quantification of this effect indicates that the percentage of cells treated with Helja showing the presence of peroxides was significantly higher (22.0 +/-5.4 %) in relation to the cells incubated with water (10.4 +/- 4.6 %) and even higher than cells treated with hydrogen peroxide (14.7 +/- 0.8) which was used as a positive control. These results reveal that the lectin induces oxidative stress in *C. albicans* cells.

Effect of Helja on the morphological transition from yeast to filamentous forms.
The transition between yeast and filamentous forms constitutes an important attribute for pathogenicity in *C. albicans.* In order to assess the effect of Helja on the cellular dimorphism, assays under optimal conditions for the induction to filamentous forms were carried out. The microscopic observation of *C. albicans* cells treated with Helja (0.1 µg/µl) revealed morphological differences compared to controls (Fig. 4). While the control samples showed abundant filamentous forms, the cells subjected to Helja treatment were present as blastoconidia (yeast forms). The inhibition of morphological

switching was visualized both at 5 h and 24 h after treatment with the lectin. To quantify this effect the percentages of filamentation were calculated relative to the control in the presence of water (100 % of filamentation). Thus, it was determined that Helja significantly inhibited filamentation, since the cells treated with the lectin showed only 20.8 +/- 10.7 % (5 h) and 4.4 +/- 3.7 % (24 h) of filamentous forms with respect to the controls. These results show the ability of Helja to inhibit the plasticity of cell morphology.

Effect of Helja on the adhesion of C. albicans to BEC.

The adhesion of *Candida* to host tissues is a critical initial step in the infection process (Zhu and Filler, 2010). Hence, for further assessment of the antifungal activity of Helja, its effect on the yeast adherence to primary host BEC was analysed. BEC and yeast cells were incubated in the absence or presence of Helja and after filtering the samples to remove the non-adhered yeasts, they were subjected to microscopic observation and quantitative analysis. The Fig. 5A shows a microscopic image of *C. albicans* adhered to human BEC. The number of yeasts bound to 100 BEC and the subsequent statistical analysis showed a significant reduction of adherent cells (46 %) in the samples subjected to 0.1 μg/μl Helja treatment compared to controls (Fig. 5B), demonstrating a clear inhibitory effect of Helja on the adhesion of *Candida* to BEC.

Effect of Helja on the biofilm development of C. albicans.

The effect of Helja on the biofilm formation on polystyrene surface at the different stages of development was assessed. Thus, Helja was added at the initial phase, in which the planktonic cells adhere to the surface, or in the intermediate stage, in which the cells are already attached to the support and actively secreting the components of the extracellular matrix. Interestingly, Fig. 6A shows that both the lectin added in the initial phase (0 h) and in the intermediate stage (8 h) caused a significant decrease in the number of cells compared to controls reaching a 40 % and 30 % respectively. Next, the biofilm structure was examined by CLSM. Based on the affinity of CFW for polysaccharides and the amphipathic nature of the FM4-64, the two fluorophores provided an effective labeling of yeast cell wall and the cell membrane system, respectively. Representative CLSM images of *C. albicans* biofilms from independent assays stained with blue and red markers are shown in Fig. 6B. The biofilm coverage

visualized by staining with both fluorophores was clearly lower in Helja treatments compared to controls. From the quantitative analysis of the images it was determined that the biofilm coverage area on the polystyrene surface was significantly lower in the samples treated with Helja (13 %) relative to controls (66 %) for the two groups of biostructures (Fig 6C).

Effect of Helja on the CSH of C. albicans.

Non-specific unions, principally attributed to hydrophobic forces, have been implicated in yeast adhesion to plastic support (Goswami et al., 2017). To explore whether Helja induces changes in the CSH, this feature was determined by the method of microbial adhesion to hydrocarbons. Interestingly, the CSH of the yeast cells was drastically reduced from 40 % in the controls to 4 % in Helja treated cells (Fig 7), suggesting that alterations in the CSH could contribute, at least in part, to the effect of the lectin on yeast adherence and consequent biofilm development.

Analysis of the binding of Helja to the cell surface of C. albicans.

To explore the direct interaction of the lectin with the cellular surface of *C. albicans*, Helja was conjugated to FITC and monitored by CLSM. As observed in Fig. 8, the yeast cells were completely labeled with green fluorescence of Helja-FITC after 1 h of incubation, revealing the binding of the protein to yeast cell surface. To assess whether Helja remained attached to the yeast surface or whether it could be internalized into the cells, we have performed a screening along the z axis of yeast cells by CLSM. The localization of Helja FITC was restricted to the surface of cells, and no internalization was observed even after 24 h of incubation (not shown). As observed in Fig. 8, the binding of Helja-FITC to *C. albicans* cells was examined incubating simultaneously with CFW for cell wall staining. Thus, the images also show the fluorescence of Helja-FITC distributed as a thin outer layer to the CFW signal, and this localization did not change after 24 h of incubation. Since Helja is a mannose binding lectin, we assessed whether the binding of the lectin to the cell surface could be attributed to its ability to recognize putative mannoproteins ligands present in outer layer of yeast cell wall. Then, after incubation of the cells with Helja-FITC for 24 h, D-mannose or ConA, a known mannose binding lectin, were added. The Fig. 9 shows that the fluorescent signal of the lectin was barely visible or totally abolished after 20 min of the mannose or ConA

addition, demonstrating the sugar and ConA ability to displace the binding of Helja-FITC to yeast surface.

Discussion

There is global consensus on the rational use of antibiotics to attenuate the emergence of resistant microorganisms, which has been recommended by the World Health Organization (WHO, 2015). According to this concern, the search for new natural antimicrobial agents has been a great challenge of the scientific community in recent years. In this sense, the previous antifungal activity associated to Helja stimulated us to deepen the study of its anti-*Candida* properties.

Our results have shown that Helja affects the viability of *C. albicans*, thus, its antifungal activity is based on a fungicidal effect. A better clinical efficiency of fungicidal agents over those fungistatic drugs remains elusive. However, a prolonged fungicidal effect (i.e., amphotericin B) was reflected in suppression of the fungus after removal of the drug, whereas a fungistatic agent (i.e., fluconazole) showed rapid regrowth of the pathogen after the antifungal therapy (Lewis and Graybill, 2008). In this sense, the fungicidal ability of Helja places it among the candidates for improving current antifungal therapies.

Although many lectins with different biological properties have been described in the literature, only a small group showed antifungal activity (Yan et al., 2015). On the other hand, most of studies have been conducted with phytopathogenic fungi and only few works involve fungi of medical importance. Among them, it has recently been reported that a chitin-binding protein from *Moringa oleifera Lam. (Moringaceae)*, Mo-CBP2, shows anticandidal activity by increasing cell membrane permeability and reactive oxygen species production on the fungal cells (Neto et al., 2017). Another example is PgTeL, a chitin-binding lectin of *Punica granatum L. (Lythraceae)* whose action mechanism involves oxidative stress, energetic collapse and damage of *C. albicans* and *C. krusei* cell wall (da Silva et al., 2018). Although the molecular mechanisms leading the antifungal activity of lectins is not well elucidated yet, it is speculated that their growth inhibition capability is related to cell wall specific carbohydrate recognition (Dang and Van Damme, 2015).

Here we show the interaction of Helja with the fungal cell wall surface, which was inhibited by mannose and ConA, suggesting that the binding could be mediated by ligands of a glycosidic nature. This finding becomes relevant since it provides an explanation for the antifungal ability of Helja. According to the knowledge assembled so far, an antifungal drug that targets the cell wall would be an efficient way to inhibit

the growth of fungi (Ansari et al., 2016). The cell wall is a dynamic structure important for maintaining the cell shape and the protection against environmental stresses. The walls must be physically robust, but flexible enough to permit cell expansion, cell division and morphogenesis (Hall and Gow, 2013). Previously Kingsbury et al. (2012) showed that the fluorescent brighteners inhibit the fungal growth disrupting cell wall through the binding to chitin and glucan. Another example is caspofungin, a fungicidal drug especially efficient towards *Candida* spp., which induces chitin accumulation and cell wall modifications determinants for its deleterious effects (Rueda et al., 2014). Similarly, the lectins from *Calliandra surinamensis Benth. (Fabaceae)* pinnulae and *P. granatum* juice induce the loss of fungal viability through cell wall rearrangements (Procopio et al., 2017; da Silva et al., 2018). It was previously demonstrated that Helja induces the permeabilization of the plasma membrane of the *C. albicans*, which was evidenced by the uptake of the SYTOX Green fluorophore by the yeast cells after the treatment with the lectin (Regente et al., 2014). Previous reports have also shown rapid structural realignments of the cell wall in response to the shrinkage of the plasma membrane during osmotic shock (Ene et al., 2015) or changes in membrane fluidity associated with changes in cell wall mechanical properties (Francois, 2016), suggesting an intimate relationship between the cell wall and the plasma membrane in the fungal cells. In this sense, the interaction of Helja with the cell wall could trigger determinant modifications for its antifungal effect at the level of the cell wall, the plasma membrane or both structures.

Here we also show that *C. albicans* cells undergo oxidative stress when treated with Helja. ROS are frequently increased when intracellular oxygen metabolism is unbalanced. When this balance is disrupted, molecules such as hydrogen peroxide, hydroxyl and superoxide radicals can be accumulated originating cellular damage. The mode of action of Helja is probably linked to its ability to increase ROS levels and to disrupt the integrity of cell structures. Although the induction of ROS is usually an early event that share different signaling pathways, the sequence of events triggered by the interaction of Helja with the cell wall which ultimately lead to yeast death remains to be elucidated.

On the other hand, the binding of Helja to cell surface seems to affect the interaction of *C. albicans* to both biotic and abiotic surfaces. The carbohydrates present on the fungal cell wall are the initial point of contact in primary interaction with host cells. Thus, Helja could counteract the infection process through the binding to carbohydrate

ligands. Here we show that this lectin decreases the adherence to BEC, biofilm formation ability and CSH, which are determining factors of the virulence of *C. albicans* (Zhu and Filler, 2010; Goswami et al., 2017; Cavalheiro and Teixeira, 2018). Adherence is a critical first step in the infection process. It is essential for both colonization and subsequent induction of mucosal disease (Zhu and Filler, 2010). Thus, it is not surprising that this microorganism expresses multiple different cell wall structures that mediate adherence to BEC. The cell wall proteins of *C. albicans* are highly decorated with elaborate carbohydrate such as α- and β-linked mannose units referred to as mannoproteins (Hall and Gow, 2013). Mutants involved in the biosynthesis of mannoproteins show attenuated virulence and most also have adhesion defects. Thus, the ability of Helja to reduce the levels of adhesion to BEC could be associated with its interaction with cell wall mannoproteins. In fact, the Helja-FITC binding with the outer layer of the cell wall surrounding the signal of CFW, and the mannose and ConA inhibitory effect on this interaction, provide strong evidences in this regard. Interestingly, the results of this work and the previous characterization of Helja activity against phytopathogenic fungi (Del Rio et al., 2018) show similarities and differences related to the mechanism of antifungal action of the lectin. The Helja antifungal effect on *S. sclerotiorum* involves interaction with the cell surface and internalization in the intracellular space (Del Rio et al., 2018). However, here we show that the localization of the lectin is restricted to the outer layer of the fungal cell wall and Helja was not able to enter into yeast cells for 24 h of incubation. On the other hand, since mannose inhibits both the uptake of Helja into *S. sclerotiorum* spores and the binding of the lectin to *C. albicans* cell wall, the antifungal action of Helja appears to be determined by its specific binding ability to ligands with mannose structures exposed towards the outer surface of the cell wall in these two different species.

On the other hand, yeast to hyphal switching in *C. albicans* is one of the major factors governing the virulence. The fungus can grow in a single-celled, budding yeast form (blastospore) or in a filamentous form (including both pseudohyphae and true hyphae). The production of germ-tubes results in conversion to a filamentous growth phase or hypha, also called the mycelial form. The formation of pseudohyphae occurs by polarized cell division when yeast cells growing by budding have elongated without detaching from adjacent cells. Hyphae appear to be the invasive form of the organisms, as the majority of intracellular organisms are hyphae, whereas yeast are typically located either between or on the surface of epithelial cells. It is known that mutants of *C.*

albicans with reduced capacity to invade epithelial cells in vitro usually have reduced virulence in experimental animal models of mucosal candidiasis (Zhu and Filler 2010). We found that fungal cells treated with Helja appeared only in yeast form in contrast to the untreated *C. albicans* cells which were efficiently able to express filamentous forms, suggesting that Helja inhibits the morphogenetic switching. Thus, it can be speculated that Helja could avoid host epithelial cell damage by disrupting its interaction on two action levels, both in the initial adhesion and in the hyphal switching-mediated invasion. Other specific feature of *Candida* species pathogenicity is the ability to form biofilms, which protects them from external factors such as host immune system defenses and antifungal drugs. Biofilms are structured communities of microorganisms surrounded by polymeric matrix and adhered to an inert or living surface. Cell adherence is a primary phase in formation of biofilm where *C. albicans* adhere to the substratum and the intermediate stage includes the secretion of components of the extracellular matrix that is assembled in the maturation step to develop the biofilm (Ganguly and Mitchell, 2011). Our results showed the antibiofilm activity of Helja, visualized as a reduction of biofilm development. Interestingly, Helja affected the progress of the biofilm both when it was added from the beginning of its formation, as in the intermediate stage of development. Some plant compounds have been evaluated for antibiofilm activities on *Candida* species. For instance, flavonoids from *Dalea elegans* and *Scutellaria baicalensis* showed antibiofilm activity against *C. albicans* (Peralta et al., 2015; Serpa et al., 2012). Among lectins tested for antibiofilm activity, the fungal growth was progressively inhibited at increasing doses of lectin PgTeL, however this was accompanied by an increase of biofilm biomass. This effect was associated to the agglutinating property of the lectin, which would facilitate cell-cell adhesion (da Silva et al., 2018). The fact that Helja maintains its antibiofilm activity on preformed biofilm structures highlights its antifungal ability since *Candida* biofilms are 30-2000 times more resistant to several drugs compared to their planktonic counterparts (Olsen, 2015). On the other hand, Helja induced a decrease of CSH in yeast cells, which is an attribute associated with fungal virulence. Under different environmental and growth conditions yeast cells can present a high degree of hydrophobicity, which helps *C. albicans* to be a successful fungal pathogen. Thus, the CSH influences several steps in pathogenesis such as nonspecific adhesion to host tissues and implanted medical devices, enhanced germ-tube induction, increased co-aggregation and colonization of cells, biofilm formation, avoidance of neutrophil mediated killing and protection from host defense

mechanisms (Goswami et al., 2017). The comparative analysis of hydrophilic and hydrophobic cells indicated that both forms are biochemically similar, however the ultrastructure of their cell walls is different (Singleton and Hazen, 2004). It could be speculated that the binding of Helja to mannoproteins of the outer cell wall is the cause of the decrease of CSH, probably limiting the exposure of hydrophobic residues to the cell surface.

Even though the occurrence of multiple putative targets of Helja in the fungal cell might be speculated on the basis of the lectin affinity for mannose, additional experimentation is required to determine the spatial and/or temporal order of reactions composing the signal transduction pathway. However, according to current knowledge, it could be speculated that both the destabilization of the plasma membrane and / or the cell wall through its interaction with constitutive mannoproteins and the induction of ROS triggered by Helja could be sufficient to trigger its cytotoxic effect.

Our results show promising antifungal activity of Helja through a mechanism that involves multiple levels of action on *C. albicans* cells, opening up novel opportunities to employ this information in improving the antifungal treatment strategies.

Conclusion

The results demonstrate the interaction of Helja with the mannoproteins of *Candida albicans* cell wall, the disruption of the cell integrity, the production of ROS, the inhibition of the morphological transition from yeast to filamentous forms and the induction of the fungal cell viability loss. The binding Helja-*Candida* also provides a possible explanation of the lectin effect on cell adherence, biofilm development and CSH, relevant features related to virulence of the pathogen.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Figure legends

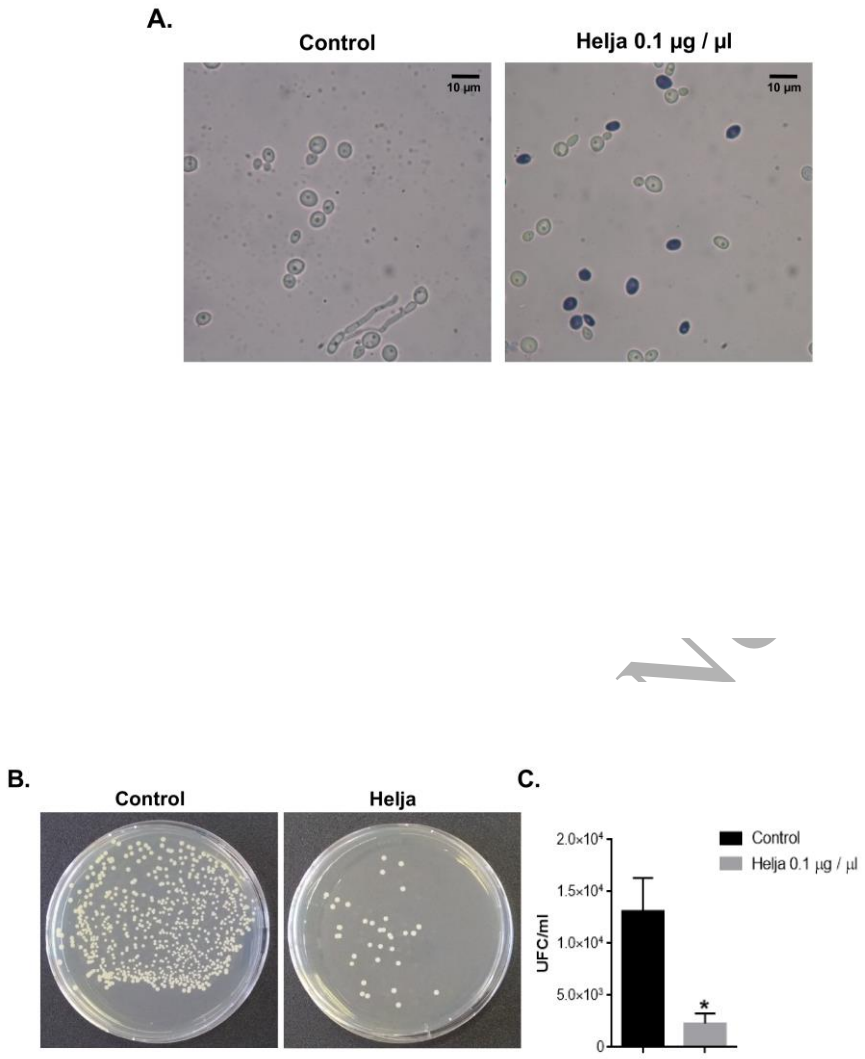


Fig. 1. Effect of Helja on cell viability of *C. albicans*. A. Yeast cells incubated in the absence or presence of Helja (0.1 µg/µl) stained with Evans Blue to detect non-viable cells. The images are representative of 3 biological replicates. **B.** Photographs of *C. albicans* cultures incubated in the absence (control) or presence of Helja (0.1 µg/µl). **C.** Percentage of cell viability expressed from count of CFU of cultures showed in **B.** The results are representative of 2 biological replicates. (*) significative difference $p<0,05$.

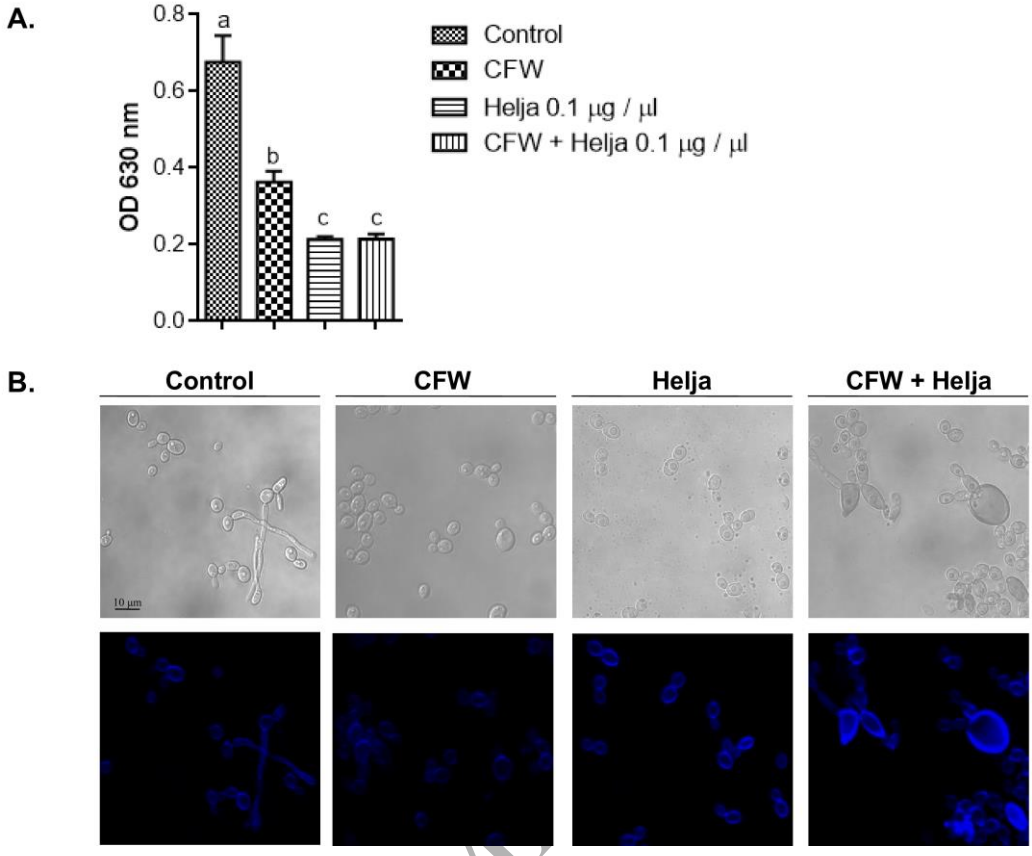


Fig. 2. Effect of Helja on cell integrity of *C. albicans.* A. Yeast growth **(A)** or CLSM images **(B)** of cells incubated with water (control), with CFW (20 µg/ml), with Helja (0.1 µg/µl), or with CFW (20 µg/ml) and Helja (0.1 µg/µl) simultaneously, in Sabouraud broth at 30ºC. The experiments were performed in triplicate, and the values are shown as the average with its standard deviation. Different letters indicate significant differences between treatments, $P < 0.05$.

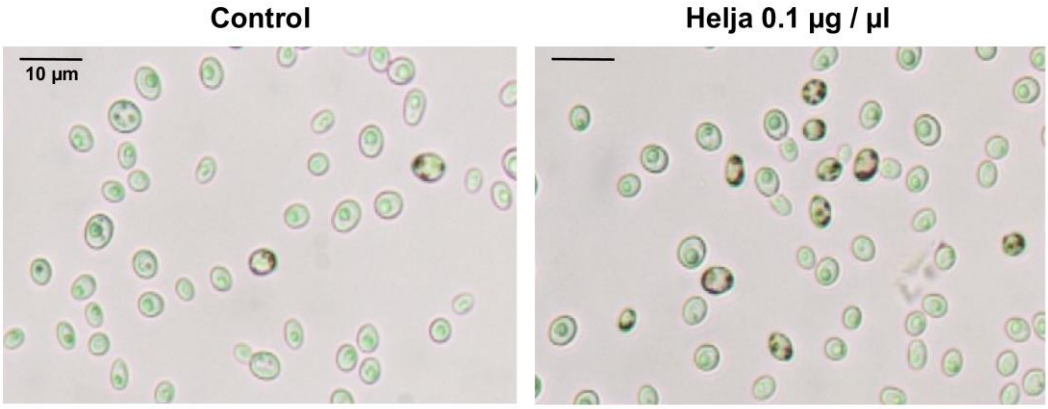


Fig. 3. Effect of Helja on oxidative status of *C. albicans.* Yeast cells incubated with water (control) or Helja (0.1 µg/µl) for 2 h in the presence of 0.5 mg/ml DAB for detection of hydrogen peroxide. The images are representative of 3 biological replicates. Scale bar: 10 µm.

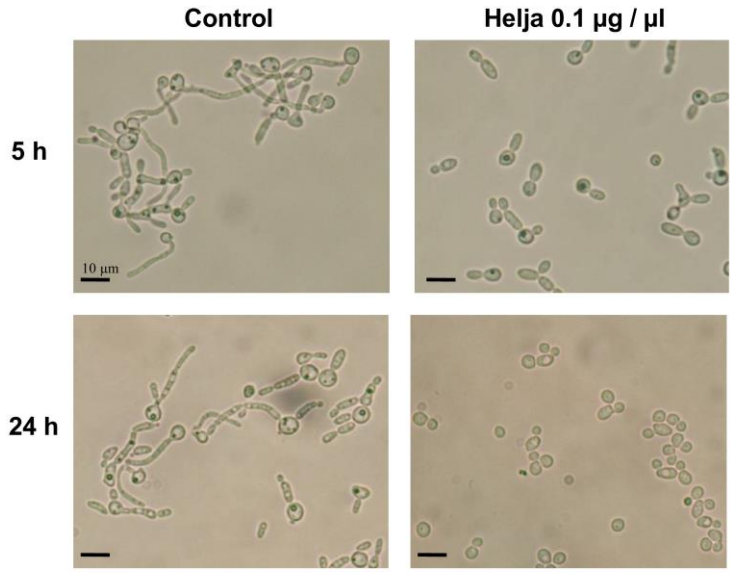


Fig. 4. Effect of Helja on morphological transition from yeast to filamentous forms. Yeast cells incubated for 5 h and 24 h in absence (control) or presence of Helja (0.1 µg/µl) as indicated in Materials and methods section, and observed by optical microscopy. The images are representative of 2 biological replicates.

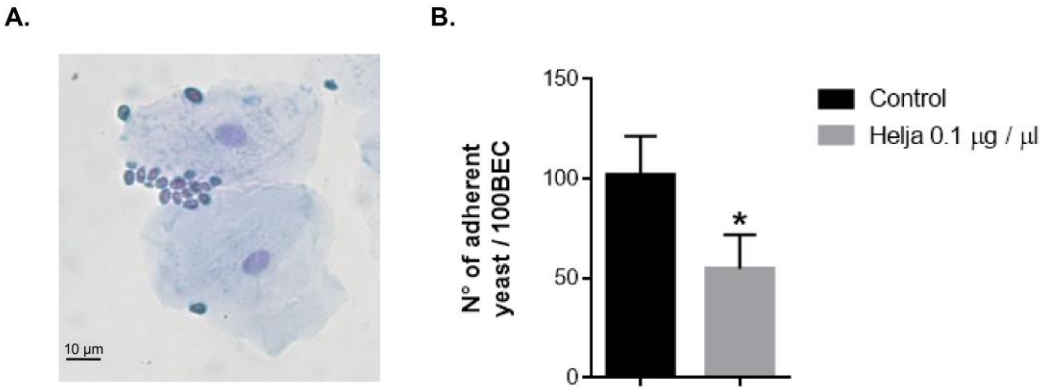
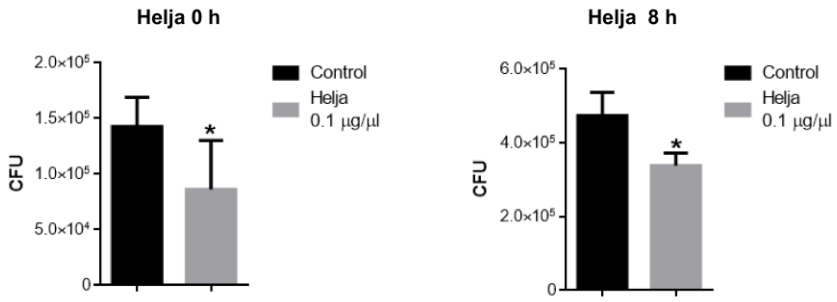
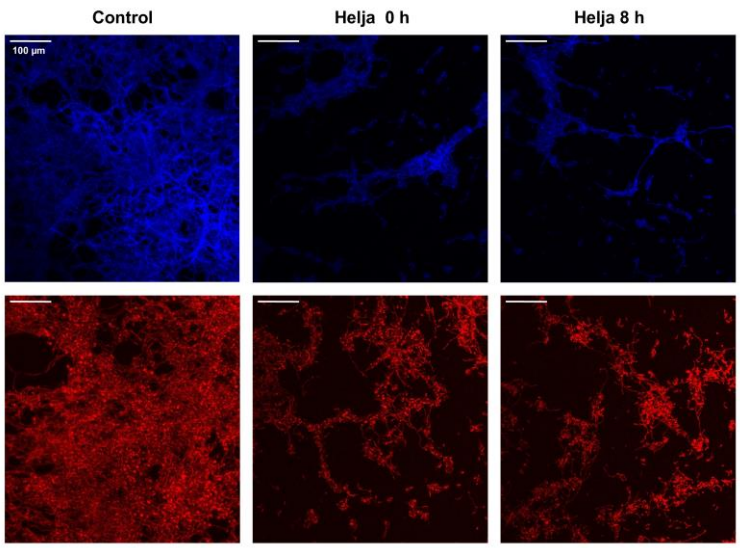


Fig. 5. Effect of Helja on *C. albicans* adherence to epithelial cells. A. Microscopic image of *C. albicans* cells adhered to BEC. **B.** Number of *C. albicans* cells adhered to 100 BEC in the absence (control) or presence of Helja (0.1 µg/µl). The experiments were performed in triplicate. (*) significative difference $p<0,05$.

A.


Helja 0 h
Helja 8 h
CFU
2.0×10⁵
1.5×10⁵
1.0×10⁵
5.0×10⁴
0
6.0×10⁵
4.0×10⁵
2.0×10⁵
Control
Helja
0.1 μg/μl
*

B.


Control
Helja 0 h
Helja 8 h
100 μm

C.

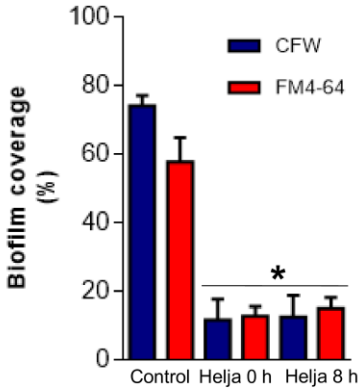


Fig. 6. Effect of Helja on biofilm development. A. Biofilm development on polystyrene microplates in the absence (control) or presence of Helja (0.1 µg/µl) added to the assay at 0 h or at 8 h. Biofilm cells were quantified as CFU. The experiments were performed in triplicate. (*) significative difference p<0,05. **B.** Biofilms developed in **A** stained with CFW (cell walls) or FM-64 (cell membranes) and observed under CLSM. The images are representative of 3 biological replicates. **C.** Biofilm coverage area quantified by image processing program ImagJ from five photographs for each experimental condition.

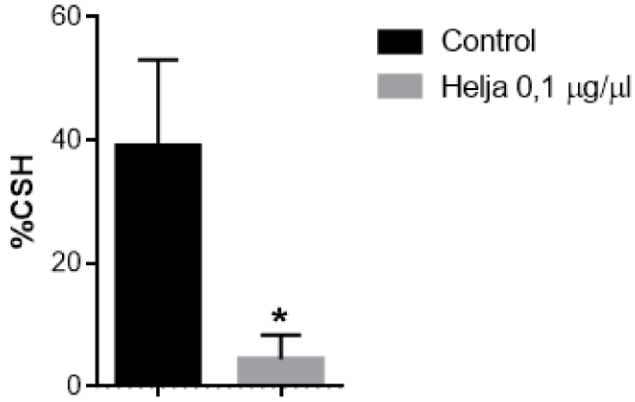


Fig. 7. Effect of Helja on cellular surface hidrofobicity of *C. albicans.* Yeast cells incubated in the absence (control) or presence of Helja (0.1 μg/μl) and subjected to determination of CSH. The percentage of CSH was calculated as: [(ODInitial _ ODFinal) / ODInitial] × 100. The experiments were performed in triplicate. (*) significative difference $p<0,05$.

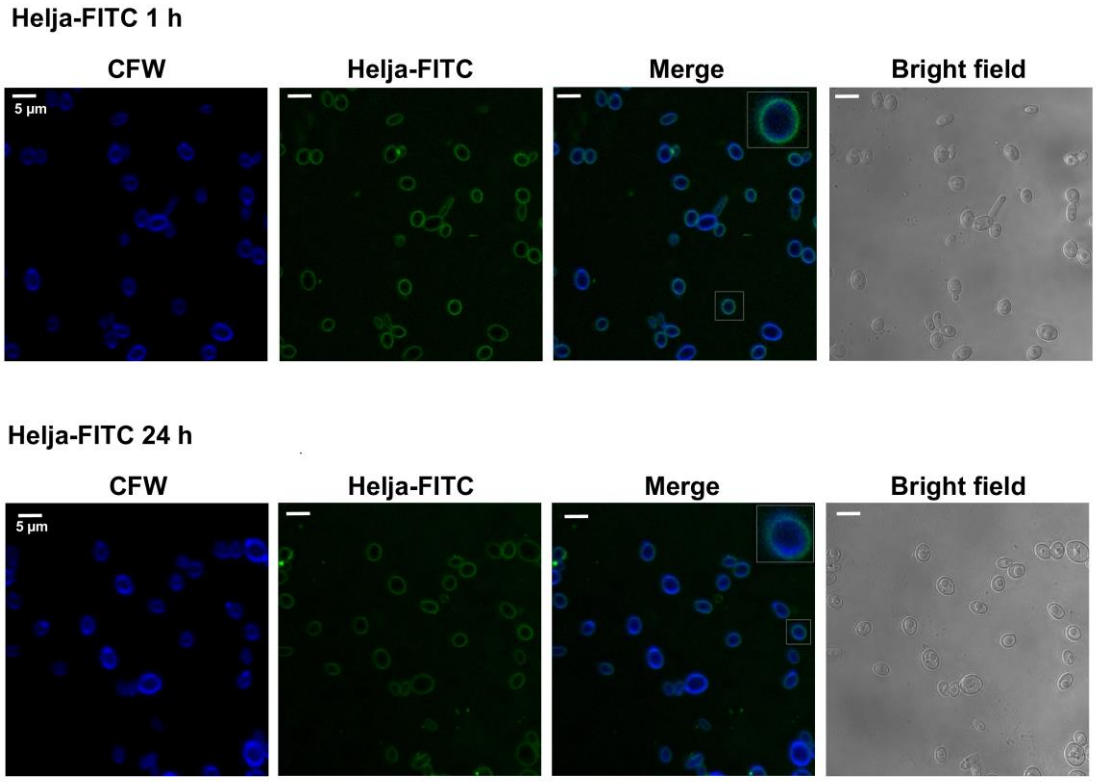


Fig. 8. Binding of Helja-FITC to *C. albicans* cells. Yeast cells incubated with 0,05 µg/µg Helja-FITC for 1h or 24 h, stained with CFW, and observed under bright field or CLSM. The images are representative of 3 biological replicates.

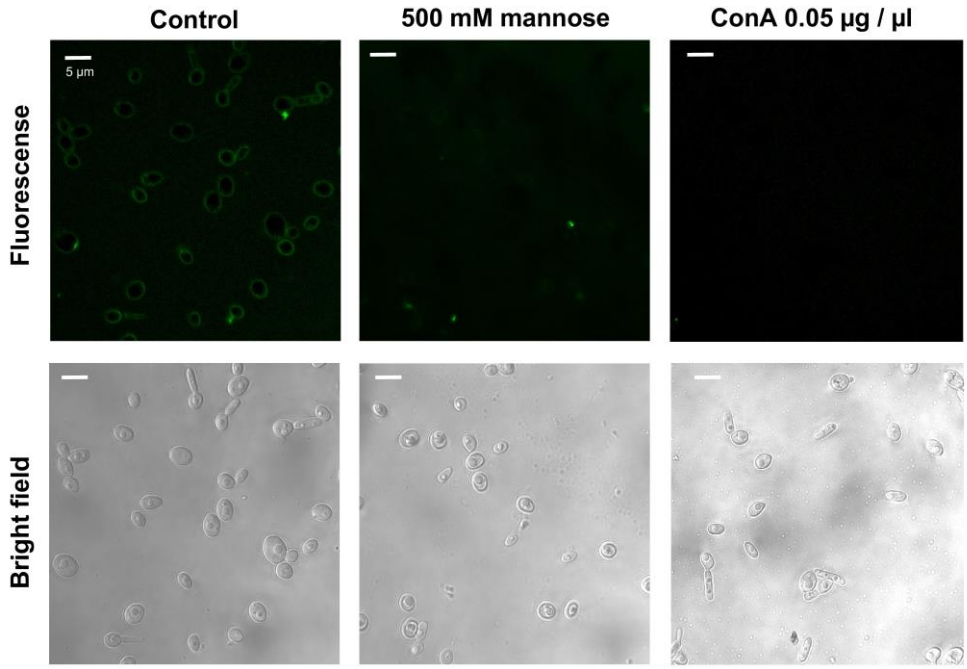


Fig. 9. Effect of D-mannose and ConA on binding Helja-FITC-*C. albicans*. Yeast cells incubated with 500 mM D-mannose or 0.05 µg/µl ConA for 20 min after incubation with 0,05 µg/µg Helja-FITC for 24 h, and observed under bright field or CLSM.

Graphical Abstract