



Short communication

Susceptibility testing of *Candida albicans* and *Candida glabrata* to *Glycyrrhiza glabra* L.

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ABSTRACT

Medicinal plants and their botanical formulations have gained a pivotal attention among scientific researchers mainly due to its action as health promoters. Licorice (*Glycyrrhiza glabra* L.) constitutes a great example, with an increasingly evidenced antimicrobial potential. Opportunistic yeast infections constitute an alarming public health problem, highly exacerbated by the inefficacy of antifungal drugs and the increase of drug-resistant microorganisms, being *Candida* species one of the most common invaders. The present work aims to assess *Candida glabrata* and *Candida albicans* susceptibility to *G. glabra* methanol: water extract by using flow cytometry and transmission electron microscopy techniques. After 5 min, licorice extract (1.5 mg/mL) altered *Candida* membrane potential. Within an hour, it induced primary damages on *Candida* species cells, causing cell cytoplasm disorganization with high evidence of cell membrane invaginations, making cells turgid. Thus, based on the current findings, licorice extract seems to be a promising anti-*Candida* agent, without presenting any toxic potential at the effective concentrations used.

1. Introduction

Medicinal plants have gained a special attention in the last years. In fact, natural matrices possess a multitude of health benefits (Faria et al., 2011; Lopes et al., 2013; Sher, 2009). Particularly, the study of the antifungal and anti-yeast properties against opportunistic fungi and yeasts, such as *Candida* species, is still being the focus of numerous researches (Martins et al., 2015b, 2014). Considered a commensal microorganism, being naturally present in gastrointestinal and genitourinary tracts of healthy population, *Candida* species have been increasingly associated with severe organic dysfunctions (Martins et al., 2014). As the fourth leading cause of systemic infections, they have markedly triggering the wellbeing involve in life-threatening situations (Martins et al., 2014).

Nowadays, there is a lack of antifungal agents with recognized efficiency, becoming urgent to provide a proper and efficient clinical intervention. Despite at several years ago these drugs exerted a potent effect, presently they are no longer effective (Martins et al., 2015b). In a general manner, microorganisms have shown increasing rates of drug resistance, and *Candida* species comprises a very good example (Martins

et al., 2015b). Aiming to control/avert this emerging epidemic problem, numerous studies have been carried out to evaluate the acquired mechanisms of drug-resistance by *Candida* species, and to provide new insights to the candidiasis treatment (Jiang et al., 2014; Kanafani and Perfect, 2008; Perlin, 2009; Sanglard and Odds, 2002). However, besides the evaluation of an efficient multi-drug therapy, by using chemicals drugs or even natural products combination, the assessment of the corresponding mechanisms of action, the occurrence of morphological changes in *Candida* cells and the determination of kinetic parameters are also important. All these achievements will provide knowledge related with the type of cellular lesion occurred in *Candida* cells, to infer the relevance and interest of licorice extract for future applications, as also to analyze the feasibility to carry out more detailed studies, such as the assessment of *in vitro* and *in vivo* cytotoxic potential in human cells. Then, it will become possible to predict the *in vivo* safety and upcoming clinical use of the studied substances and the likelihood of side effects occurrence, both at short and middle-long terms.

Considering the previous results obtained to licorice extract (completely characterized in terms of phenolic composition), namely antioxidant (Martins et al., 2015a) and anti-*Candida* (Martins et al., 2016)

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activities, the present study aims to access the effect of licorice extract on cell integrity and cultivability of the most problematic and virulent *Candida* species, namely *Candida albicans* and *Candida glabrata* species (Martins et al., 2015c). The main aspects that triggered the development of this experiment was to understand in which manner licorice exerted its anti-*Candida* potential, once no visible effects were observed when isolated phenolic compounds present in the extract were tested separately, while with the whole extract a prominent effect was reached. Cell changes occurred after licorice application was assessed by flow cytometry and transmission electron microscopy.

2. Material and methods

2.1. Plant material and phenolic extracts

Glycyrrhiza glabra L. rhizomes and roots, supplied by Soria Natural (Garray – Soria, Spain), were obtained in autumn 2012, after reaching three years. Methanol: water extraction of licorice was performed by stirring the sample (1 g) with 30 mL of methanol/water (80:20, v/v) at 25 °C and 150 rpm for 1 h, and filtered through Whatman no. 4 paper. The obtained residue was again extracted with an additional 30 mL portion of the extraction solvents mixture. Then, combined extracts were evaporated at 35 °C under reduced pressure (rotary evaporator Büchi R-210, Flawil, Switzerland) to remove the entire methanol used to perform phenolic extraction, and subsequently lyophilized (FreeZone 4.5, Labconco, Kansas City, MO, USA). Finally, the lyophilized extracts were re-dissolved in water, performing a stock solution with a concentration of 50 mg/mL, from which several dilutions were prepared (Martins et al., 2015a).

The concentration tested in this study (1.5 mg/mL) was based on the obtained results for minimal inhibitory (MIC) and minimal fungicidal (MFC) concentrations (Martins et al., 2016), and prepared from a licorice stock solution of 50 mg/mL. Sterile water was used as solvent.

2.2. Standards and reagents

Sabouraud Dextrose Broth (SDB) and Agar were from Merck (Darmstadt, Germany). Propidium iodide (PI) cell dye and HyClone Dulbecco's modified eagles medium (DMEM) were from Thermo Fisher Scientific (Wilmington, USA). Hank's balanced salt solution (HBSS), fetal bovine serum (FBS) and penicillin/streptomycin solution (100 U/mL and 100 mg/mL, respectively) were purchased from Gibco Invitrogen Life Technologies (California, USA). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA).

2.3. Yeast strains

In this study two different *Candida* species from the American Type Culture Collection (ATCC) were selected, namely *Candida albicans* ATCC 90028 and *C. glabrata* ATCC 2001 strains. The licorice extract concentration (1.5 mg/mL) was selected taking into consideration the previous results obtained (Martins et al., 2016) on the assessment of the anti-*Candida* activity in planktonic and their respective biofilms cells: *C. albicans* ATCC 90028 (MIC/MFC \geq 1.5 mg/mL), *C. glabrata* ATCC 2001 (MIC = 0.75 mg/mL; MFC = 1.5 mg/mL). Both strains were from the archive collection of the biofilm group of the Centre of Biological Engineering, University of Minho, Braga – Portugal.

2.4. Assessment of licorice anti-*Candida* mechanism of action

To perform the assessment of the effective mode of action of licorice extract on *Candida* species, a standard concentration of 1.5 mg/mL was used, based on the results obtained in the previous study (Martins et al., 2016).

2.4.1. Cytometry experiments

2.4.1.1. Staining procedures for flow cytometry experiments. The fluorescence probe PI was used to access the occurrence of cell membrane lesions in *Candida* species. Thus, two colonies from the sabouraud dextrose agar (SDA) were grown in SDB at 37 °C, 150 rpm, until late exponential growth phase and collected by centrifugation (2655g, for 5 min). Pellets were resuspended in water and the cell concentration adjusted to a final density of 0.5 McFarland, and then incubated with 1.5 mg/mL licorice methanol: water extract for 5, 15, 30 and 60 min, without agitation, at 37 °C. After this time, *Candida* cells were collected by centrifugation, resuspended in water and stained with 1 μ g PI/mL in the dark, during 30 min. Positive (i.e. cells treated with ethanol 90%) and negative (i.e. cells without licorice methanol: water extract) controls were also included.

The staining cells were acquired in a FACSCalibur Cytometer (BD Biosciences, Sydney) standard model equipped with 3 photomultipliers (PMTs), standard filters (FL1: BP 530/30 nm; FL2: BP 585/42 nm; FL3: LP 650 nm) and a 15 mW 488 nm Argon Laser. Then, *Candida* cells were analyzed and the percentage of stained cells was determined, by using cell Quest Pro software (version 4.0.2, BD Biosciences, Sydney).

2.4.1.2. Assessment of kinetic curves. Parallel to flow cytometry approaches, kinetic growth studies were also performed, by cultivability assay methodology after treating *Candida* cells with licorice extract, for 5, 15, 30 and 60 min, at 37 °C. Thus, at predetermined time points, serial dilutions of yeasts suspensions were prepared and plated on SDA medium for colony-forming units (CFU) determination. Following incubation at 37 °C for 24 h, the number of CFU was determined and results presented as Log reduction (CFUs/mL) – Log reduction = \log_{10} (number of viable cells before treatment) – \log_{10} (number of viable after treatment).

2.4.2. Transmission electron microscopy (TEM)

The effect of licorice extract on *Candida* cells morphological structure was observed by transmission electron microscopy (TEM). For that, *Candida* cells treated with 1.5 mg/mL of licorice extract during 30 min, were prepared as described in previously section (2.4.1.1.). In brief, samples were fixed in 2.5% glutaraldehyde (Electron Microscopy sciences, Hatfield, USA) and 2% paraformaldehyde (Merck, Darmstadt, Germany) in phosphate buffer 0.1 M with 0.5 mM MgCl₂ (pH 6.5), dehydrated and embedded in Epon resin (TAAB, Berks, England). Ultrathin sections (40–60 nm thickness) were prepared on a RMC Ultramicrotome (PowerTome, USA) using diamond knives (DDK, Wilmington, DE, USA). The sections were mounted on 200 mesh copper or nickel grids, stained with uranyl acetate and lead citrate for 5 min each, and examined under a JEOL JEM 1400 TEM (Tokyo, Japan). Images were digitally recorded using a CCD digital camera Orious 1100W Tokyo, Japan at the HEMS/I3S from the University of Porto. ImageJ software (Rasband, 2017) was used to measure cell walls of untreated and treated *Candida* cells. For that, five different cells derived from different micrographs were measured in five different cell wall regions.

2.5. Evaluation of the cytotoxic activity

Liver tissues were rinsed in Hank's balanced salt solution containing 100 U/mL of penicillin and 100 μ g/mL of streptomycin, and subsequently divided into 1 \times 1 mm³ explants. Some explants were placed in 25 cm³ tissue flasks, containing DMEM and supplemented with 10% fetal bovine serum, 2 mM of non-essential amino acids, 100 U/mL of penicillin and 100 mg/mL of streptomycin, and then incubated at 37 °C with a humidified atmosphere containing 5% CO₂. This medium was replaced every 2 days, and cells cultivation continued under direct monitoring every 2–3 days using a phase contrast microscope. Prior to confluence, cells were sub-cultured and plated in 96-well plates, with a

Table 1

Kinetic study of the candidacidal potential of licorice extract against *Candida albicans* ATCC 90028 and *Candida glabrata* ATCC 2001 strains by flow cytometry using propidium iodide staining.

<i>Candida species</i>	Licorice extract (mg/mL)	% of stained cells						
		(–) Control	(+) Control	5 min.	10 min.	15 min.	30 min.	60 min.
<i>C. albicans</i> ATCC 90028	1.5	7.7	83.1	70.0	73.5	76.8	78.6	81.3
<i>C. glabrata</i> ATCC 2001	1.5	4.8	88.9	97.2	97.6	98.4	98.8	98.9

(–) Control – negative control, i.e. cells without licorice treatment; (+) Control – positive control, i.e. cells treated with ethanol 90%.

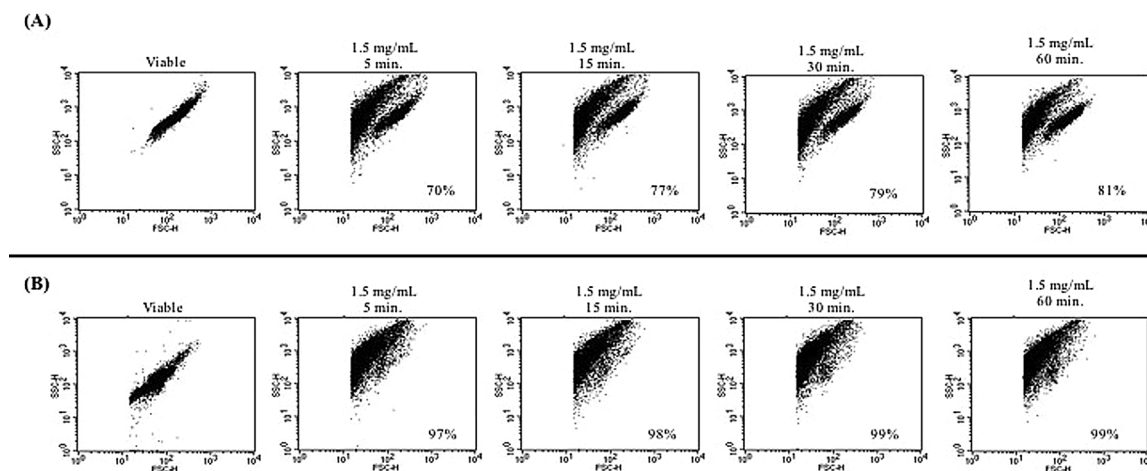


Fig. 1. Flow cytometry scattergram obtained to *Candida albicans* ATCC 90028 (A) and *Candida glabrata* ATCC 2001 (B), before (viable cells) and after 5', 15' and 30' treatment with licorice methanol: water extract; % corresponds to the % of PI stained cells.

final density of 1.0×10^4 cells/well and cultivated in DMEM medium, containing 10% FBS, 100 U/mL of penicillin and 100 μ g/mL of streptomycin (Abreu et al., 2011). Then, cells were treated for 48 h with the different diluted sample solutions. Three independent experiments were carried out and the results expressed in GI_{50} values (mg/mL), i.e. the extract concentration that inhibited 50% of net cell growth. Ellipticine was used as positive control.

2.6. Statistical analysis

All statistical tests were performed using IBM SPSS Statistics for Windows, version 22.0. Means were compared using Tukey's honestly significant difference (HSD) multiple comparisons test and further coupled to Welch to verify the equality of means.

3. Results and discussion

Candida species are responsible for several opportunistic disorders in human, which are difficult to treat mostly due to their increasing resistance to antifungals (Martins et al., 2015b). Therefore, it is very important to search and to develop efficient antifungal agents. Considering the highly effective *in vitro* anti-*Candida* activity evidenced by licorice methanol: water extract in previous experiments (Martins et al., 2016) and that the mechanism of action of this plant extract is poorly understood, it becomes essential to deepen knowledge on biocidal effect of licorice. In this sense, reference strains from *C. albicans* and *C. glabrata* species were selected to determine the effect of licorice extract on its cells by flow cytometry and transmission electron microscopy. For that, a standard concentration of licorice extract was tested based on the previous results (Martins et al., 2016) and the biocidal effect evaluated by flow cytometry (Table 1 and Fig. 1).

Flow Cytometry has been used to determine the quantitative properties of single cells, measuring cell size, cell granularity, the amounts of cell components, among other aspects in living cells, in less than one minute. With the use of fluorescent probes, flow cytometry allows to

distinguish living from dead cells, to infer about membrane integrity and other indicators of microbial viability. The monitoring of transmembrane electrochemical potential and membrane damages provides rapid and feasible indications of the antimicrobial-agent-induced injury (Gokahmetoglu et al., 2003). For this reason, this technique has been markedly highlighted by several authors as a rapid and sensitive *in vitro* method for anti-yeasts susceptibility assessment on different *Candida* species isolates (Gokahmetoglu et al., 2003). This assay was based on the detection of increased permeability of *Candida* cells to PI, after licorice extract application.

After 5 min-licorice applications, nearly to 97% of *C. glabrata* ATCC 2001 were already PI stained (Table 1 and Fig. 1B). The effect of licorice extract on *C. albicans* cells (Table 1 and Fig. 1A) is relatively lower than that observed for *C. glabrata* cells, with a 70% of PI stained cells after 5 min and no pronounced alterations after 10 (73%), 15 (77%), 30 (79%) and 60 min (81%) of licorice application. This suggests that the loss of cell integrity due to the licorice extract is not time dependent and the effects are evident immediately after application. Moreover, the fact that the cell damage to be more pronounced in *C. glabrata* cells, comparatively to *C. albicans* might be related with the different levels of complexity between *Candida* species, displaying *C. albicans* a more complex cellular envelope than *C. glabrata* species (Groot et al., 2008).

Previous experiments carried out by Gokahmetoglu et al., 2003, aiming at assessing the *in vitro* antifungal susceptibility of *C. albicans* to fluconazole and amphotericin B using flow cytometry observed an increasing resistance of *Candida* species to both anti-yeast drugs. The authors stated that were necessary at least 24-h incubation for reading microdilution assays, while to observe noticeable cell membrane damages by flow cytometry were required 4- and 2-h incubation, respectively, to fluconazole and amphotericin B (Gokahmetoglu et al., 2003). Moreover, MIC value corresponded to the lowest drug concentration used, 1 μ g/mL and 0.25 μ g/mL respectively, that resulted in an increase of mean channel fluorescence by 80 and > 90% of the growth control for fluconazole and amphotericin B (Gokahmetoglu

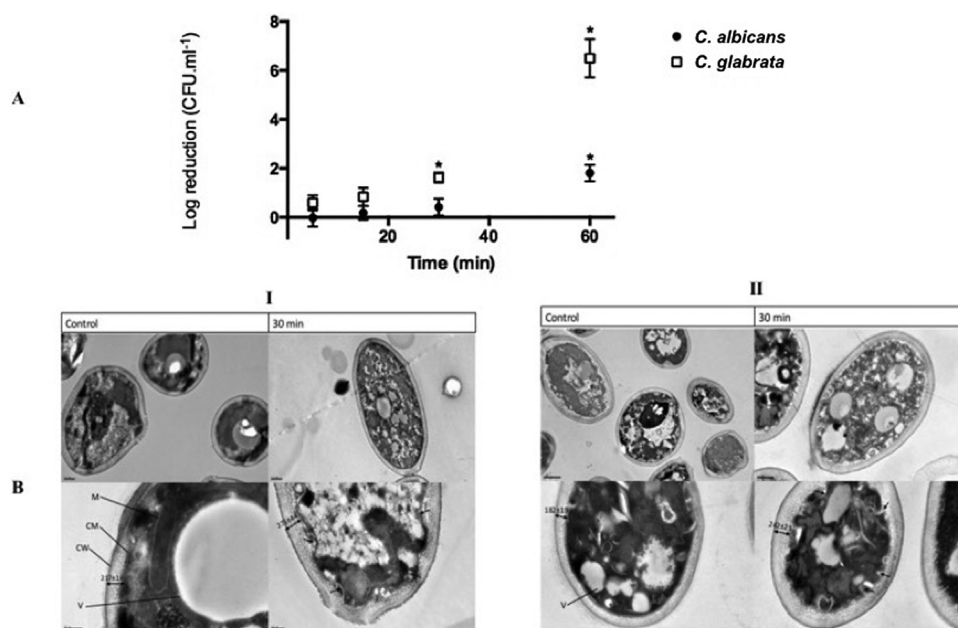


Fig. 2. Effect of licorice methanol: water extract on *Candida albicans* ATCC 90028 (I) and *Candida glabrata* ATCC 2001 (II) cells. A) Logarithm of reduction of colony forming units (CFUs) and B) Morphological cells changes evaluated by transmission electron microscopy (TEM). Error bars represent standard deviations (SD). Statistical p value (represented by *) indicates concentrations that are significantly different from control. * $p < 0.05$. M corresponds to mitochondria; CW corresponds to cell wall; CM corresponds to cellular membrane and V corresponds to vacuole. Arrows evidenced the presence of cell membrane invaginations.

et al., 2003). Interestingly, in this study after 5 min of licorice application at 1.5 mg/mL, 70% of *C. albicans* ATCC 90028 cells presented their cell membrane compromised, while after 60 min nearly to 81% and 99% of *C. albicans* ATCC 90028 and *C. glabrata* ATCC 2001 strains were already stained with PI, respectively. These results show that this extract does not require the high exposing time that is reported for the treatments with the traditional antifungals (Martins et al., 2015c).

Based on these findings, licorice extract seems to cause serious primary cellular damages on *Candida* cells, once the passage of PI into the cells occurred in a fast and significant manner from the first moments, being this aspect indicative of the existence of membrane damages/lesions. In fact, after membrane destabilization, cells lack its proper homeostasis, and consequently, organic unbalance blocks its metabolic functions and cells lost functionality (Li et al., 2014; Sanglard, 2002). Notwithstanding, it is also convenient to highlight that in several cases, cells may only suffer reversible damages, and subsequently can adapt and re-acquire its functionality. Aiming to evaluate if cell membrane integrity would affect cultivability, the number of CFUs was determined. Fig. 2A shows the CFU reduction of *C. albicans* ATCC 90028 and *C. glabrata* ATCC 2001 strains, treated with licorice extract (1.5 mg/mL) over time. With our results, it is possible to conclude that licorice extract exerts not only reversible but also irreversible damages, leading to a significant decrease of viable cells from both *Candida* species populations. Moreover, it is also important to highlight that despite cell integrity loss, some *Candida* cells remain cultivable. The most significant reduction was observed to *C. glabrata* ATCC 2001, where no cultivable cells were detectable at 60 min-treatment with a reduction of approximately 7 orders of magnitude. On the other hand, a reduction of 1.8 orders of magnitude was observed, from 0 to 60 min of treatment of *C. albicans* ATCC 90028. Thus, taking it account these findings, it is possible to confirm that licorice extract presents a marked ability to exert irreversible cellular damages in both *Candida* strains tested.

On the other hand, and despite the scarcity of studies reporting the mechanism of action of licorice extract against *Candida* species, through the present study it was expected that licorice lead to microorganisms' toxicity and consequent unviability by primary cellular membrane damages. In fact, it was already reported that licorice extracts mainly acts by inhibition of enzymatic activity and consequent establishment of non-specific interactions with membrane proteins (Sher, 2009). Moreover, and not least interesting to emphasize is that Wyk et al., 2009

observed that the antifungal activity evidenced by licorice extracts against *C. albicans* was mainly conferred by flavonoids and iso-flavonoids, which act in a synergistic manner with the other bioactive compounds presents in the samples (Jatav et al., 2011; Wyk et al., 2009), being this aspect in agreement with the results obtained in the present study.

Nevertheless, taking into account the advances reached in the last years, the number of studies assessing the type of morphological changes occurred in *Candida* cells still continue being scarce (Martins et al., 2015b,c). Thus, aiming to clarify the type of morphological cellular damages occurred in *Candida* cells, microscopic analyses using TEM were carried out, being the obtained results presented in Fig. 2B. The micrographs of the internal morphology of licorice treated *C. albicans* (Fig. 2I B) and *C. glabrata* (Fig. 2II B) cells confirms the hypothesis of the dramatic changes at 30 min of treatment, with the cytoplasmic disorganization coupled with notable alterations in the cytoplasmic membrane size and structure resulting in evident invaginations (see arrows). It is also important to highlight the increased of the cell membrane thickness from to 217 ± 1.9 nm up to 275 ± 4.4 nm, and from to 182 ± 1.9 nm up to 242 ± 2.3 nm in case of *C. albicans* and *C. glabrata* respectively, after licorice application. The results suggest that the plant extract induced dysfunctions on the cell membrane and consequently, the cell lost its metabolic functions leading to viability lost as proved by the cultivability assays after 60 min (Fig. 2A).

Finally, the assessment of the cytotoxic potential of licorice extract was also performed using liver tissues. Interestingly, licorice extract exerts neither toxic effects nor morphological changes up to a concentration of 1.5 mg/mL (data not shown). Therefore, licorice seems to be an upcoming effective, efficient and safer anti-*Candida* drug.

4. Conclusion

Overall, licorice methanol: water extract exerts a prominent candidacidal effect, being this pronounced effect observable both in *C. albicans* and *C. glabrata* strains, which means that licorice extract acts efficiently in a non-selective manner.

Flow Cytometry and TEM observations revealed that this extract causes fast and irreversible damages to the cell walls of both *Candida* species. Moreover, it was also shown that this extract is non-toxic to liver tissues. Based on this findings licorice extract may be considered

as promising anti-yeast agent, exerting fast and irreversible primary cellular damages both in *C. glabrata* and *C. albicans* cells, being those effects non-time-dependent.

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