aIn vitro anti-*Candida* activity of *Glycyrrhiza glabra* L.

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Abstract

The severity and frequency of opportunistic fungal infections still growing, concomitantly to the increasing rates of antimicrobial drug's resistance. Natural matrices have been used over years due to its multitude of health benefits, including antifungal potential. Thus, the present work aims to evaluate the anti-*Candida* potential of the phenolic extract and individual phenolic compounds of *Glycyrrhiza glabra* L. (licorice), by disc diffusion assay, followed by determination of the minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC) for both planktonic cells and biofilms.

Licorice extract evidenced inhibitory potential against the nineteen tested *Candida* strains, but no pronounced effect was observed by testing the most abundant individual phenolic compounds. *Candida tropicalis* strains were the most sensible, followed by *Candida glabrata, Candida parapsilosis* and, then, *Candida albicans*. Lower MIC and MFC values were achieved to *C. glabrata* and *C. tropicalis*, which confirms its susceptibility to licorice extract; however, for *C. tropicalis* strains a higher variability was observed. Anti-biofilm potential was also achieved, being most evident in some *C. glabrata* and *C. tropicalis* strains. In general, a twice concentration of the MIC was necessary for planktonic cells to obtain a similar potential to that one observed for biofilms. Thus, an upcoming approach for new antifungal agents, more effective and safer than the current ones, is stablished; notwithstanding, further studies are necessary in order to understand its mechanism of action, as also to assess kinetic parameters.

Keywords: Glycyrrhiza glabra L.; anti-*Candida* potential; biofilms; phenolic extracts/compounds.

1. Introduction

Considered as commensal microorganisms, *Candida* species have been causing a multitude of organic disturbances (Martins et al., 2014; Vázquez-González et al., 2013). Local infections are the most common, but their frequency and severity, together with the increasing rates of systemic infections, alarm the medical community (Mayer et al., 2013; Wächtler et al., 2012). Numerous antifungal agents were developed, but, currently, high rates of inefficacy have been observed (Kanafani and Perfect, 2008; Sanglard and Odds, 2002). Despite the mechanisms of acquired-drug resistance are not completely understood, several virulence factors have been described as playing an important role in the occurrence of the present invasive fungal infection. The ability of biofilm formation needs a particular attention, once recent studies showed that the majority of *Candida* infections are associated with biofilm growth (Sardi et al., 2013).

Natural matrices comprise a multitude of bioactive properties, not only conferred by isolated compounds, but mainly due to the occurrence of synergistic and polyvalence reactions between them. In particular, phenolic extracts have evidenced significant antimicrobial properties against a multitude of opportunistic invaders, including *Candida* species (Barros et al., 2013; Gallucci et al., 2014; Martins et al., 2015b, 2015c). *Glycyrrhiza glabra* L. (licorice) is commonly recommended as antitussive, mucolytic, expectorant, antiulcer, anti-inflammatory, antimicrobial, cytostatic, immunostimulant, and hepatoprotective, as well as flavor enhancer, due to its sweetening properties (Vanaclocha and Cañigueral, 2003). Considering the pronounced antioxidant activity of the licorice hydromethanolic extract previously reported by the authors, mainly attributed to the presence of phenolic compounds such as flavones, flavanones and chalcones (Martins et al., 2015a), in the present work, it was

investigated the anti-*Candida* activity of the phenolic extract and individual compounds against planktonic cells and biofilms.

2. Materials and Methods

2.1. Plant material, phenolic extract and individual phenolic compounds

Rhizomes and roots of *G. glabra* L., previously dried and reduced in smaller pieces, supplied by Soria Natural (Garray – Soria, Spain), were obtained in autumn 2012, after reaching three years. The samples were clean products with monitored parameters of pesticides, herbicides, heavy metals and radioactivity.

To obtain the phenolic extract, a hydromethanolic extraction was carried out. The sample (1 g) was extracted with 30 mL of methanol: water (80:20, v/v) at 25ºC and 150 rpm, during 1 h, and then filtered through Whatman No. 4 paper. The obtained residue was again extracted with an additional 30 mL portion of the hydromethanolic mixture. Both extracts were combined, evaporated at 35ºC under reduced pressure (rotary evaporator Büchi R-210, Flawil, Switzerland) and then lyophilized (FreeZone 4.5, Labconco, Kansas City, MO, USA). The lyophilized extracts were re-dissolved in water, performing a stock solution of 50 mg/mL, from which several dilutions were prepared to evaluate anti-*Candida* activity.

The extract was previously characterized by the authors using HPLC-DAD-ESI/MS (Martins et al., 2015a), being formononetin, liquiritigenin and apigenin derivatives the most abundant compounds. Therefore, these molecules were also tested (individually and combined in the relative proportions found in the extract) for anti-*Candida* activity, after dissolution in water: dimethyl sulfoxide (DMSO) (50:50, v/v), to a stock solution

of 1 mg/mL. The same procedure was adopted by using a standard concentration of 1 mg/mL of each one of the molecules and respective proportional combinations.

2.2. Standards and reagents

Methanol was of analytical grade purity and supplied by Pronalab (Lisbon, Portugal). RPMI 1640 medium and apigenin were purchased from Sigma Chemical Co., (St. Louis, MO, USA). Sabouraud Dextrose Broth (SDB) and Agar were from Merck (Darmstadt, Germany). Formononetin and liquiritigenin were from Extrasynthese (Genay Cedex, France). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA).

2.3. Evaluation of the anti-Candida activity

2.3.1. Disc diffusion assay

Nineteen *Candida* strains were used during this study (**Table 1**), belonging to the *Candida albicans, Candida glabrata, Candida parapsilosis* and *Candida tropicalis* species. Four of them were from the American Type Culture Collection (ATCC), and the others fifteen were clinical isolates from vaginal and urinary tracts and oral cavity. The clinical isolates were obtained from the archive collection of the biofilm group of the Centre of Biological Engineering, University of Minho, Braga – Portugal. Before each experiment, all strains were grown in Sabouraud Dextrose Agar (SDA) for 24 h at 37ºC. After that time, one loop of each colony of cells was transferred to Sabouraud Dextrose Broth (SDB) and incubated under stirring at 37ºC during 24 h. An aliquot of each species (300 μL), containing approximately 1×10^5 cells/mL was spread in SDA

Petri dishes. Then, an aliquot (25 μL) of the licorice extract, with a known concentration (50 mg/mL), was placed on a sterile blank disc, previously placed on the inoculated petri dishes. Sterile water was used as negative control. The plates were incubated at 37ºC, during 24–48 h. The evaluation of inhibitory properties was performed measuring the corresponding zones of inhibition (mm).

2.3.2. Minimal inhibitory (MIC) and minimal fungicidal (MFC) concentrations

Minimal inhibitory (MIC) and minimal fungicidal (MFC) concentrations of the licorice extract, in planktonic cells was determined based on the guidelines from the Nature Protocols (Wiegand et al., 2008), with some modifications. Only the *Candida* species in which pronounced results were reached in the disc diffusion assay, were selected to determine MIC and MFC values. Afterwards, a colony recovered from the SDA was suspended in 5 mL of sterile saline solution (0.85% NaCl) and vortexed for 15 s. The resulting suspension was adjusted by adding saline solution to reach the value of 0.5 in McFarland scale. Successive dilutions of the plant extract (0.1875, 0.375, 0.75, and 1.5 mg/mL) were prepared in RPMI 1640 medium, at pH 7. An aliquot of licorice extract (100 μL) was dispensed into a 96-well plates (Orange Scientific, Braine-l'Alleud, Belgium) and further incubated with aliquots (100 μL) of the selected *Candida* species. Sample- and yeast- free controls were also included. The 96-well plates were incubated at 37ºC for 48 h. After visualization of the resultant plate, the MIC values were determined, corresponding to the lower concentration of antifungal agent in which no visible growth was observed, by comparison with the control (cells grown without extracts). The number of viable cells was assessed by the determination of number of colony forming units (CFUs), through several dilutions. After 24 h of incubation at

37ºC, the number of colonies formed was counted. The MFC values were considered the antifungal agent concentration in which no CFUs were counted. The experiment was repeated in triplicate in three independent occasions, and the results presented as the total of CFUs (Log CFUs).

2.3.3. Individual phenolic compounds activity

The evaluation of the anti-*Candida* potential of individual phenolic compounds was carried out using the disc diffusion assay. Sterile water was used as negative control and licorice extract (50 mg/mL) as positive control, from which relative proportions of the most abundant phenolic compounds, formononetin (F), liquiritigenin (L) and apigenin (A), were tested. At the same time, a standard concentration (1 mg/mL) of each one of those phenolic compounds was also used to assess the existence of dose-dependent anti-*Candida* activity. All the possible combinations of the compounds (F+L; F+A; L+A; F+L+A) were also evaluated, considering their relative abundances in the licorice extract (Martins et al., 2015a), in order to assess the occurrence of synergistic reactions.

2.4. Effect on biofilm formation

The effect of licorice hydromethanolic extract against *Candida* biofilms was only determined for the strains in which positive results in planktonic cells were achieved. A standardized cell suspension (200 μL containing $1x10^5$ cells/mL in RPMI 1640 medium) was placed into 96-wells polystyrene microtiter plates and incubated at 37ºC on a shaker at 120 rpm/min, during 24 h. Sample and yeast-free controls were also included.

Then, biofilm medium was aspirated and non-adherent cells were removed by washing the biofilms with 200 μL of PBS. 200 μL of each tested concentration of licorice hydromethanolic extract (0.75; 1.5; 3 mg/mL) was added. The biofilms were further incubated during 24 h, at 37ºC on a shaker at 120 rpm/min. The effect of licorice extract on biofilm formation was assessed through quantification of the number o CFUs. These experiments were performed in triplicate, in three different occasions, and the results presented in terms of Log (CFU).

2.5. Statistical analysis

Three samples were used for each preparation and all the assays were carried out in triplicate. The results were analyzed using one-way analysis of variance (ANOVA) and means were compared using Tukey's honestly significant difference (HSD) multiple comparisons test, and further coupled to Welch to verify the equality of means. In the case of the graphs of *Candida tropicalis*, a Student´s t-test was used to determine the significant difference among two different samples, with $\alpha = 0.05$. This treatment was carried out using SPSS v. 22.0 program (IBM Corp., Armonk, NY, USA).

3. Results and discussion

Table 1 shows the anti-*Candida* potential of licorice extract evaluated by disc diffusion assay. Considering the inhibition zones, among the tested *Candida* species, *C. tropicalis* appeared to be the most susceptible (10-13 mm), followed by *C. glabrata* (12 mm), *C. parapsilosis* (10-12 mm) and *C. albicans* (10-12 mm). Karomi et al., (2012), evaluated the antifungal potential of different parts of licorice methanolic extract against *C. albicans*, and obtained similar inhibition zones when compared with the present experiment. The authors also observed that the antifungal potential was dose-dependent

to the licorice concentration, as well as to the type of plant part used: the roots evidenced the most pronounced anti-*C. albicans* potential, followed by stems, leaves and then seeds (Karomi et al., 2012). In the same line, Irani et al., (2010) evaluated the antimicrobial potential of licorice ethanolic and aqueous extracts, and also achieved similar results. Those authors observed an antimicrobial potential, including against *C. albicans*, dependent on the tested concentration, on the type of plant part and on the solvent extract used. Once again, the roots of licorice presented a most pronounced effect than leaves, and aqueous extract was most effective against *C. albicans* than ethanolic extract. All these results were in part confirmed in the present work and by the subsequent determination of MICs and MFCs values.

Despite in the initial screening (disc diffusion assay), *C. tropicalis* and *C. glabrata* presented a higher susceptibility to the licorice extract, through using microdillution method, the most pronounced sensibility was observed to *C. glabrata* strains, confirmed by the lower MIC and MFC values, which varied, respectively, between 0.1875-0.75 mg/mL and 0.375-1.5 mg/mL. *C. glabrata* D1 (MIC=0.1875-0.375 mg/mL; MFC=0.375 mg/mL) was the most sensible, followed by 513100 (MIC=0.375-0.75 mg/mL; MFC=0.75 mg/mL) and ATCC2001 (MIC=0.375-075 mg/mL; MFC=1.5 mg/mL). Notwithstanding, *C. tropicalis* also showed a significant susceptibility to licorice extract, but a higher variability between tested strains was observed: AG1 and T2.2 were the most sensible (MIC=0.375 mg/mL; MFC=0.75 mg/mL), followed by 75 (MIC=0.375-0.75 mg/mL; MFC=1.5 mg/mL), ATCC750, 12 and 519468 (MIC=0.75-1.5 mg/mL; MFC=1.5 mg/mL) and, lastly, to 544123, no activity was observed in planktonic cells. Among the *C. parapsilosis* strains, only the reference strain ATCC22019 evidenced to be susceptibility (MIC=1.5 mg/mL), but no fungicidal effect was achieved at the tested concentrations (0.1875; 0.375; 0.75; 1.5 mg/mL). To the *C.*

albicans strains no antifungal effect was observed. In contrary, Irani et al., (2010) detected activity in *C. albicans* (on both MIC and MFC). Notwithstanding, the tested concentrations were higher than the used in the present work, excepting to the licorice leaves (MIC/MBC=0.625 mg/mL) and roots (MIC=1.25 mg/mL; MBC=2.5 mg/mL) derived from the aqueous extract. Extrapolating these results to the present experiment, in which a relative proportion of methanol: water (80:20, v/v) was used, it is possible to infer that the active constituents of the licorice extract present a higher solubility in water, than using other solvent extracts.

In relation to the evaluation of the anti-*Candida* potential of the individual phenolic compounds (apigenin, formononetin, liquiritigenin) no pronounced effects were achieved, neither at the relative concentration present in the licorice extract (50 mg/mL), nor at higher concentrations - 1 mg/mL of each molecule, and respective combinations (data no shown). Fatima et al., (2009) evaluated the antifungal potential of ethanol extract and ethyl acetate fraction of licorice roots, and an active constituent of this plant part, glabridin, against wild type, resistant mutants and clinical isolates of *Candida* species. Glabridin showed pronounced anti-*Candida* effects: the inhibition zones varied between 4-9 mm for the wild type and resistant mutants, and between 6-8 mm for the clinical isolates. MIC values were also determined for the present strains, being for the wild type and resistant mutants 0.03125-0.125 mg/mL, and for the clinical isolates 0.0625-0.125 mg/mL. It is very important to highlight that, despite glabridin belongs to the class of isoflavonoids, being an isoflavane, the type of solvent extract used highly affects the type and quantity of phenolic compounds present in the final extract.

For the *Candida* strains in which considerable antifungal effect were observed, the effect on biofilm formation was carried out, namely to *C. glabrata* strains (ATCC2001, D1 and 513100) and *C. tropicalis* (ATCC750 and T2.2). Despite *C. tropicalis* strains,

namely AG1 and T2.2, as well as ATCC750, 12 and 519468 have evidenced a similar response to the extract, a representative strain of each group was selected. **Figure 1** shows the obtained results by using licorice extract against biofilms of *C. glabrata* and *C. tropicalis*, expressed as logarithm of number of CFUs. In general, a twice concentration of licorice extract was necessary towards to obtain a similar antifungal effect in biofilms. For *C. glabrata* ATCC2001, the MFC value for planktonic cells was 1.5 mg/mL, while for biofilms was 3 mg/mL; in the same line, to D1 the obtained MFC values in planktonic cells and biofilms were, respectively, 0.375 mg/mL and 3 mg/mL. Lastly, for 513100 the MFC values in planktonic cells and biofilms were 0.75 mg/mL and 3 mg/mL, respectively. Concerning to *C. tropicalis*, the obtained MFC values for ATCC750 strain were, respectively, 1.5 mg/mL and 3 mg/mL in planktonic cells and biofilms. On the other hand, while the obtained MFC value in T2.2 planktonic cells was 0.75 mg/mL, no MFC value was determined in biofilms, since it was higher than 3 mg/mL.

4. Conclusions

Considering all the obtained results, it is possible to conclude that despite some *Candida* strains appears to be sensible to the licorice extract, by using disc diffusion assay, similar results and sensibility were not achieved through the determination of MIC and MFC, in planktonic cell and biofilms. Furthermore, and despite phenolic extract evidenced a promissory anti-*Candida* potential, no positive effects were obtained by using the most abundant individual phenolic compounds, which means that probably vestigial molecules improve the final effect of the extract and/or activate some of the active constituents.

In this sense, further experiments are necessary towards to determine the real mechanism of action of the present plant extract, to evaluate the type of lesion caused in *Candida* species, as also to assess kinetic parameters. This would open perspectives regarding new antifungal agents, much more effective and safer than the currently available.

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Table 1. Anti-*Candida* activity of the hydroalcoholic extract from licorice, in planktonic cells

Species	Strains	Origin	Inhibition zone (mm)	MIC (mg/mL)	MFC (mg/mL)
C. albicans	ATCC 90028	Reference	10	>1.5	>1.5
	558234	Vaginal	11	>1.5	>1.5
	575541	Urinary	12	>1.5	>1.5
	557834	Vaginal	11	>1.5	>1.5
C. glabrata	ATCC 2001	Reference	12	0.375-0.75	1.5
	D1	Oral	12	0.1875-0.375	0.375
	513100	Urinary	12	0.375-0.75	0.75
C. parapsilosis	ATCC 22019	Reference	12	1.5	>1.5
	AM2	Oral	10	>1.5	>1.5
	AD	Oral	10	>1.5	>1.5
	513143	Vaginal	12	>1.5	>1.5
	491861	Vaginal	12	>1.5	>1.5
C. tropicalis	ATCC 750	Reference	10	0.75-1.5	1.5
	AG1	Oral	10	0.375	0.75
	75	Vaginal	10	0.375-0.75	1.5
	12	Vaginal	10	0.75-1.5	1.5
	544123	Urinary	13	>1.5	>1.5
	519468	Urinary	10	0.75-1.5	1.5
	T2.2	Oral	12	0.375	0.75

Figure captions

Figure 1. Logarithm of number of colony forming units (CFUs) of different *C. glabrata* (A) and *C. tropicalis* (B) biofilms treated within different concentrations of the hydromethanolic extract of licorice. Error bars represent standard deviations (SD). ** $P<0.05$ and *** $P<0.005$, extract concentrations results that are significantly different fom the positive control. In each strain different letters mean significant differences ($p<0.05$).