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In 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 established; notwithstanding, further studies are necessary in order to understand its mechanism of action, as also to assess kinetic parameters.

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

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Table 1
Anti-*Candida* activity of the hydroalcoholic extract from *Glycyrrhiza glabra L.* (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

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 25 °C 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 35 °C 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 redissolved 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 37 °C. After that time, one loop of each colony of cells was transferred to Sabouraud Dextrose Broth (SDB) and incubated under stirring at 37 °C 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 37 °C, 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 were 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 37 °C 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

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