Antifungal activity of phenolic compounds identified in flowers from North Eastern Portugal against Candida species

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\textbf{ABSTRACT:} Aim: To evaluate the antifungal effect of gallic acid, catechin, luteolin and quercetin, phenolic compounds identified from flowers of North Eastern Portugal, against Candida planktonic and biofilm cells. \textbf{Materials & methods:} The MICs were determined in Candida planktonic cells and the effect of phenolic compounds on Candida biofilms was assessed through quantification of CFUs. \textbf{Results:} MIC values demonstrated that gallic acid presented the highest effect against all Candida species. Catechin showed a similar effect against Candida albicans American Type Culture Collection (ATCC) 90028 cells. In addition, gallic acid and quercetin had demonstrated only a minimal effect against Candida species biofilms. \textbf{Conclusion:} Gallic acid affected the growth of the different planktonic Candida species in all concentrations used; still, catechin showed a similar effect against C. albicans ATCC 90028 and Candida glabrata ATCC 2001 cells. In addition, only gallic acid and quercetin demonstrated a slight effect against all Candida species biofilms.

\textbf{KEYWORDS} • antifungal effect • biofilms • Candida species • medicinal flowers • phenolic compounds

\textbf{Background}  
Candida species are normal commensal microorganisms of the human biota, found in the oral, gastrointestinal, urinary and vaginal mucosa [1], and are opportunistic pathogens, with the ability to cause superficial and serious systemic infections. Indeed, the Candida genus is the most frequently recovered from fungal hospital infections, named candidosis [2]. The Candida genus is composed of an extremely heterogeneous group of over 150 species [2], but it is well established that only a minority are implicated in human candidosis. Moreover, a major virulence factor of Candida is its ability to adapt to a variety of different habitats, with the consequent formation of surface-attached microbial communities known as biofilms [3–5]. Candida yeasts, which can live in a biofilm, can have significantly different properties from free-floating microorganisms, due to the existence of an extracellular matrix. This extracellular matrix allows different microorganisms to cooperate and interact among themselves in various ways and confers a certain degree of protection against drugs. Biofilms can be found on different surfaces, such as biotic (mucosal surfaces) and abiotic (invasive medical devices) [6,7]. These communities present a high resistance to typical antifungal drugs, such as amphotericin B and fluconazole [8,9]. The biomedical significance of biofilms is considerable, as most infections result from preformed biofilms [10,11].

In clinical practice, most cases of candidosis have been attributed to Candida albicans. However, more recently, non-\textit{C. albicans} Candida (NCAC) species have been identified as common pathogens [12], and the prevalence of these species in human infections has been changing in recent years. In European countries, an analysis showed that the incidence rates for NCAC candidosis were 14% each for Candida glabrata and Candida parapsilosis, 7% for Candida tropicalis and 2%
for Candida kru sei [13]. This increased incidence can be attributed to improvements in diagnostic methods and the emergence of molecular techniques. However, it can also be a reflection of the high level of resistance often exhibited by NCAC species to antifungal therapies, such as the azole drugs and their derivatives, which continue to dominate as the choice against Candida-related infections [14–17]. Candidosis can be treated, not only by theazole class, but also by echinocandins and polyenes antifungal classes. The selection of the antifungal agent depends on the local epidemiology, percentage of strains resistant to fluconazole and even origin of infection [18]. In addition, at least 70% of the antifungal drugs are prescribed empirically [19] and, consequently, a decrease in susceptibility to fluconazole, along with cross-resistance to other azoles, have been noted, as, for example, in the case of C. glabrata [20]. Thus, in order to overcome this clinical problem, an enlarged interest in finding new effective natural drugs, such as plant extract compounds (specifically some phenolic compounds) and essential oils, has been observed [21–23]. In this context, the main objective of the present work was to evaluate the potential antifungal effect of gallic acid, catechin, luteolin and quercetin, phenolic compounds identified in flowers of the North Eastern Portugal, against Candida planktonic and biofilm cells (C. albicans American Type Culture Collection [ATCC] 90028, C. tropicalis ATCC 750, C. parapsilosis ATCC 22019 and C. glabrata ATCC 2001).

Materials & methods

• Phenolic compounds

The extraction, identification and quantification of phenolic compounds from flowers of Castanea sativa, Filipendula ulmaria, Rosa micrantha [24] and Cytisus multiflorus [25], and fresh leaves of Cistus ladanifer [26] were previously described by the authors using a high-performance liquid chromatography-diode array detector/electrospray source mass spectrometer. This work was focused on four different phenolic compounds that seemed more promising against Candida species: one phenolic acid (gallic acid) and three flavonoids (catechin, luteolin and quercetin).

• Strains & growth conditions

Four Candida reference strains from the ATCC, namely C. albicans (ATCC 90028), C. glabrata (ATCC 2001), C. parapsilosis (ATCC 22019) and C. tropicalis (ATCC 750), were used in this study. Cells were grown on Sabouraud dextrose agar (SDA; Merck, Munich, Germany) for 24 h at 37°C, then inoculated in Sabouraud dextrose broth (Merck) and incubated for 18 h at 37°C under agitation at 120 rpm/min. After incubation, the cells were harvested by centrifugation at 3000 x g for 10 min at 4°C and washed twice in 15 ml of phosphate-buffered saline (PBS; pH 7; 0.1 M). Pellets formed were suspended in 10 ml Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma, MO, USA) buffered to pH 7 and the cellular density was adjusted to 2 × 10⁷ cells/ml using a Neubauer chamber.

• Phenolic compound activity against planktonic Candida cells (MIC)

The MICs of all the species under study were determined according to the guidelines of the National Committee for Clinical Laboratory Standards M27-A2 document [27], with some modifications. Previously to these experiences, twofold final concentration serial dilutions of each compound stock were prepared in RPMI 1640 medium ranging from 0.156 to 1.5 mg/ml and maintained in a freezer. Aliquots of each phenolic compound (100 µl), at a twofold final concentration, and Candida species suspensions (100 µl at 2 × 10⁷ cells/ml, for a final concentration of 1 × 10⁶ cells/ml) were mixed in the 96-well plates (Orange Scientific, Braine-l’Alleud, Belgium). The 96-well plates were incubated at 37°C for 48 h and then the MIC value was determined, firstly by direct observation and secondly by determination of the number of CFUs. The number of CFUs was determined after appropriate serial dilutions in PBS and by plating 10 µl of each cell dilution onto SDA. After 24 h of incubation at 37°C, the number of colonies was enumerated. These experiments were performed three-times and, at least, in triplicate. Yeast cultures without phenolic compounds and negative controls (only RPMI) were also included.

• Phenolic compounds activity against Candida biofilms

Phenolic compounds were tested against Candida species biofilms. For that, standardized Candida cell suspensions (200 µl containing 1 × 10⁷ cells/ml in RPMI 1640 medium) were placed into wells of 96-well polystyrene microtiter plates (Orange Scientific) and incubated at 37°C on a shaker at 120 rpm/min for 24 h. The 96-well plates used in this study are
often applied to form biofilms, since they possess properties completely different from the plates used for MIC determination assays. In addition, according to our previous results, it was possible to confirm that after 24 h of growth on those plates, there was matrix production, therefore confirming the presence of a biofilm [Fonseca E et al. Effects of fluconazole in Candida glabrata biofilms and its relation with ABC transporters genes expression (2014). Manuscript in Preparation]. Negative controls (200 µl of RPMI 1640 medium) were also included.

At 24 h, biofilm medium was aspirated and nonadherent cells removed by washing the biofilms once in 200 µl of PBS. Then, 200 µl of each phenolic compound (prepared in RPMI 1640 medium), ranging from 0.625 to 5 mg/ml, were added. The biofilms were incubated for a further 24 h at 37°C on a shaker at 120 rpm/min. The effect of phenolic compounds on Candida biofilms was assessed through quantification of the number of CFUs. It is important to emphasize that cells initially used to produce a biofilm are free floating and only some of them form the biofilm. Therefore, the numbers of the initial inoculum and cells within a biofilm cannot be directly correlated. For that, the volume of biofilm was aspirated once with 200 µl of PBS. Then, the biofilms were scraped from the respective wells and the suspensions vigorously vortexed for approximately 2 min to disaggregate cells from the matrix. Serial dilutions were made in PBS, plated onto SDA and incubated for 24 h at 37°C. These experiments were performed in triplicate and, at least, in three independent assays. The results were presented in terms of Log of CFUs.

- **Statistical analysis**

Results were compared using two-way analysis of variance by applying the Bonferroni post-test for means comparisons, using GraphPad Prism 6 (GraphPad Software, CA, USA).

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### Results & discussion

In nature, phenolic compounds are involved in plant growth and reproduction, and, curiously, provide resistance to plant pathogens and even predators, protecting crops and seed from diseases [28,29]. With over 9000 natural antimicrobials identified, the flavonoid family is the largest group of phenolic compounds [30]. It is important to emphasize that the phenolic compounds used in this study, gallic acid (phenolic acid), catechin (flavan-3-ols), luteolin (flavone) and quercetin (flavonol), were previously identified in different medicinal flower species [24–26]. The MIC values were determined and ranged from 0.156 to 1.250 mg/ml, as can be observed in Table 1. In addition, the MIC values were also confirmed measuring Candida planktonic cells (CFU determination) viability (Figure 1).

The results presented in Table 1 clearly demonstrate that gallic acid was the most effective (<0.156 mg/ml) against the planktonic Candida cells for all the studied species. In addition, catechin demonstrated a similar effect against C. albicans ATCC 90028 cells. It is important to highlight that the catechin, for example, demonstrates a higher effect than the one presented by Haghighi et al. in 2011, which found a MIC value of 9.47 mg/ml against C. albicans [31], even though the actual mechanism of action of gallic acid on yeast cells has not been widely studied. In 2011, Hong et al. proved that gallic acid present in a hydrolysable tannin extracted from the bark of Rhizophora apiculata possessed anti-C. albicans activity [32]. Although luteolin has been previously reported to exhibit antimicrobial activity against Bacillus cereus and Salmonella enteritidis [33], and quercetin against Staphylococcus aureus, Escherichia coli and Pseudomonas fluorescens [34], in this work, these phenolic compounds demonstrated a lower effect against all Candida species cells (≥0.625 mg/ml).

The highest resistance of C. tropicalis ATCC 750 cells to all the flavonoids in this study (MIC:
1.250 mg/ml), with the exception of gallic acid (MIC: <0.156), should be pointed out. In fact, the MIC values were higher than expected. However, in accordance with MIC values that we have obtained for traditional antifungal agents (C. glabrata: 0.625–1.250 mg/ml of fluconazole [Fonseca E et al. Effects of fluconazole in Candida glabrata biofilms and its relation with ABC transporters genes expression (2014), Manuscript in Preparation]), we consider the MIC values acceptable to explore as potential future candidates in the treatment of candidosis. Furthermore, many studies have been focused on natural compounds and plant-derived active principles as possible alternative treatments of Candida infections [35–37]. Measuring Candida planktonic cell (CFU determination) viability is of greatest importance for distinguishing between fungicidal and fungistatic effects. The viability results confirmed that gallic acid demonstrated the highest antifungal activity (p < 0.01 at all concentrations) against Candida planktonic cells (Figure 1A–D). It should be noticed that gallic acid is in fact a causative agent of at least 2 Log of reduction for all species, at the lowest concentration tested (0.156 mg/ml). Interestingly, this phenolic acid also possessed the capability to totally eradicate C. parapsilosis ATCC 22019 (Figure 1C) and C. tropicalis ATCC 750 (Figure 1D) planktonic cells at concentrations higher than 0.625 (p < 0.001) and 1.25 mg/ml (p < 0.001), respectively. Despite the fact that the mechanism of action of gallic acid on yeast cells has not been widely understood, it can be proposed that it acts by disrupting the structure of the cell membrane and inhibiting the normal budding process [38–40]. Candida glabrata ATCC 2001 was the species that, in general, presented the highest initial reduction for all

Figure 1. Logarithm of number of cells of Candida species grown in the presence of increased concentrations of gallic acid, catechin, luteolin and quercetin, after 48 h. (A) Candida albicans American Type Culture Collection (ATCC) 90028; (B) Candida glabrata ATCC 2001; (C) Candida parapsilosis ATCC 22019; and (D) Candida tropicalis ATCC 750. Error bars represent standard deviation. Statistical p value (represented by *, ** or ***) indicate concentrations that are significantly different from control. *p < 0.05; **p < 0.01; ***p < 0.001.
phenolic compounds tested, with more than 2 Log of reduction, at 0.156 mg/ml (p < 0.001) (Figure 1B). However, in opposition to C. tropicalis ATCC 750 and C. parapsilosis, gallic acid was unable to eradicate, at any concentrations tested, C. glabrata ATCC 2001 cells. Furthermore, this fungistatic effect was also observed against C. albicans ATCC 90028 (Figure 1A). Catechin and luteolin presented a similar effect against C. parapsilosis ATCC 22019, causing more than 3 Log of reduction at 1.25 mg/ml (p < 0.001) (Figure 1C). In this study, C. tropicalis ATCC 750 was the species that showed the lowest inhibition for all flavonoids, with less than 1 Log of reduction, even for the highest concentration tested (Figure 1D). So, despite the highest genetic similarity between C. tropicalis ATCC 750 and C. albicans ATCC 90028 [41], no similarities were found in terms of the phenolic compound effect.

In most natural environments, microorganisms exist predominantly as biofilms, rather than planktonic or free-floating cells [42]. Therefore, the second aim of this work was to test the phenolic compounds against Candida species pre-formed biofilms. For that, the relative numbers of viable cells within the biofilm were evaluated by CFU counts (Figure 2). Biofilm drug resistance is a phenomenon consistently expressed across model microbial systems [3,43] and likely to be of great clinical relevance [44]. Hawser and Douglas, in 1995, firstly demonstrated a similar resistance effect of Candida biofilms to traditional antifungal agents [45]. As such, any evidence of activity against biofilm-associated organisms would represent an important new finding.

The effects of the phenolic compounds on Candida biofilms (Figure 2) revealed a decreased susceptibility to these microorganisms comparatively with planktonic counterparts (Figure 1). Gallic acid, the phenolic compound that demonstrated the highest effect for planktonic cells, luteolin and quercetin, was only able to reduce

![Figure 2. Logarithm of number of Candida biofilms treated with increased concentrations of gallic acid, catechin, luteolin and quercetin, after 24 h, formed in Roswell Park Memorial Institute 1640. (A) Candida albicans American Type Culture Collection (ATCC) 90028; (B) Candida glabrata ATCC 2001; (C) Candida parapsilosis ATCC 22019; and (D) Candida tropicalis ATCC 750 cells. Error bars represent standard deviation. Asterisks indicate concentrations that are significantly different from control. *p < 0.05.](image-url)
**EXECUTIVE SUMMARY**

**Objectives of the study**
- The main aim of this study was to evaluate the antifungal effect of gallic acid, catechin, luteolin and quercetin, a set of phenolic compounds identified from flowers of North Eastern Portugal, against planktonic and biofilm cells of four of the most pathogenic *Candida* species.

**Methods**
- Four reference strains from the American Type Culture Collection (ATCC), namely *Candida albicans* (ATCC 90028), *Candida glabrata* (ATCC 2001), *Candida parapsilosis* (ATCC 22019) and *Candida tropicalis* (ATCC 750), were used. The MIC of each phenolic compound was determined for planktonic cells and its effect against *Candida* biofilm quantified by CFUs.

**Conclusion**
- Overall, in this work, gallic acid showed antifungal activity against the growth of all planktonic *Candida* species. Similar antifungal effect was obtained with catechin against *C. albicans* ATCC 90028 and *C. glabrata* ATCC 2001 cells.
- Gallic acid and quercetin also demonstrated a slender effect against *Candida* species biofilms.

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**Future perspective**
Candidosis treatment is difficult, especially due to the eukaryotic nature of fungal cells. Thus, there are few effective antifungal agents available for clinical use (azoles, polyenes or echinocandins). Moreover, abrupt changes in the way drugs are prescribed and the use of newer antifungal drugs induced *Candida* species to develop resistance. In order to overcome this problem, there will be an increasing interest in natural compounds, specifically in phenolic compounds. So, in the future, we will continue to seek new potential anti-*Candida* compounds from the North Eastern Portugal flowers.
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References

Papers of special note have been highlighted as:

- of interest
- of considerable interest


- Excellent article for understanding the potential of natural compounds.


- Excellent article for understanding the full characterization of phenolic compounds.


- Excellent article for understanding the full characterization of phenolic compounds.


- Excellent article for understanding the MIC technique.


Excellent article for understanding the characterization of biofilms of non-*Candida albicans* *Candida* species.