PRELIMINARY COMMUNICATION

Future MICROBIOLOGY

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

Carlos Tiago Alves[1], Isabel CFR Ferreira[2], Lillian Barros[2], Sónia Silva[1], Joana Azeredo[1] & Mariana Henriques*[,1]

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. **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. **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. **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.

KEYWORDS
• antifungal effect • biofilms • *Candida* species • medicinal flowers • phenolic compounds

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 fluconazol [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-*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%

[1]IBB–Institute for Biotechnology & Bioengineering, Centre of Biological Engineering, University of Minho, 4710-057 Braga, Portugal
[2]CIMO/School of Agriculture, Polytechnic Institute of Bragança, Campus de Santa Apolónia, 172, 5301-855 Bragança, Portugal
*Author for correspondence: Tel.: +351 253604408, Fax: +351 253604429; mcrh@deb.uminho.pt

Future Medicine part of fsg

for *Candida krusei* [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 the azole 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 × *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 2×10^7 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 2×10^7 cells/ml, fmaor a final concentration of 1×10^7 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 24h 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 1×10^7 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.* Eeffects 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 total medium was removed and the biofilms were washed 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).

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 enteritidi* [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:

Table 1. MIC values obtained with gallic acid, catechin and luteolin against *Candida* species.

Phenolic compounds	MIC (mg/ml)			
	Candida albicans ATCC 90028	*Candida glabrata* ATCC 2001	*Candida parapsilosis* ATCC 22019	*Candida tropicalis* ATCC 750
Gallic acid	<0.156	<0.156	<0.156	<0.156
Catechin	<0.156	0.625	0.625	1.250
Luteolin	0.625	0.625	0.625	1.250
Quercetin	0.625	1.250	1.250	1.250

ATCC: American Type Culture Collection.

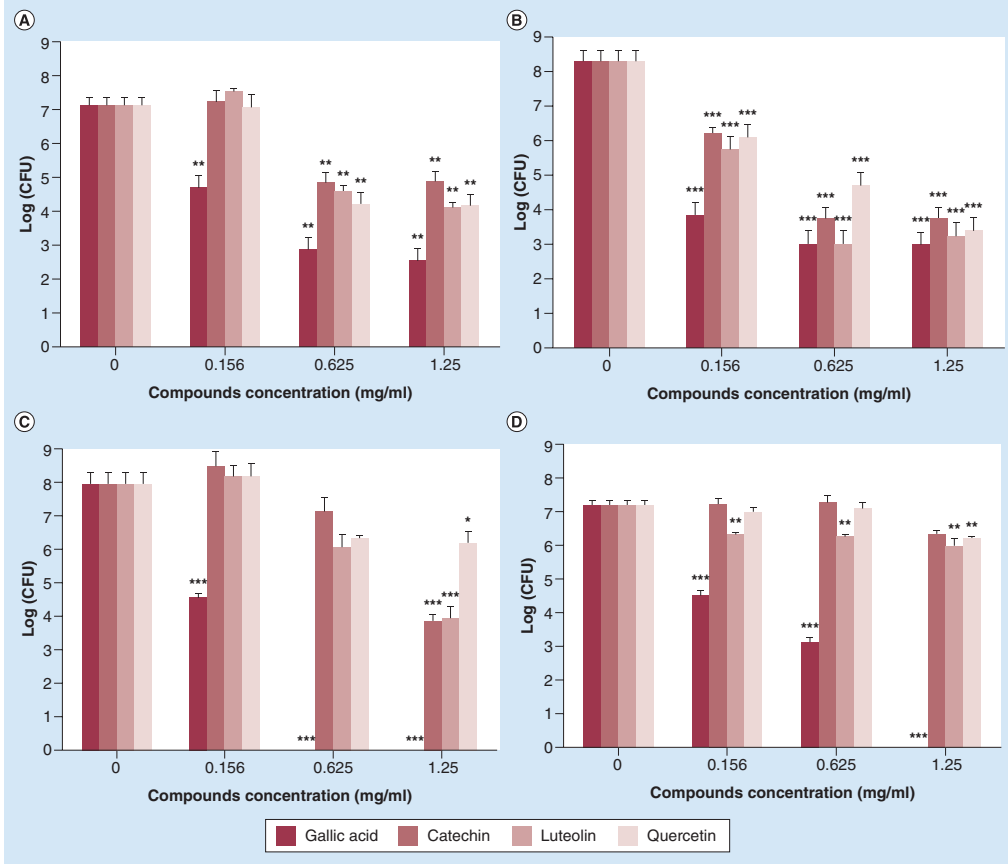

Figure 1. Logarithm of number of cells of *Candida* species grown in the presence of increased concentrations of gallic acid, catechin, luteolin and quercetin, after 48 h. (A) *Candida albicans* American Type Culture Collection (ATCC) 90028; **(B)** *Candida glabrata* ATCC 2001; **(C)** *Candida parapsilosis* 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.

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

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. parapsiloisis*, 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 preformed 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)** reveled 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

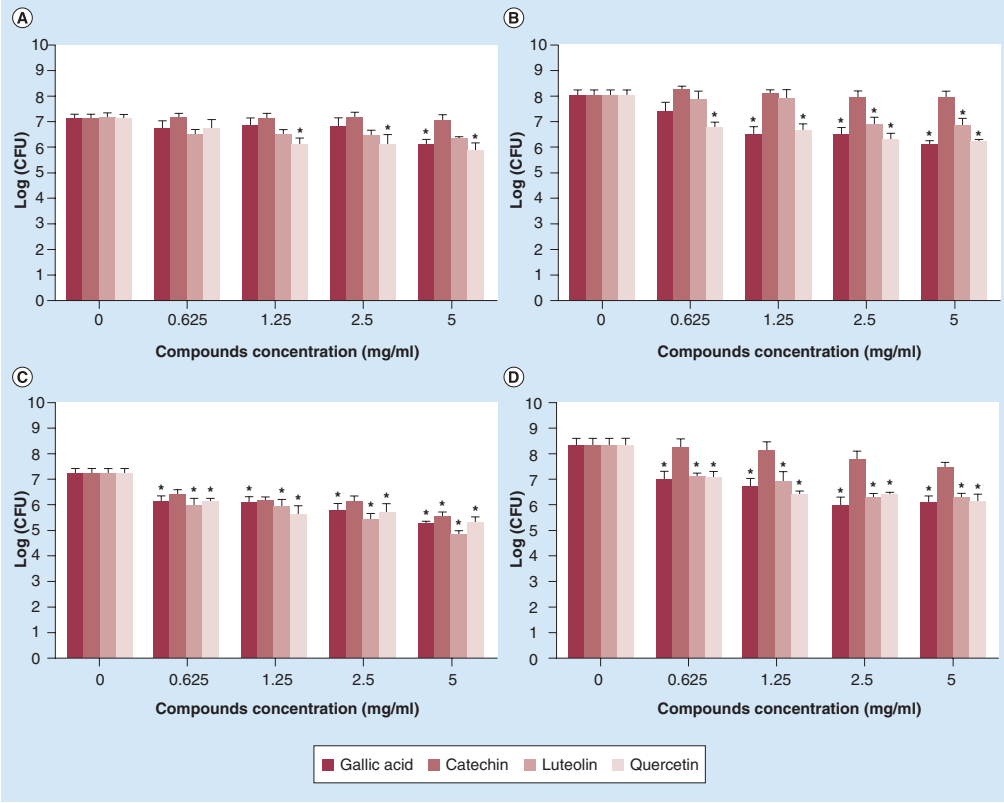

Figure 2. Logarithm of number of *Candida* biofilms treated with increased concentrations of gallic acid, catechin, luteolin and quercetin, after 24 h, formed in Roswell Park Memorial Institute 1640.
(A) *Candida albicans* American Type Culture Collection (ATCC) 90028; **(B)** *Candida glabrata* ATCC 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.
*$p < 0.05$.

C. glabrata ATCC 2001 **(Figure 2B)**, *C. parapsilosis* ATCC 22019 **(Figure 2C)** and *C. tropicalis* ATCC 750 **(Figure 2D)** biofilm cells in 2 Log for the highest concentration tested ($p < 0.05$). In terms of species, *C. albicans* ATCC 90028 biofilms were the most resistant to all compounds, where the best results were obtained with gallic acid and quercetin ($p < 0.05$) **(Figure 2A)**. In 2009, Wang *et al.* also showed a great antifungal effect of gallic acid against *C. albicans* biofilms [46]. Moreover, catechin was the phenolic compound under study, which had demonstrated the lowest effect, with the exception of 1 Log of reduction at the highest concentration, tested in the case of *C. parapsilosis* ATCC 22019 ($p < 0.05$) **(Figure 2C)**. As it is known, biofilms are organized, structured communities embedded within a matrix of extracellular material [42]. Moreover, *Candida* biofilm matrix structure and composition is strongly species dependent [42,46]. For example, *C. albicans* ATCC 90028 biofilm structure involves, generally, two distinct layers: a thin, basal yeast layer and a thicker, less compact hyphal layer, while *C. parapsilosis* ATCC 22019 biofilms are thinner and less structured, and consist exclusively of aggregated blastospores [47], which could justify the difference of results obtained for each *Candida* species.

Conclusion

Overall, this work demonstrates that the phenolic compounds, especially gallic acid, affected the growth of different planktonic *Candida* species. Catechin showed a similar effect against *C. albicans* ATCC 90028 and *C. glabrata* ATCC 2001 cells at higher concentrations. In addition, gallic acid and quercetin demonstrated only a slight effect against *Candida* species biofilms.

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.

Acknowledgements

The group would like to acknowledge our beloved Rosário Oliveira for her precious contributions to the beginning of this work.

Financial & competing interests disclosure

The authors are grateful to strategic project PEst-OE/AGR/UI0690/2011 for financial support of the research centers. CT Alves and L Barros thank FCT, POPH-QREN and FSE for their grants SFRH/BD/72742/2010 and SFRH/BPD/4609/2008, respectively. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

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.

References

Papers of special note have been highlighted as:

- • of interest
- •• of considerable interest

1 Soll DR. *Candida* commensalism and virulence: the evolution of phenotypic plasticity. *Acta Trop.* 81, 101–110 (2002).

2 Calderone RA. Introduction and historical perspectives. In: *Candida and Candidiasis*. Calderon RA (Ed.). ASM Press, Washington DC, USA, 15–25 (2002).

3 Chandra J, Kuhn D, Mukherjee P *et al.* Biofilm formation by fungal pathogen *Candida albicans*: development, architecture, and drug resistance. *J. Bacteriol.* 183, 5385–5394 (2001).

4 Douglas LJ. *Candida* biofilms and their role in infection. *Trends Microbiol.* 11, 30–36 (2003).

5 Ganguly S, Mitchell AP. Mucosal biofilms of *Candida albicans*. *Curr. Opin. Microbiol.* 14(4), 380–385 (2011).

6 Finkel JS, Mitchell AP. Genetic control of *Candida albicans* biofilm development. *Nat. Rev. Microbiol.* 9, 109–118 (2011).

7 Fux CA, Costerton JW, Stewart PS *et al.* Survival strategies of infectious biofilms. *Trends Microbiol.* 13, 34–40 (2005).

8 Ramage G, Saville SP. *Candida* biofilms: an update. *Eukaryot. Cell* 4(4), 633–638 (2005).

9 Pereira Gonzales F, Maisch T. Photodynamic inactivation for controlling *Candida albicans* infections. *Fungal Biol.* 116(1), 1–10 (2012).

10 Harriott MM, Lilly EA, Rodriguez TE *et al.* *Candida albicans* forms biofilms on the vaginal mucosa. *Microbiology* 156, 3635–3644 (2010).

11 Hasan F, Xess I, Wang X *et al.* Biofilm formation in clinical *Candida* isolates and its association with virulence. *Microbes Infect.* 11, 753–761 (2009).

12 Martins M, Henriques M, Ribeiro AP *et al.* Oral carriage of patients attending a dental clinic in Braga, Portugal. *Rev. Iberoam. Micol.* 27, 119–124 (2010).

13 Tortorano AM, Kibbler C, Peman J, Bernhardt H, Klingspor L, Grillot R. Candidaemia in Europe: epidemiology and resistance. *Int. J. Antimicrob. Agents* 27, 359–366 (2006).

14 Redding S, Kirkpatrick W, Coco B *et al.* *Candida glabrata* oropharyngeal candidiasis in patients receiving radiation treatment for head and neck cancer. *J. Clin. Microbiol.* 40, 1879–1881 (2002).

15 Ruhnke M. Epidemiology of *Candida albicans* infections and role of non-*Candida-albicans* yeasts. *Curr. Drug Targets* 7, 495–504 (2006).

16 Gonzalez GM, Eliondo M, Ayala J. Trends in species distribution and susceptibility of bloodstream isolates of *Candida* collected in Monterrey, Mexico, to seven antifungal agents: results of a 3-years (2004 to 2007) surveillance study. *J. Clin. Microbiol.* 46, 2902–2905 (2008).

17 Negri M. Henriques M, Svidzinski TI *et al.* Correlation between Etest®, disk diffusion, and microdilution methods for antifungal susceptibility testing of *Candida* species from infection and colonization. *J. Clin. Lab. Anal.* 23, 324–330 (2009).

18 Muñoz P, Burillo A, Bouza E. Criteria used when initiating antifungal therapy against *Candida* spp. in the intensive care unit. *Int. J. Antimicrob. Agents* 15, 83–90 (2000).

19 Leon C, Ruiz-Santana S, Saavedra P *et al.* Usefulness of the '*Candida* score' for discriminating between *Candida* colonization and invasive candidiasis in non-neutropenic critically ill patients: a prospective multicenter study. *Crit. Care Med.* 37, 1624–1633 (2009).

20 Sobel JD. Changing epidemiology of invasive candidiasis in intensive care units – much ado about nothing? *Crit. Care Med.* 36, 2188–2189 (2008).

21 Dai YC, Yang ZL, Cui BK *et al.* Species diversity and utilization of medicinal mushrooms and fungi in China (review). *Int. J. Med. Mushrooms* 11, 287–302 (2009).

22 Saleem M, Nazir M, Ali MS *et al.* Antimicrobial natural products: an update on future antibiotic drug candidates. *Nat. Prod. Rep.* 27, 238–54 (2010).

- • **Excellent article for understanding the potential of natural compounds.**

23 Palaniappan K, Holley RA. Use of natural antimicrobials to increase antibiotic susceptibility of drug resistant bacteria. *Int. J. Food Microbiol.* 140(2–3), 164–168 (2010).

24 Barros L, Dueñas M, Carvalho AM *et al.* Characterization of phenolic compounds in flowers of wild medicinal plants from Northeastern Portugal. *Food Chem. Toxicol.* 50, 1576–1582 (2012).

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

25 Barros L, Alves CT, Dueñas M *et al.* Characterization of phenolic compounds in wild medicinal flowers from Portugal by HPLC-DAD-ESI/MS and evaluation of antifungal properties. *Indust. Crops Prod.* 44, 104–110 (2013).

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

26 Barros L, Dueñas M, Alves CT *et al.* Antifungal activity and detailed chemical characterization of *Cistus ladanifer* phenolic extracts. *Indust. Crops Prod.* 41, 41–45 (2013).

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

27 Pfaller MA, Chaturvedi V, Espinel-Ingroff A *et al.* Reference method for broth dilution antifungal susceptibility testing of yeasts; approved standard – second edition. National Committee for Clinical Laboratory Standards, PA, USA, 22(15) (2002).

- • **Excellent article for understanding the MIC technique.**

28 Naeini A, Khosravi AR, Chitsaz M *et al.* Anti-*Candida albicans* activity of some Iranian plants used in traditional medicine. *J. Mycol. Med.* 19, 168–172 (2009).

29 Ross J, Kasum C. Dietary flavonoids: bioavailability, metabolic effects, and safety. *Ann. Rev. Nutr.* 22(1), 19–34 (2002).

30 Whiting D. Natural phenolic compounds 1900-2000: a bird's eye view of a century's chemistry. *Nat. Prod. Rep.* 18(6), 583–606 (2001).

31 Haghighi F, Roudbar Mohammadi SH, Farhadi Z. The effect of catechin on fungal biofilm formation of standard susceptible and resistant strains of *Candida albicans*. *Armaghan Danesh* 16(4 (64)), 340–332 (2011).

32 Hong LS, Ibrahim D, Kassim J *et al.* Gallic acid: an anticandidal compound in hydrolysable tannin extracted from the barks of *Rhizophora apiculata* Blume. *J. Appl. Pharm. Sci.* 01(06), 75–79 (2011).

33 Lv PC, Li HQ, Xue JY *et al.* Synthesis and biological evaluation of novel luteolin derivatives as antibacterial agents. *Eur. J. Med. Chem.* 44, 908–914 (2009).

34 Arima H, Ashida H, Danno G. Rutin-enhanced antibacterial activities of flavonoids against *Bacillus cereus* and *Salmonella enteritidis*. *Biosci. Biotechnol. Biochem.* 66, 1009–1014 (2002).

35 Ozdemir Z. Growth inhibition of *Clavibacter michiganesis* subsp. *michiganensis* and *Pseudomonas syrigae* pv. *Tomato* by olive mill wastewaters and citric acid. *J. Plant Pathol.* 91(1), 221–224 (2009).

36 Saravanakumar A, Venkateshwaran K, Vanitha J *et al.* Evaluation of antibacterial activity, phenol and flavonoid contents of *Thespesiapopulnea* flower extracts. *Pak. J. Pharm. Sci.* 22(3), 282–286 (2009).

37 Sudjana AN, D'Orazio C, Ryan V *et al.* Antimicrobial activity of commercial *Olea europaea* (olive) leaf extract. *Int. J. Antimicrob. Agents* 33(5), 461–463 (2009).

38 Kim KJ, Woo SS, Bo KS *et al.* Activity and mode of action of silver nano-particles on *Candida albicans*. *Biometals* 22, 235–242 (2009).

39 Endo EH, Cortez DA, Ueda-Nakamura T *et al.* Potent antifungal activity of extracts and pure compound isolated from pomegranate peels and synergism with fluconazole against *Candida albicans*. *Res. Microbiol.* 61(7), 534–540 (2010).

40 Ghannoum MA, Rice LB. Antifungal agents: mode of action, mechanisms of resistance, and correlation of these mechanisms with bacterial resistance. *Clin. Microbiol. Rev.* 12(4), 501–517 (1999).

41 Butler G, Rasmussen MD, Lin MF *et al.* Evolution of pathogenicity and sexual reproduction in eight *Candida* genomes. *Nature* 459, 657–662 (2009).

42 Donlan RM, Costerton JW. Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin. Microbiol. Rev.* 15, 167–193 (2002).

43 Baillie GS, Douglas L. *Candida* biofilms and their susceptibility to antifungal agents. *Methods Enzymol.* 310, 644–656 (1999).

44 Costerton JW, Stewart P, Greenberg E. Bacterial biofilms: a common cause of persistent infections. *Science* 284, 1318–1322 (1999).

45 Hawser SP, Douglas LJ. Resistance of *Candida albicans* biofilms to antifungal agents *in vitro*. *Antimicrob. Agents Chemother.* 39, 2128–2131 (1995).

46 Wang C, Cheng H, Guan Y *et al.* [*In vitro* activity of gallic acid against *Candida albicans* biofilms]. *Zhongguo Zhong Yao Za Zhi* 34(9), 1137–1140 (2009).

47 Silva S, Henriques M, Martins A *et al.* Biofilms of non-*Candida albicans Candida* species: quantification, structure and matrix composition. *Med. Mycol.* 46, 1–9 (2009).

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