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BACTERIAL AND FUNGAL PATHOGENESIS - REVIEW

# *Candida albicans* antibiofilm molecules: analysis based on inhibition and eradication studies

**Carolina do Rosário Esteves Guimarães[1] · Humberto Fonseca de Freitas[1] · Tânia Fraga Barros[1]**

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## Abstract

Biofilms are communities of microbial cells surrounded by an extracellular polysaccharide matrix, recognized as a fungal source for local and systemic infections and less susceptible to antifungal drugs. Thus, treatment of biofilm-related *Candida* spp. infections with popular antifungals such as fluconazole is limited and species-dependent and alternatively demands the use of expensive and high toxic drugs. In this sense, molecules with antibiofilm activity have been studied but without care regarding the use of important criteria such as antibiofilm concentration lower than antifungal concentration when considering the process of inhibition of formation and concentrations equal to or lower than 300 μM. Therefore, this review tries to gather the most promising molecules regarding the activity against the *C. albicans* biofilm described in the last 10 years, considering the activity of inhibition and eradication. From January 2011 to July 2021, articles were searched on Scopus, PubMed, and Science Direct, combining the keywords "antibiofilm," "candida albicans," "compound," and "molecule" with AND and OR operators. After 3 phases of selection, 21 articles describing 42 molecules were discussed in the review. Most of them were more promising for the inhibition of biofilm formation, with SM21 (24) being an interesting molecule for presenting inhibitory and eradication activity in biofilms with 24 and 48 h, as well as alizarin (26) and chrysazine (27), with concentrations well below the antifungal concentration. Despite the detection of these molecules and the attempts to determine the mechanisms of action by microscopic analysis and gene expression, no specific target has been determined. Thus, a gap is signaled, requiring further studies such as proteomic analyses to clarify it.

**Keywords** Synthetic molecules · Natural product · Review · Activity · *Candida albicans*

## Introduction

*Candida albicans* is still considered one of the most relevant species of the human microbiota [1–3] and is part of the vaginal, gastrointestinal, and oral mycobiota of most healthy individuals. It can become pathogenic when there are any changes in the local mycobiota, host immunity, or normal tissue barriers [4]. To aid in the expression of pathogenicity, some mechanisms are activated, such as the production of proteolytic enzymes and the formation of biofilms, which are produced by *Candida* spp., *C. albicans* being the most studied [5–11].

Set up as communities of microbial cells, biofilms are surrounded by an extracellular matrix of polysaccharides, which confers protection to their structure. This community is formed from the adhesion and growth of cells on abiotic surfaces, such as air–liquid interfaces or solid materials, and sometimes on biotic surfaces, such as tissues [8, 12, 13]. Its cells communicate through signaling molecules in the process called quorum-sensing [14]. For fungal biofilms, the formation comprises four steps: adherence, initiation, maturation, and dispersal, lasting 24 to 48 h (Fig. 1) [15].

In the adherence step, the base layer of the biofilm is formed from the adhesion (of fungal cells suspended in the medium (planktonic cells)) onto biotic or abiotic surfaces containing organic substances that allow yeast cell proliferation into microcolonies, which expand and fill the entire surface over time [16]. Cell anchorage depends on nonspecific

✉ Tânia Fraga Barros
tfbarros@ufba.br

Carolina do Rosário Esteves Guimarães
carolina.guimaraes@ufba.br

Humberto Fonseca de Freitas
hffreitas@ufba.br

[1] Post-Graduation Program in Pharmacy, Pharmacy College, Federal University of Bahia, Barão de Geremoabo Street, 147, Ondina, Salvador, Bahia CEP 40170115, Brazil

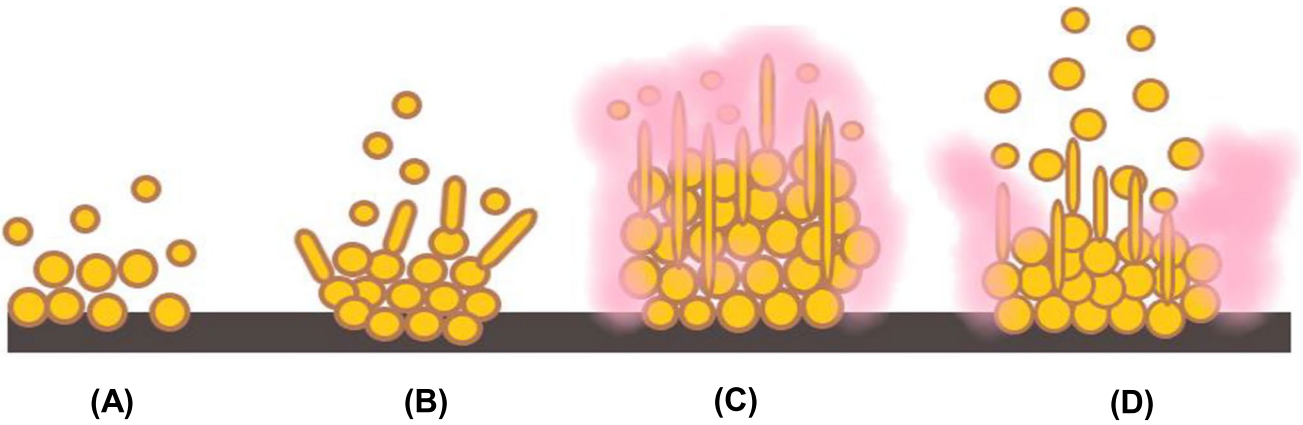



**Fig. 1** Steps of *Candida albicans* biofilm development. **A** Adherence. **B** Initiation. **C** Maturation. **D** Dispersal

factors, for example, hydrophobicity of the cell surface and electrostatic forces, and specific factors, such as the production of fungal surface proteins recognized by whey proteins and salivary factors [17–19]. To increase adhesion to the surface and between cells, blastoconidia form filamentous structures (hyphae) and pseudohyphae, which will form part of the new layers of the biofilm, providing support to the structure and allowing its growth. Thus, this initiation step, similar to the previous adherence step, is critical for the development, activity, and maintenance of the biofilm [7]. In the third step (maturation), the extracellular matrix accumulates in the cell layers of the biofilm, functioning as a "shield" for the cells that make up the community [16]. Finally, in the dispersal stage, cells or pieces of biofilm detach from the structure and new foci of infection form. Therefore, this step is crucial for the development or maintenance of local and systemic infections [8, 9, 20, 21].

The importance of biofilms in the clinical field was reported by the US Centers for Disease Control and Prevention in 2000, which classified biofilm-related diseases as two of the seven major health safety challenges facing the medical community [22]. This is justified, for example, by the high ratio (up to 70%) between the formation of these structures in central venous catheters and the onset of bloodstream fungal infections [23], which cause 100,000 deaths per year in the USA [7]. Soldini et al. [24] observed that patients with fungemia of *Candida* spp. that were high biofilm producers had a shorter survival (57.5%) than those with low-producing isolates (33.3%).

For the treatment of infections by *Candida* spp. associated with biofilms, the use of popular antifungals such as fluconazole depends on the species, so echinocandins and liposomal amphotericin B are options. To inhibit or eradicate biofilms requires concentrations of these antifungals up to 1000 times higher than those used against planktonic cells [25]. This lower susceptibility is attributed to the difficulty of penetration of these antimicrobials in the biofilm structure due to their composition of multiple layers of cells and, even more so, by the extracellular matrix (where proteins that function as efflux pumps can still be found) [11, 26, 27]. However, these drugs at higher doses for longer-than-usual treatment periods are usually more expensive and can trigger hepatotoxicity and nephrotoxicity [28–31]. This can lead to longer hospital stays and increased costs to health systems by almost 6.5 million dollars per year, as observed in the USA [7, 27].

Given the above challenges, alternatives have been sought for the treatment of fungal infections associated with biofilms through drug repositioning, combination with antifungals, and the use of natural and synthetic molecules, which are still under study [32]. Such molecules are used to eradicate planktonic cells, neutralize virulence factors, weaken the cellular matrix, and block important processes for biofilm maturation and maintenance, such as mechanisms of quorum-sensing detection and hyphal production [33]. Depending on when these molecules are applied to the biofilm, at the beginning of its formation or during its development, their activity can be classified as inhibition or eradication, respectively [34, 35].

To measure the blockade of formation (biofilm inhibitory concentration or BIC), the tested molecule is added together with the microorganism inoculum [36] or 90 min after the addition of the microorganism [37]. In the eradication test, the biofilm eradication concentration (BEC) is determined for the molecule added after biofilm formation, also considering different formation times, depending on the step to be investigated [37–39]. Such concentrations can be determined by direct quantitative methods (counting of the number of viable cells through plate culture and confocal laser scanning microscopy), indirect quantitative methods (dry weight, crystal violet staining, and tetrazolium salt assays), or qualitative methods (scanning electron microscopy and the Congo red agar method) [40].

The evaluation of the inhibitory activity against the formation of biofilms requires special care. In the inhibition

assays, the molecule is added at the same time as the microorganism that will form the biofilm. This makes it necessary to distinguish between antifungal and antibiofilm activity. Molecules with a BIC lower than the minimum inhibitory concentration (MIC) suggest a specific action on the biofilm, while the opposite is difficult to define. In addition to the difficulty of definition, a BIC greater than the MIC could influence the selection of resistant cells, making it impossible to use the molecule as an antifungal [41–44]. Although this distinction is needed, many studies do not apply this rule when selecting molecules with inhibitory activity against biofilm formation.

This review aims to gather the most promising molecules against the antibiofilm activity of *C. albicans* described in the last 10 years whose BIC is lower than their MIC. In addition, the discussed molecules will be characterized in relation to the application in the biofilm (preventive or eradication) and the possible mechanisms of action and cellular toxicity, going over the gaps that need to be filled on this topic in the search for new molecules.

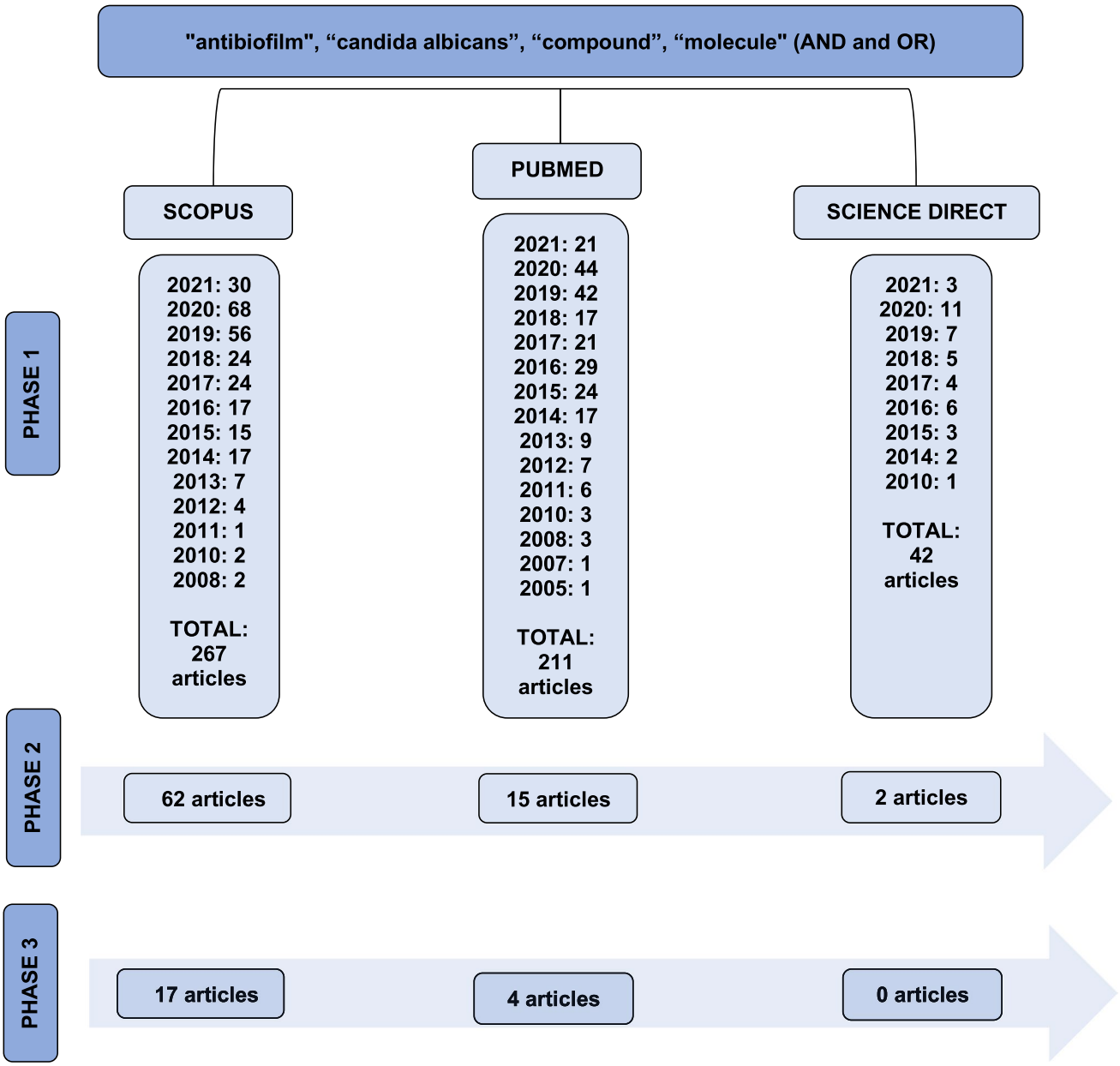



**Fig. 2** Selection flow of articles used in the review

## Methods

The search was conducted in three electronic databases, Scopus, PubMed, and Science Direct. We searched for articles published from January 2011 to July 2021 by combining the keywords "antibiofilm," "candida albicans," "compound," and "molecule" with the AND and OR operators. Without any exclusion criteria, phase 1 of the search generated 520 articles. In phase 2, a filter was applied to include only original articles in English, Portuguese, or Spanish and to exclude review articles, studies without isolation of the molecule, studies without antibiofilm activity for *C. albicans*, articles with polymicrobial biofilms, and repeat articles. The result of this filter was 79 articles, whose abstracts we read for the next filter (Phase 03), which entailed the reading of each full article. In this last phase, we considered only those with a description of a BIC lower than the MIC, antibiofilm activity greater than or equal to 50%, and BIC less than or equal to 300 µM. Thus, 21 articles that characterized 42 molecules were chosen for review (Fig. 2).

## Results and discussion

Although the first scientific publication on biofilm dates back to 1683 by Antonie van Leeuwenhoek [45], the real knowledge of fungal biofilms, especially *Candida* spp., began in 1984 [46]. Interest in the study of antibiotic agents against *Candida* became common only starting in 2011, after which 96.7% of the articles on this subject have been published, according to the PubMed database [47], including the articles that describe molecules with potential antibiofilm against *Candida* spp.

Among the analyzed molecules, we observed a similar distribution between molecules of natural and semisynthetic origin, which were not observed for their distribution over the biofilm steps in which they act. The action on inhibition was predominant among the evaluated molecules (Fig. 3), only one molecule being detected with activity on all steps. This can be explained by the fact that the inhibition assay is shorter than eradication assays, and the evaluation of inhibition is the first step of evaluating the antibiofilm activity of a molecule, since negative inhibition results generally reflect negative results for eradication due to the complexity of a preformed biofilm.

The determination of the biofilm step affected by the molecule may help to conceive its mode of application. If the goal is to avoid biofilm formation, the molecule should be used before the adherence step, for example, administered to the patient before placing a medical device or coating the inside of the medical device. However, to eradicate the biofilm, the molecule can be used both in the biofilm formation step (initial phase of adherence and intermediate phase of initiation) and in the mature phase (maturation step). With this in mind, we grouped 29 molecules of the review according to their step of action: molecules 1 to 18, classified as promising for the inhibition of biofilm formation (Table 1); molecules 19 to 23, as promising for biofilm eradication (Table 2); and molecules 24 to 29, as promising for the inhibition of biofilm formation and eradication (Table 3).

### Promising molecules for inhibiting biofilm formation

For biofilm development, the mechanisms involved in the adherence and initiation steps must be present, such as maintenance of cell surface hydrophobicity, hyphae formation, and adhesin expression [32]. In fact, the selected molecules (Table 1) act primarily on these mechanisms.

In this context, these molecules were classified as promising because they inhibit the formation of *C. albicans* biofilms at concentrations ($BIC_{50}$) lower than the $MIC_{50}$ in in vitro experiments. Among the mechanisms presented,

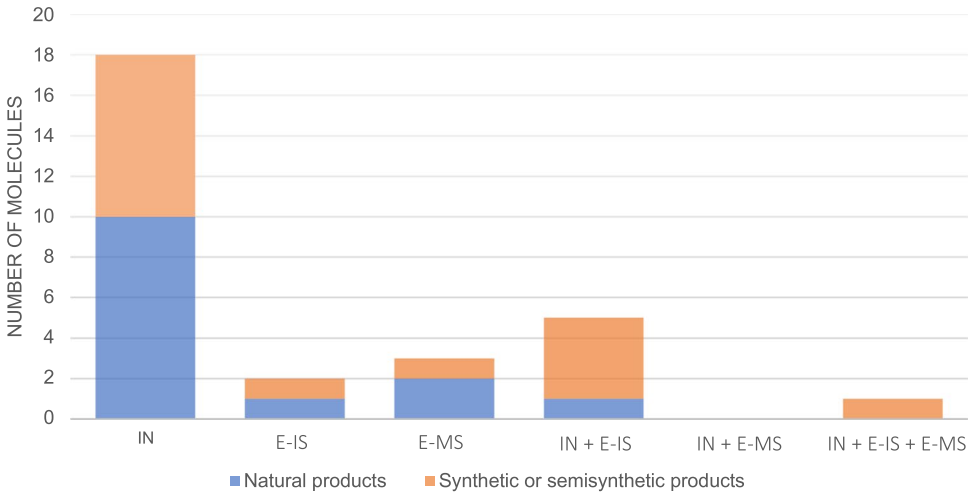



**Fig. 3** Distribution of molecules with 50% or more activity according to application step and source. IN, inhibition in biofilm formation; E-IS, biofilm eradication on initiation step; E-MS, biofilm eradication on maturation step; IN + E-IS, inhibition in biofilm formation and biofilm eradication on initiation step; IN + E-MS, inhibition in biofilm formation and biofilm eradication on maturation step; IN + E-IS + E-MS, inhibition in biofilm formation and biofilm eradication on initiation and maturation step

**Table 1** Promising molecules for inhibiting biofilm formation

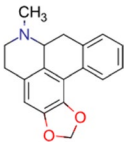
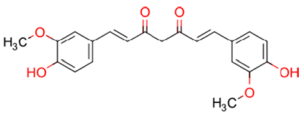
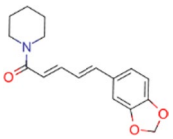
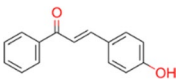
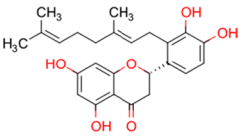
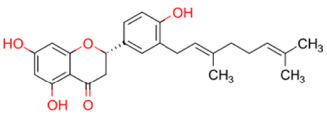
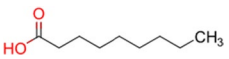
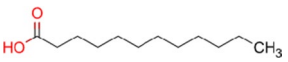
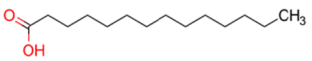
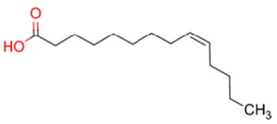
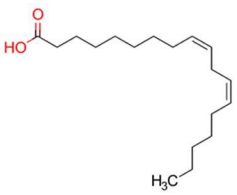
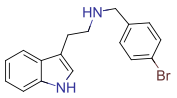
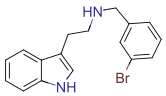
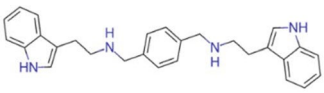
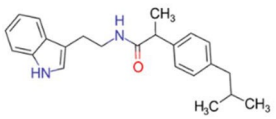
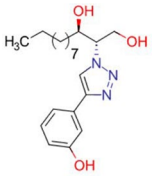
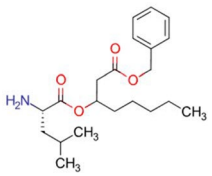
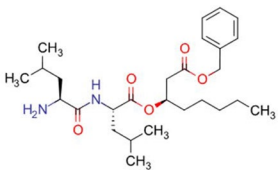
| N° | Molecule | Chemical structure | $BIC_{50/80}$ [a] | $MIC_{50/80}$ [b] | Mecanism | Reference |
|---|---|---|---|---|---|---|
| 1 | Roemerine |  | 28.6 µM | 916 µM | Inhibition of metabolic activity and hyphae formation; Reduction of fungal cell surface hydrophobicity. | [48] |
| 2 | Curcumin |  | 135.7 µM | 271.5 µM | Inhibition of metabolic activity, hyphae formation and expression of adhesins. | [52] |
| 3 | Piperine |  | < 112 µM | > 3589 µM | Inhibition of the formation and elongation of hyphae and the expression of adhesins. | [44] |
| 4 | 4'-hydroxychalcone |  | 69.6 µM | 279 µM | Inhibition of fungal cell wall formation. | [56] |
| 5 | Propolin D |  | < 11.8 µM | 117.8 µM | Mimics the effect of farnesol (component of quorum-sensing). | [59] |
| 6 | Propilin H |  | < 12.2 µM | 122.4 µM | | |
| 7 | Nonanoic acid |  | < 12.6 µM | 632 µM | | [43] |
| 8 | Lauric acid |  | < 10 µM | > 2496 µM | | |
| 9 | Myristic acid |  | < 43.8 µM | > 2189 µM | | [60] |
| 10 | Myristoleic acid |  | < 44.2 µM | > 2209 µM | | [60, 61] |
| 11 | Linoleic acid |  | < 35.7 µM | > 1782 µM | | |

**Table 1** (continued)

| N° | Molecule | Chemical structure | $BIC_{50/80}$ [a] | $MIC_{50/80}$ [b] | Mecanism | Reference |
|---|---|---|---|---|---|---|
| 12 | Derivative 3 |  | 24.3 µM | 194 µM | Mimics the effect of tryptophol (component of quorum-sensing). | [62] |
| 13 | Derivative 4 |  | 12.1 µM | 194 µM | | |
| 14 | Derivative 24 |  | 37.9 µM | > 303 µM | | |
| 15 | Derivative 36 |  | 45.9 µM | > 367 µM | | |
| 16 | 1,2,3-triazole analog 2b |  | 5.3 µM | 10.8 µM | Production of reactive oxygen species (ROS). | [73] |
| 17 | Amino acid derivative 2 |  | 22 µM | 137.6 µM | No defined mechanism of action. | [42] |
| 18 | Dipepitide derivative 27 |  | 21.1 µM | 126.4 µM | | |

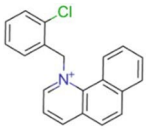
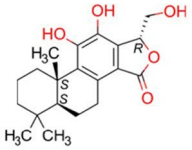
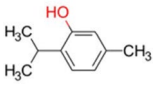
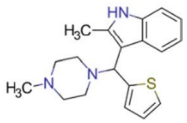
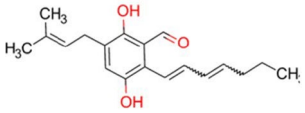
[a]$BIC_{50/80}$: concentration that inhibits 50 or 80% of biofilm formation; [b]$MIC_{50/80}$: concentration that inhibits 50 or 80% of growth

most of the molecules act on the hyphae and/or adhesins, preventing both the adhesion process and its amplification, and a reduction in cellular metabolism is often observed. This last point, however, is a source of doubt about the cause–effect relationship, as it is not clear whether the inhibition of metabolism reduces the formation/development of hyphae and adhesins or the negative regulation of genes related to these processes slows cellular metabolism. Roemerine **(1)** and curcumin **(2)** are molecules in this situation.

The effect of the apomorphic alkaloid roemerine on the filamentation of sessile cells of *C. albicans* was observed by Ma et al. [48] in a dose-dependent manner. It was also found that 28.6 µM roemerine inhibited the metabolic activity of cells by 80%, revealing a specific action on sessile cells because it showed low antifungal action against planktonic cells ($MIC_{80}$ = 916 µM), reducing cell density from 57.3 µM. In addition to the effects on adhesion and possibly on the previously discussed metabolism, roemerine decreased the fungal cell surface hydrophobicity. Cell surface hydrophobicity is an important factor for biofilm formation and dispersion and depends on the protein content and hydrophobic amino acid content of the proteins on the cell surface [49, 50]. A potential for in vivo use is added to the antibiofilm effect, which is justified by the low toxicity observed in trials with healthy worms of *Caenorhabditis elegans* ($IC_{50}$ = 229 µM and $LD_{50}$ = 14,663 µM) and nonsignificant toxicity to endothelial cells ($IC_{50}$ = 154 µM).

To better understand the antibiofilm mechanism of roemerine, Ma et al. [48] investigated changes in gene expression, and after treatment with 28.6 µM of the molecule,

**Table 2** Promising molecules for biofilm eradication

| N° | Molecule | Chemical structure | $BEC_{50/80}$ [a] | $MIC_{50/80}$ [b] | Mecanism | Reference |
|---|---|---|---|---|---|---|
| 19 | (1)-N-2-methoxybenzyl-1,10-phenanthrolinium bromide (FEN) |  | 10.2 µM (48 hours) | 4.1 µM | Rupture of membrane integrity. | [79] |
| 20 | Simplexene D |  | 72.2 µM | 92.4 µM | Rupture of membrane integrity (plasma and nuclear); Disorganization of cytoplasmic content. | [80] |
| 21 | Thymol |  | 41.6 – 166 µM (48 hours) | 20.3 – 41.6 µM | Deformation and disaggregation of biofilm cells; Reduction in hyphae formation. | [81] |
| 22 | MMV688768 |  | 0.4 µM (24 hours) | 3.12 µM | Blockage of hyphal elongation. | [85] |
| 23 | Dihydroauroglaucin |  | 52 µM (24 hours) | 13 µM | No defined action mechanism. | [86] |

[a]$BEC_{50/80}$: concentration that eradicates 50 or 80% of the biofilm formed; [b]$MIC_{50/80}$: concentration that inhibits 50 or 80% of growth

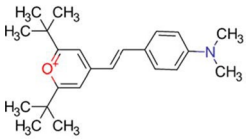
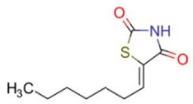
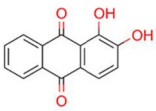
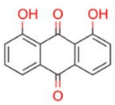
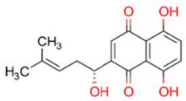
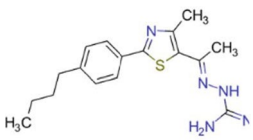
specific biofilm and hyphae genes, such as *YWP1* (yeast-form wall protein 1), *SAP5* and *SAP6* (secreted aspartic proteinase), *HWP1* (hyphal wall protein), and *ECE1* (endothelin converting enzyme), were upregulated, while the *EFG1* (enhanced filamentous growth protein) gene was negatively regulated. The expression of the *SAP5*, *SAP6*, *HWP1*, and *ECE1* genes depends on the *EFG1* gene, which encodes a transcription factor at the end of the Ras/cAMP/PKA pathway, which is important for the development of hyphae. This suggests that roemerine represses the formation of hyphae by negatively modulating the Ras/cAMP/PKA pathway [51].

Curcumin, a natural polyphenolic derivative, inhibited biofilm metabolic activity ($BIC_{50}$ = 135.7 µM) with a low reduction in biomass (12%, $p$ = 0.19) at the same concentration, suggesting a preferential blocking of biofilm formation at the expense of antifungal action ($MIC_{50}$ = 271.5 µM). This action was associated with reduced cell adhesion due to inhibition of filamentation, according to scanning electron microscopy (SEM) analyses of sessile cells but also due to lower adhesin expression. Both effects corroborate evidence of significant downregulation (more than threefold) of key genes for adhesins (*ALS*3) and hyphae formation (*HWP*1) [52].

Despite its potential, curcumin has limited application value due to its low solubility in aqueous media. As an alternative, Palmieri et al. [53] associated curcumin with a hydrophilic molecule, polyethylene glycol, allowing the release of curcumin–polyethylene glycol nanomolecules locally in aqueous environments, and the action of these molecules on cells initiated biofilm formation. To increase the bioavailability of curcumin, other nanoparticles have been developed, such as those associated with chitosan molecules [54]. This carrier molecule has positively charged amines that can bind to negatively charged biofilm polymers, such as eDNA [55], facilitating its diffusion and the action of curcumin within the biofilm structure.

Similar to what was observed with curcumin, Priya and Pandian [44] observed potent antibiofilm activity of piperine **(3)** ($BIC_{90}$ = 112 µM) related to the inhibition of adhesion and adhesin expression, with a low effect (32 times lower) on planktonic cell viability ($MIC_{50}$ = 3589 µM). The initial light microscopy and SEM studies indicated interference by piperine on the yeast transition to the hyphal form and on hyphal extension. Later, there was positive regulation of the *TUP*1 gene, a transcriptional repressor of genes that initiate the filamentation process, along with a strong negative regulation of several genes encoding adhesins and

**Table 3** Promising molecules for inhibition of biofilm formation and eradication

| N | Molecule | Chemical structure | $BIC_{50/80}$ [a] | $BEC_{50/80}$ [b] | $MIC_{50/80}$ [c] | Mecanism | Reference |
|---|---|---|---|---|---|---|---|
| 24 | SM21 |  | 85% inhibition at < 0.6 µM | 9.5 µM (24 hours) 73.9 µM (48 hours) | 0.6 µM | Inhibition of hyphae formation. | [87] |
| 25 | S-8 |  | 37.5 µM | 4.7 – 18.8 µM (24 hours) | 300 µM | Inhibition of hyphae formation and expression of adhesins; Reduction of fungal cell surface hydrophobicity. | [88] |
| 26 | Alizarin |  | 82% inhibition at 2.1 µM | > 90% inhibition at 8.3 µM (24 hours) | > 8,325 µM | Inhibition of hyphae formation and expression of adhesins. | [90] |
| 27 | Chrysazin |  | 2.1 µM | < 8.3 µM (24 hours) | > 8,325 µM | Inhibition of hyphae formation. | [90] |
| 28 | Shikonin |  | 65,4% inhibition at 13.9 µM | 80% inhibition at 55.5 µM (24 hours) | 13.9 µM | Inhibition of hyphae formation and expression of adhesins; Regulation of farnesol synthesis (component of quorum-sensing). | [37] |
| 29 | Compound 1 |  | 61,7% inhibition at 0.4 µM | 66,3% inhibition at 6.1 µM (24 hours) | 1.5 µM | No defined mechanism of action. | [91] |

[a]$BIC_{50/80}$: concentration that inhibits 50 or 80% of biofilm formation; [b]$BEC_{50/80}$: concentration that eradicates 50 or 80% of the biofilm formed; [c]$MIC_{50/80}$: concentration that inhibits 50 or 80% of growth

hyphal formation, for example, *ALS*3 (5×), *HWP*1 (4×), *EFG*1 (5×), and *CPH*1 (4.5×). This molecule also showed no acute toxicity in human oral epithelial cells or nematode models at higher concentrations (2–3×) than the $BIC_{90}$. Thus, piperine seems to be a potential candidate for the treatment of *C. albicans* infection associated with biofilms, especially for oral candidiasis.

With a more related action on the activity of adhesins, Lobo, Lopes, and Klein [56] studied an intermediate flavonoid metabolite called 4′-hydroxychalcone (**4**). This metabolite inhibited biofilm formation ($BIC_{50}$ = 69.6 µM) at sub-MIC concentrations ($MIC_{50}$ = 279 µM) associated with inhibition of fungal cell wall formation and adhesion. Considering that the cell wall contains proteins with adhesin activity (Als1p and Als4p), changes in its formation may interfere with the adhesion process between cells, which is necessary for biofilm formation [57, 58].

Several molecules also act on the initial stage (or adhesion) of the biofilm by interfering with quorum-sensing pathways, for example, prenylated flavones [59], long- and medium-chain fatty acids [43, 60, 61], and tryptamine analogs [62]. The phenomenon of quorum-sensing is a cell–cell communication process that depends on cell density and occurs at all stages of biofilm formation. Through signaling molecules produced by the cells themselves, such as farnesol, tyrosol, phenylethanol, and tryptophol [63], cells respond to internal and external stimuli. These responses include changes in morphogenesis (transition from spherical

to hyphae), onset of programmed cell death, apoptosis, and production of virulence factors [14], which occur through the activation or repression of genes in the cells of the community in each phase of biofilm formation.

Among the signaling molecules, farnesol and tyrosol are the most studied. Farnesol was the first molecule known to participate in quorum-sensing isolated from *C. albicans* [64]. One of its main functions is to inhibit the yeast–hypha transition, thus influencing cell adhesion to substrates, the structure of the mature biofilm, and the biofilm cell dispersion stage. When the concentration of farnesol is low, tyrosol can exert an influence on biofilm cells, stimulating the production of hyphae during the early stages of biofilm formation [65]. In addition to these two signaling molecules, tryptophol, another component of *C. albicans* quorum-sensing, shows effects similar to farnesol on the filamentation process [64].

With structural similarity to farnesol and therefore with the ability to mimic the effect of this molecule on the biofilm, five prenylated flavanones (propolin C-H) extracted from *Macaranga tanarius* (Okinawa propolis) were studied by Lee et al. [59]. Among these flavanones, propolin D **(5)** (11.8 μM) and propolin H **(6)** (12.2 μM) stood out for inhibiting 95% and 65–75% of the biofilm formation of *C. albicans* ATCC 10,231, respectively. According to the authors, the reduction of biofilm was related to lower cell aggregation and negative regulation of genes essential for the development and elongation of hyphae and intercellular adhesion, such as *HWP*1 and *ECE*1 [66–68]. In addition, the most promising compound (propolin D) did not interfere with the growth of planktonic cells ($MIC_{50} = 118$ μM) nor was it cytotoxic in *C. elegans* models ($IC_{50} > 1178$ μM), making it an important prototype for more studies.

The same group of researchers [43] investigated 31 other molecules (saturated and unsaturated fatty acids) that also mimic farnesol. Six medium-chain fatty acids were promising for inhibition greater than 75% on the biofilm formation of *C. albicans* ATCC 10,231 at concentrations ranging from 10 to 15.4 μM: heptanoic acid, octanoic acid, nonanoic acid **(7)**, decanoic acid, undecanoic acid, and lauric acid **(8)**. All these fatty acids showed antifungal activity ($MIC_{50}$) 50–250 times higher than their $BIC_{50}$, suggesting a very specific action on the biofilm. As a control, farnesol also inhibited hyphal growth and cell aggregation but at high concentrations (449.7 μM), suggesting that these acids have a high antibiofilm potential.

The effect of subinhibitory concentrations of these fatty acids was analyzed at the molecular level, revealing that they negatively regulate multiple genes, for example, transcriptional regulators of filament growth (*CPH*1, *UME*6 and *EFG*1), hyphae-specific (*EAP*1, *HWP*1, *HST*7, *RAS*1, and *ECE*1), and adhesins (*ALS*3 and *ALS*1). The most potent molecules (heptanoic acid, nonanoic acid, and lauric acid) also reduced the production of farnesol, which may represent a negative feedback from the quorum-sensing system due to their structural similarities with farnesol. In addition, nonanoic acid reduced the virulence of *C. albicans* in *C. elegans* models and showed no cytotoxicity at 632 μM, making it an interesting prototype for the design of inhibitors for clinical purposes.

Although little highlighted by the authors, myristic acid **(9)**, myristoleic acid **(10),** and linoleic acid **(11)** also showed high antibiofilm activity (> 85%) at 35.7–44.2 μM, corroborating the findings on the antibiofilm activity of these molecules described by Prasath, Sethupathy, and Pandian [60] and Kim et al. [61]. The myristic acid activity was attributed to the downregulation of the *MTS*1 gene, which encodes the C9-methyltransferase enzyme. The downregulation of this enzyme affects the glycosyl ceramide biosynthesis pathway, associated with the hyphal elongation process [60, 69]. By another regulatory route, linoleic acid reduced the concentrations of all lipid raft components, microdomains rich in sphingolipids and ergosterol that are important for maintaining the integrity of the plasma membrane and in the segregation of proteins to the plasma membrane. The greater presence of these microdomains in biofilm cells than planktonic cells highlights that changes in the composition of lipid rafts reduce the stability of these structures and interfere with the formation of biofilms [61, 70, 71].

The findings by Hara et al. [72] on the application of medium-chain and unsaturated fatty acid salts to preformed biofilms in dental prostheses suggest that potassium oleate and sodium linoleate efficiently highlight the biofilm of dental resins. This result opens a field of study for the investigation of mouthwashes with these compounds for the inhibition of biofilm formation.

Using their structural similarity with quorum-sensing molecules to suggest a mechanism of action, Pandolfi et al. [62] investigated the inhibition of biofilm formation for a series of 36 tryptamine analogs structurally similar to tryptophol. Although the reference molecule, tryptamine, did not show antibiofilm or antifungal activity (≥ 799 μM), three amine derivatives **(12–14)** and one amide derivative **(15)** showed antibiofilm activity ($BIC_{50} = 12.1–45$, 9 μM) up to 16 times stronger than their antifungal activities ($MIC_{50} = 194$ to > 367 μM) against planktonic cells of *C. albicans* ATCC 10,231. An amine derivative **(13)** with $BIC_{50} = 12.1$ μM and $MIC_{50} = 194$ μM stands out.

According to the authors, the precise mechanism of action of the derivatives has not been elucidated, but they believe that these molecules can prevent cell adhesion during biofilm formation or favor the release of the planktonic

form, probably because they actually mimic the effect of tryptophol. In vivo toxicity tests with wax moth (*Galleria mellonella*) larvae indicated that analogs **12**, **14**, and **15** were nontoxic after 72 h of exposure at concentrations above 1183 µM and could be clinically useful. Thus, this study suggests that tryptamine derivatives are promising for the design of fungal biofilm inhibitors.

In addition to the mechanisms described above, this review also identified another pathway of action of molecules with antibiofilm potential: the production of reactive oxygen species (ROS). This mechanism was verified by Reddy et al. [73] in assays with the 1,2,3-triazole analogs of C12-sphinganine, of which a derivative **(16)** with a $BIC_{50}$ (5.3 µM) lower than the $MIC_{50}$ value (10.8 µM) had action related to the reduction in biofilm biomass as intracellular ROS production increased in sessile cells. These substances include superoxides ($O_2^{-}\bullet$), peroxides ($H_2O_2$ and ROOH), and free radicals (HO• and RO•), which cause oxidative damage in the cell, including damage to DNA, mitochondria, and enzymes important to metabolism, inducing cell apoptosis [74, 75]. As the formation of biofilms by adhered cells (sessile cells) requires the maintenance of cell multiplication capacity, molecules that inhibit cell growth by inducing ROS production, for example, may interfere with the biofilm formation process.

Unlike the previous molecules, molecules **17** and **18** seem to act against the adhesion process but without a defined mechanism of action, a worthy topic for further research. Developed by Jovanoci et al. [42] and inspired by rhamnolipids, these molecules blocked the formation of *C. albicans* biofilm at concentrations of 22 and 21.1 µM, respectively, concentrations 6 times smaller than their antifungal $MIC_{50}$ (137.6 and 126.4 µM, respectively), with no cytotoxic effect ($IC_{50} > 211$ µM) in healthy human fibroblasts (cell line MRC5).

Both compounds seem to preserve the rounded shape typical of most yeasts in the treated cells, while the control cells exhibit an elongated mycelial network. However, these compounds did not inhibit the formation of hyphae or reduce their length during the cell adhesion process, suggesting, according to the authors, that rhamnolipid derivatives prevent cell adhesion to abiotic and biotic surfaces.

In fact, cell adhesion depends on nonspecific factors, such as cell surface hydrophobicity and electrostatic forces, and specific factors, such as the production of fungal surface proteins [17–19]. Although many of these molecules can act similarly to biosurfactants, reducing the cell surface hydrophobicity, they have critical micellar concentrations higher than those of rhamnolipids, refuting this hypothesis [42]. Thus, the authors believe that both molecules can act in other pathways of the adhesion process, possibly on specific targets such as cell wall proteins, especially adhesins or mannoproteins.

## Promising molecules for biofilm eradication

Unlike the studies on inhibiting biofilm formation, eradication studies are important because they allow the identification of active molecules from the biofilm with extracellular matrix that is in formation or is fully formed. Thus, the influence of molecules on cells in the early stage of biofilm formation is ignored. Consequently, the BEC may be equal to or greater than the MIC. It is important to consider that in the more advanced stages of biofilm development, for example, proliferation (12–30 h) and maturation (38–72 h), the sessile cells have a lower susceptibility to the molecules tested. In these stages, multiple layers of cells with hyphae and pseudohyphae surrounded by the extracellular matrix, together with efflux pumps present in this matrix, hinder the access of these molecules to fungal cells [76]. In the last 10 years, few molecules have been able to exclusively eradicate already formed biofilms. Furthermore, accurately determining the antibiofilm mechanisms of these compounds is one of the greatest current challenges. Considering this context and the selection criteria used in this review, the molecules with the activity of eradicating a biofilm are much fewer than those with the activity of preventing its formation, their mechanism of action being suggested by cellular assays rather than complementary studies, such as on gene-level or proteomic expression.

Among the mechanisms described for the selected molecules, two caused the rupture of membrane integrity: (1)-N-2-methoxybenzyl-1,10-phenanthrolinium bromide (FEN) **(19)** and simplexene D **(20)**.

Previous studies of the antifungal activity of FEN ($MIC_{50} = 4.1$ µM) against *C. albicans* 10,231 [77, 78], according to Nuryastuti et al. [79], investigated the antibiofilm properties of this molecule. Although FEN had $BEC_{50} = 20.5$ µM for the mature biofilm (48 h), only concentrations higher than 656 µM were required for eradication (> 80%) of the mature biofilm. According to SEM, the eradication promoted by FEN comes from the mechanism of antifungal action of the molecule, such as the disruption of the membrane integrity of the microorganism.

For the simplexene D molecule, there was no overlap between the antifungal effect and the antibiofilm effect. This molecule, studied by Favre-Godal et al. [80], had more potent action against the mature biofilm (48 h) of *C. albicans* (CAF2-1) than five other cassano-type diterpenoids isolated from *Swartzia simplex* root bark. Transmission electron microscopy studies concluded that treatment with simplexene D causes the rupture of the plasma membrane of biofilm cells, as well as other cytological effects, for example, the rupture of the nuclear membrane and disorganization of the cytoplasmic content, in which the mitochondria, Golgi complex, and ribosomes are affected. However, the accumulation of vacuoles with lipid bodies

in the cells was not observed, suggesting a mechanism of action different from that of classic antifungals, such as azole derivatives. Despite the lack of accurate information on the molecular mechanisms involved in these cytological effects, knowing that the simplexene D molecule may have a different mechanism from the classic antifungal agents already gives this agent an advantage over FEN due to the lower likelihood that a mechanism of resistance to antifungal agents will influence the antibiofilm action of the molecule.

In addition to membrane rupture, a second mechanism highlighted among the selected molecules was related to cytological effects, such as the deformation and disintegration of biofilm cells, promoted by thymol **(21)**. This effect was described by Jafri and Ahmad [81] in mature biofilms of *C. albicans* strains sensitive to fluconazole (CAJ-01) and resistant to fluconazole (KGMU028). According to the authors, a significant eradication of the biofilm ($BEC_{80}$ = 41.6 and 166 µM) occurred at concentrations equal to or greater than the antifungal concentrations (MIC = 20.8–41.6 µM). However, exposure of mature biofilms to sub-MIC concentrations of thymol caused disintegration and deformation of biofilm cells, in addition to a reduction in hyphal formation, as also observed by Dalleau et al. [82] and Miranda-Cadena et al. [83]. Cytotoxicity studies have evaluated the viability of the compound for in vivo assays, and no general toxic effect was observed, according to the hemolysis assays with erythrocytes ($IC_{50}$ > 15.310 µM) [34]. In addition to its potential as an antibiofilm agent and low cytotoxicity, thymol acted synergistically with fluconazole and amphotericin B in sessile cells, reducing the MIC of these agents by up to 32 times, making it a potential adjuvant in the treatment of patients infected with isolates resistant to the first-choice antifungal agents.

Despite the promising results of thymol, the use of phytocomposites of essential oils for drug development has limitations that lie in their low solubility in aqueous solution, their volatility, their instability, and possible hypersensitivity reactions [83]. To circumvent these factors, Al-Ani and Heaselgrave [84] investigated the antibiofilm activity and biocompatibility of thymol formulations in carrier molecules, such as hydroxypropyl methylcellulose and poloxamer 407 (P407). Among the combinations, thymol-P407 showed the best antibiofilm activity and the best biocompatibility with human cell lines (embryonic kidney cells), paving the way for the development of viable drugs containing thymol as an active ingredient.

Like thymol, the synthetic indole derivative MMV688768 **(22)** has antibiofilm action related to the blockage of hyphal elongation, leading to the formation of shorter structures [85]. This effect was determined by morphological and substructural analyses of the biofilm exposed to the molecule by SEM and can partially explain the eradication of the biofilm in the proliferation stage (24 h) ($BEC_{50}$ = 0.4 µM). Its $BEC_{50}$ is lower than its $MIC_{50}$ (3.12 µM), so the antifungal effect is separate from the antibiofilm effect, avoiding the selection of resistant cells. This factor, associated with the fact that MMV688768 also showed low toxicity in the hepatocellular carcinoma model (HepG2 cells) at a concentration 64 times higher ($CC_{50}$ = 32.04 µM) than the $BEC_{50}$, makes the molecule a very promising candidate for antibiofilm drugs.

Unlike the above molecules, dihydroauroglaucine **(23)** did not have any investigated mechanism. This natural product obtained from the endophytic fungus *Aspergillus amstelodami* combined with the fruit of *Ammi majus* L. could eradicate the preformed biofilm (24 h) of *C. albicans* ATCC 10,231 at a concentration of 52 µM [86]. Even so, the toxicity of the molecule in normal human fibroblast cell lines (BHK) was considered moderate (40% cell death at 166 µM). Further toxicity studies in animal models are needed for a more thorough evaluation, including with the concentrations close to the BEC, before discarding it as a possible antibiofilm drug.

## Promising molecules for the inhibition of biofilm formation and eradication

Compounds that act both for inhibition and eradication of the biofilm are even more valued because they can prevent the formation of and destroy preformed biofilms, weakening the existing infection and reducing the chance of dispersion of biofilm cells for the formation of new infections. Thus, these molecules can be applied at any time in the treatment of the patient. However, for the molecules that act in eradication, finding molecules that act in biofilm formation and in preformed biofilm formation at the same time is not an easy task, as few molecules have these properties.

Among the few molecules found in our selection, most of them showed interference with the processes of adhesion and hyphae formation, important both for the early stages of biofilm formation and for the maintenance of this structure in its mature phase, corroborating the findings of molecular analyses. In this profile, we found the SM-21, a derivative of dimethylamino phenyl-vinyl-pyril **(24)**; S-8, a thiazolidinedione derivative **(25)**; alizarin **(26)** and chrysazin **(27)**, two anthraquinones; and shikonin **(28)**.

SM-21, the molecule with the best antibiofilm properties among the more than 50,000 compounds, was available from ChemBridge Corporation and screened by Wong et al. [87]. The authors observed, in addition to a significant decrease in biofilm formation (85%) at a concentration below the MIC (BIC < 0.6 µM; MIC = 0.6 µM), a 50% reduction in the preformed biofilm after 24 h (initiation step) and 48 h (maturation step) at concentrations of 9.5 and 73.9 µM, respectively. This impact can be attributed to the ability to inhibit the formation of hyphae at low concentrations of the compound

(1.3 μM) through the modulation of the *HWP*1 gene and, to a lesser extent, to the damage caused to the membrane at sub-MIC concentrations (0.3 μM).

In addition to the antibiofilm potential, SM-21 showed low toxicity ($CC_{50}$ = 10 μM) in models of human oral keratinocytes, the concentration of 5.9 μM being capable of preventing the invasion of *C. albicans* in these cells, which was corroborated by the prolonged survival of mouse models with invasive candidiasis after administration of SM21 at a concentration of 0.1 mg/kg.

The modulation of the *HWP*1 gene is also related to the effect of biofilm cell exposure to S-8 [88] and shikonin [37]. These molecules also caused downregulation of the agglutinin genes (*ALS*3) and increased the expression of hypha-specific gene expression repressors (*TUP*1 and *NRG*1). Other genes were regulated differently by the two molecules. While S-8 caused negative regulation of extracellular adhesion proteins (*EAP*1), genes related to long-term maintenance (*UME*6), and transcriptional regulators of hyphae (*CST*20, *HST*7, and *CPH*1), shikonin promoted downregulation of genes *EFG*1, *CPH*1, *RAS*1, and *ALS*1.

Although the molecules regulate gene expression in similar ways, they inhibit biofilm formation and eradicate biofilms by different cellular mechanisms. For example, S-8 appears to act on yeast adhesion to abiotic surfaces and host cells because it reduces the cell surface hydrophobicity index by 79% and, up to 63%, the interaction (adhesion) with host macrophages. Shikonin, however, activated a quorum-sensing mediator, farnesol, through upregulation of the *DPP*3 gene, which encodes a phosphatase responsible for the synthesis of this mediator [89]. This was confirmed by the combination of 3.5 μM shikonin and 25 μM farnesol, which allowed the inhibition of biofilm formation by 40%, probably due to the presence of enough farnesol to see the effect, given that 25 μM farnesol alone did not generate any effect. Even so, S-8 seems to be more advantageous than shikonin due to the absence of an antifungal effect on planktonic cells ($MIC_{50}$ = 300 μM), which reveals a unique effect on the biofilm.

Two quinone derivatives studied by Manoharan et al. [90], alizarin and chrysazin, are structurally similar to shikonin; however, they are more advantageous because they eradicate the biofilm at low concentrations and exclusively affect the biofilm. At a concentration of 2.1 μM, alizarin and chrysazin reduced biofilm formation by 82 and 50%, respectively, and prolonged the survival rate of *C. elegans* infected with *C. albicans* by > 50% when their concentrations were 2 and 1 μM, respectively. In the eradication studies, 8.3 μM reduced the preformed biofilm by more than 90% after 24 h. Similar to shikonin, these two molecules acted strongly on the expression of specific hyphal genes, with downregulation of the genes *ECE*1, *ECE*2, and *RBT*1. Alizarin also caused downregulation of the *ALS*3 gene (adhesin), which effect was associated with the hydroxyl portion at the C1 position of anthraquinones, according to preliminary studies of the structure–activity relationship. These molecules are even more promising because they are not toxic to *C. elegans* models at the concentrations of 4163 μM chrysazin and 8326 μM alizarin.

Although promising, some molecules have no indication of their mechanism of action, such as molecule 37 **(29)**, which was tracked by Mohammad et al. [91] among a series of phenylthiazole derivatives. Despite showing antifungal activity ($MIC_{50}$ = 1.5 μM) against the *C. albicans* (P60002) strain resistant to fluconazole ($MIC_{50}$ > 194 μM), the molecule could prevent biofilm formation at subinhibitory concentrations (61.7% at 0.4 μM) and eradicate preformed biofilm at 24 h at concentrations close to the MIC (66.3% to 6.1 μM).

## Conclusions

Throughout the process of writing this review, we observed that among studies of the inhibition of biofilm formation, many consider molecules with antibiofilm activity at concentrations above their MIC. In fact, this may mask the actual activity of the molecule, since there is an intersection between antifungal and antibiofilm activities. This observation suggests a lack of clear interpretive criteria regarding the relationship between activities, with negative and misleading consequences for the analyses. Therefore, to reduce the influence of possible antifungal activities, only molecules whose BIC was lower than the MIC were considered in this review.

Thus, we found the existence of molecules derived from natural and synthetic products that are promising both for the prevention of biofilm formation and eradication and are mainly useful for prevention. We highlight molecules in advanced stages of study, with promising results in cytotoxicity assays and in vivo assays, such as 1, 3, 5, 6, 7, 12, 14, 15, 17, 18, 21, 22, 24, 26, 27, and 29. Alizarin (26) and chrysazin (27) showed inhibition activity, as well as eradication of biofilms with 24 h of formation at concentrations up to 1000 times lower than the MIC. SM21 (24) caught our attention because it exhibits, in addition to its inhibitory action, eradication activity in biofilms at 24 and 48 h. However, the eradication action of this molecule required concentrations 10 to 100 times higher than the MIC, unlike what was observed for alizarin (26) and chrysazin (27).

Gene expression studies and microscopic analyses are the main methods used to find the targets of inhibition or eradication of fungal biofilms. Both methods suggested that most molecules alter the adhesion process by interfering in the transition from yeast to hyphae, in hyphal elongation, in the expression of adhesins, in the hydrophobicity of the

cell surface, and in the production of quorum-sensing components, thus slowing growth and biofilm ripening. Nevertheless, these techniques have limitations in identifying the proteins involved, so no specific target (protein/DNA) has been identified, especially in biofilm eradication studies. This limitation indicates the need for further studies, such as proteomic analyses and detailed analyses of each pathway associated with biofilm production to reach a biomolecular target.

**Author contribution** Carolina Guimarães, conceptualization, methodology, and writing — original draft. Humberto de Freitas, conceptualization, methodology, software, writing — review and editing, and visualization. Tânia Barros, writing — review and editing, and visualization. All authors contributed to the article and approved the submitted version.

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## Declarations

**Ethics approval** Not applicable.

**Consent to participate** Not applicable.

**Conflict of interest** The authors declare no competing interests.

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