Mini-review – *Candida albicans* biofilms: characteristics, clinical relevance, and drug susceptibility

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Candida albicans is a commensal fungus found on mucosal surfaces. However, it can cause severe and recurrent mucosal infections, as well as fatal invasive infections in both immunocompromised and immunocompetent individuals. Most of these infections are caused by the organization of the fungal cells in a sessile community encased within an extracellular matrix adhered to a biotic or abiotic substratum. *C. albicans* biofilm is composed of yeasts, pseudohyphae and hyphae forms and can be associated with other fungal species and bacteria. The organization in biofilm contributes to defence against attack by host immunity and resistance to antifungal drugs. Therefore, the knowledge of its molecular mechanisms and development can help discover new strategies of control and improve the set of conducts to manage biofilm infections. Thus, the aim of this mini-review is to gather the main information about the characteristics, clinical relevance and drug susceptibility of the biofilm formed by this important human pathogen.

Keywords: *Candida albicans*; Biofilm; Oral Candidosis; Antifungal Drugs

1. Introduction

Candida albicans is a commensal fungus found on oral cavity, gastrointestinal tract, and genitourinary tract. However, it is able to cause severe and recurrent mucosal infections, such as oral and vaginal candidosis, as well as invasive and fatal infections in both immunocompromised and immunocompetent individuals [1,2]. *C. albicans* is the most pathogenic and prevalent specie of the *Candida* genus, followed by *C. tropicalis*, *C. glabrata*, and *C. krusei* [3-6]. Estimates reports that around 75% of women experiment at least one episode of vaginal candidosis and 90% of untreated- HIV patients are affect by oral candidosis [7,8]. In addition, *C. albicans* is the third organism responsible for nosocomial bloodstream infections with mortality rates over 40% [9]. As an opportunistic microorganism, candidal infections are more common in individuals with compromised immune system or long antibiotic treatment that affect the fine balance between host and pathogen [10]. The unbalanced of normal microbiota, disruption of epithelial barriers and dysfunction of immune system, favor the transition from commensal to pathogenic fungi. The pathogenic condition is recognized by the host cells when the fungal burden increases and yeasts turn into hyphal forms activating immune response of the first line of defense, epithelial cells. The morphogenesis is one of the most important virulence factors of *C. albicans* producing filamentous forms which are more adherent to host cells and abiotic surfaces, more resistant to phagocytosis by immune cells and are also implicated in invasion through active penetration and endocytosis mechanisms causing damage, whereas yeast form is required to dissemination [11-14]. The process involved in infection is initiated by the contribution of other virulence factors, such as expression of adhesins that mediate adherence to host cells and abiotic surfaces, hydrophobicity related to adherence to abiotic surfaces of medical devices, secretion hydrolytic enzymes (proteinases and phospholipases) promoting adherence and host tissue destruction, and biofilm formation [16-18].

Biofilms are structures composed of a community of microorganisms belonging to the same or different species adhered to a substratum and encased within a self-produced extracellular matrix. Bacteria, yeasts and filamentous fungi are able to form sessile communities on both biotic and abiotic surfaces causing complex medical situations, since over 65% of all microbial infections are related to the presence of biofilms on host tissues and medical devices [19,20].

C. albicans forms a heterogeneous biofilm structured in an extracellular matrix containing yeasts, pseudohyphae and hyphae on mucosal surfaces and medical devices that cause diseases and dissemination of infection [21]. The tridimensional structure and extracellular matrix protect the cells against the action of antifungal drugs and host immune system by mechanisms not fully understood yet. Thus, the aim of this mini-review is to gather the main information about the characteristics, clinical relevance and drug susceptibility of the biofilm formed by this important human pathogen.

2. *Candida albicans* biofilm structure

The sessile community formed by the fungus *C. albicans* is composed of three morphological types, yeasts, pseudohyphae, and hyphae, embedded in extracellular polymeric substances forming pores and channels showing different phenotypic characteristics than its planktonic counterpart (Figure 1, A) [22]. The polymeric substances are constituted by polysaccharide, proteins, hexosamines, uronic acid, and DNA, and are required to promote adherence

and biofilm formation, protect the fungal cells against phagocytosis, maintain biofilm integrity and limit substances diffusion [22,23].

The biofilm growth condition is coordinated by events didactically divided into four stages: early stage, the yeasts adhere to the substrate forming the biofilm scaffold, following by cell coaggregation and colonization [17,22]. Then, at intermediate stage, the cells grow and proliferate forming the basal layer anchoring the cells. Next, the adherence to the surface stimulates the transition from yeast to hyphae and production of pseudohyphae and, finallly, at the mature stage the tridimensional structure enlarges up to 450 μm and yeasts cells on the top of biofilm disseminate to distant sites to initiate the cycle [17,21,22,24,25].

The advantages of biofilm formation include protection in the environment, resistance to chemical and physical removal, metabolic cooperation and regulation of gene expression based on necessity of the community, and ability of colonizing and causing infection due to drug susceptibility reduction and evasion to host immune action [21,26].

Candida biofilms can grow in the environment or on hosts, as well as on medical and odontological devices aided by the presence of serum and saliva [27-29]. The main difference between *Candida* biofilms grown on abiotic and biotic surfaces is that the last is more sensitive to antifungal drugs, probably, ought to the action of immune system concomitantly. Despite the negative action of immune system on biofilm, neutrophils are not able to destroy mature *C. albicans* biofilms, because β-glucans on the cell wall bind to the immune cells blocking them and mature biofilms also block oxidative immune response [30]. Furthermore, monocytes and macrophages produce factors that enhance biofilm formation resulting in thicker biofilm and apparently no fungal cell damage [31]. *Candida* cells are also able to protect themselves from complement system through expression of proteins on the surface, like Pra1p and Gpd2p, that bind to H and I factors mimicking the host cells [32].

As a community of microorganisms, the fungal cells are able to communicate and, then, coordinate the activities and behavior of the biofilm by means of secretion of signaling molecules in a process named quorum sensing [21]. Quorum sensing contributes to control the competition for nutrients, to protect the needlessly super population and has important clinical implication for dissemination and establishment of infection on distant sites [33]. Farnesol and tyrosol are the two most studied signaling molecules. They have contrary functions, for example, when farnesol accumulates beyond the threshold level, it inhibits the yeast-to-hyphae conversion, but it is unable to block hyphae extension or reverse germ tube formation [34,35]. Farnesol is secreted at later stages of biofilm formation and may stimulate dispersion of cells [36,37]. This signaling molecule also contributes to oxidative stress resistance, activation of clamydospores formation, inhibition of adherence of yeasts to the substrate, and reduction of IL-12 production by the host cells [21,38]. On the other hand, tyrosol acts at the first stage of biofilm formation stimulating filamentation and biofilm formation, but it do not affect the farnesol dominant effect on cell morphogenesis [36,38,39].

The transition from yeast to hypha form is crucial for biofilm formation and pathogenicity, but the reverse process allows the colonization of distal sites by dispersion of yeast cells in case of nutrient starvation, super population, and presence of toxic products. The dispersion of cells initiates around 3 hours after incubation and reaches the maximum value at 24 h of growth, but at 48 h the dispersion did not cease. Besides supplying yeasts cells to conquer new sites, the dispersal cells are more adherent to polystyrene and epithelial cells, thus, the biofilms are more robust, they are able to cause more damage on endothelial and epithelial cells and are more pathogenic to mouse model, and are less susceptible to antifungal drugs than the planktonic cells [40,41].

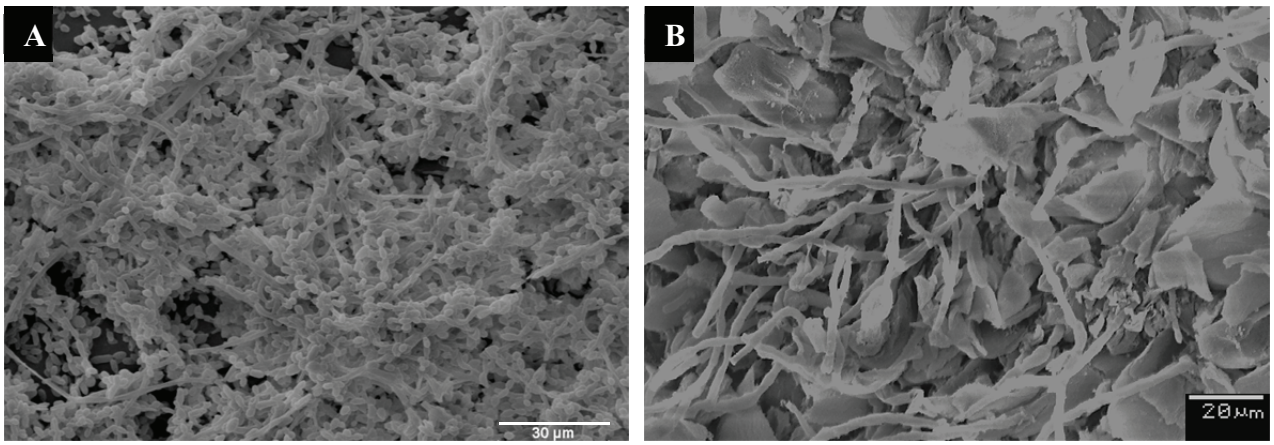

Fig. 1 (A) *Candida albicans* biofilms *in vitro* composed of yeasts, pseudohyphae and hyphae grown on polystyrene disk in Spider medium at 37°C/60 h. (B) *In vivo C. albicans* biofilm on mouse tongue dorsum showing entwined hyphae and desquamate epithelial cells.

3. Molecular mechanisms of biofilm formation

C. albicans biofilm formation is regulated by a circuit of six transcription factors, *EFG1, TEC1, BCR1, NDT80, ROB1*, and *BRG1*, which have recently evolved to adapt precisely to host environment. The transcription factors control the expression of each other and are activators and repressors of their target genes, except *TEC1* which is activator of genes involved in biofilm formation and filamentation. Together the six regulators are responsible for the regulatory network of around 15% of the genes in the *C. albicans* genome [42].

Initially, to form biofilm, yeast cells rely on expression of adhesins on the cell surface to adhere to host tissues or abiotic surfaces. The Eap1p and Csh1p confer adherence to inanimate surfaces, whereas the second one also mediates nonspecific adherence to host cells through hydrophobicity of cell surface [21,43,44]. The agglutinin-like sequence (*ALS*) family comprises 8 genes (*ALS1-7* and *ALS9*) that belong to immunoglobulin family. *ALS* genes interact specifically with host cells and mediate cell-cell interaction. Als3p is expressed only on the hypha surface and has multiple functions, it mediates adherence to oral epithelial cells, fibronectin, fibrinogen, collagen type 4, laminin and salivar pellicle. It is also an invasin, because it binds to N- cadherin and E- cadherin on the surface of endothelial and epithelial cells required to endocytosis of hyphae. Furthermore, Als3p uptakes iron from intracellular stocks of ferritin [45,46]. The most up regulated adhesin Als1p during biofilm formation is totally required to cell-substrate adherence regulated by *BCR1* [47,48]. *HWP1* is the first adhesin required to *in vivo* biofilm formation. It encodes a cell-cell adhesin expressed only by hyphae that adheres tightly to oral epithelial cells binding to mammalian transglutaminases, which cross-link covalently to host cells surface proteins [49,50]. The adhesins interact with each other promoting cell-substrate adherence mediated by Als1p and Eap1p and cell- cell interaction orchestrated by Als3p and Hwp1p [46,51].

After adherence step, yeast cells form germ tubes that will grow to form true hyphae. In biofilm phase, this process is mainly mediated by the *EFG1* gene involved in regulation of morphological transition and ability to form adherent structure on polystyrene, polyurethane, and glass. Yeast- to- hyphal transition is also mediated by other genes, such as *CPH1, TEC1, SUV3, NUP85, UME6, MDS3*, and *KEM3* [21,49].

The extracellular matrix production has many steps and regulatory networks. *ZAP1* is one of the major negative regulator of the major matrix component, soluble β-glucan. The zinc- response transcription factor Zap1 regulates *GCA1, GCA2,* and *ADH5* genes that are activators of matrix production and also binds to the promoters of *CSH1* and *IFD6* involved in inhibition of matrix production [52]. Equally important, the *FKS1* synthesizes β-1,3 glucan in the plasma membrane level and the predicted glucan transferases *BGL2* and *PHR*1 and the exo-glucanase *XOG1* are responsible for the delivery and arrangement of β-1,3 glucan in the matrix. This pathway works independently from *ZAP1* matrix formation regulatory pathway [53].

After the establishment of mature biofilm, yeasts cells are release in order to control super population and to colonize remote sites. The dispersion of cells is controlled negatively by expression of *UME6* and *SUR7* and positively by *PES1*, *NRG1*, and *HSP90* [46,54-56].

The gene expression control and the role of cells in the community is regulated in response to quorum- sensing molecules [46,56,57].

4. Clinical relevance of *Candida* biofilm on host

The infections caused by *C. albicans* are related to biofilm formation on the host tissue or on medical or odontological devices. The fungi adhere to the substrate and initiate biofilm growth and, then, invade and destroy host tissue (Figure 1, B). Oral candidosis is an example of mucosal infection caused by *C. albicans* biofilm and other species of *Candida*. The clinical manifestations can be characterized as pseudomembranous candidosis that affect newborns, elders, and HIV-positive individuals. The lesions on the mucosal surfaces are characterized by the presence of superficial white plaques that are easily removed revealing erythematous surface and the histological aspects reveal the presence of desquamated epithelial cells together with yeasts, hyphae, and bacteria [27]. The another acute form is erythematous candidosis that occurs after long treatment with broad-spectrum antibiotic, which facilitates overgrowth of *Candida* due to decrease of competitive pressure producing reddened lesions on the dorsum of the tongue and also the palate [58].

The chronic form of *Candida* infection hyperplastic candidosis is associated with smoking habit and differently from pseudomembranous and erythematous candidosis, the pseudomembrane formed in hyperplastic candidosis is not easily removed by gentle rubbing. The histological analyses shows hyphae penetrating the inflammatory infiltrate in lamina propria and alterations in the epithelium thickness [18].

As the oral cavity is colonized by many microorganisms, the *Candida* oral infections can be associated with other species of *Candida* and bacteria. There are three *Candida*- associated lesions: angular cheilitis, median rhomboid glossitis, and denture stomatitis. Angular cheilitis manifests at the angles of the mouth causing soreness, erythema and fissuring and is commonly associated with denture- induced stomatitis or existing forms of intraoral candidosis related to high load of *Candida* in combination with the bacterium *Staphylococcus aureus* [18,22].

Mediam rhomboid glossitis produces a diamond-shaped lesion on the posterior midline on the dorsum of the tongue. This chronic condition is characterized by high levels of *Candida* and is common in steroid inhalers or who are smokers [18].

The other form of oral candidosis is denture stomatitis that affects most of denture wearers, over 65%, containing a community of *Candida* cells and bacteria on the surface of the prosthesis. Wearing denture is the most predisposing factor for oral infection, particularly in cases of poor hygiene and ill-fitting denture, because oral microorganisms can adhere to the prosthesis forming biofilms and disturb protective mucosal barrier allowing penetration of *Candida* cells into the tissue promoting infection [18,58].

The *Candida* cells are able to adhere to materials, such as denture acrylic, silicon soft liners, the inner lumen of the cathethers, prosthetic heart valves, and replacement joints, forming biofim and also polimicrobial biofilm that serves as reservoir of microorganisms to spread and cause infection in other tissues, such a candidemia with high mortality rate [9]. The dispersal cells from the biofilm constitutes another challenge beyond sessile community, because they are more virulent and less susceptible to antifungal drugs than the planktonic cells [40,41].

5. Interaction of *Candida albicans* with non-*albicans* species and bacteria

Most of *Candida* infections are associated with polimicrobial biofilms in interactions among *C. albicans* and non-*albicans* species and/ or bacteria. Oral infections caused by *Candida* spp. are usually composed of two or more species of this gender, especially in individuals with important risk factors such a HIV virus, that contribute to form a biofilm more robust and with different levels of antifungal susceptibility depending on the species involved [4]. The multi-specie biofilms are result of commensalism and antagonism interactions that may be affected by the nutritional and physical environment, availability of oxygen, and other componentes that can interfere with biofilm biomass production and the behavior of microbial community [59]. *C. albicans* can interact with bacteria through physical, chemical, and metabolic relationship [60].

Candida- associated lesions are examples of infections caused by multi-specie biofilms. Denture stomatitis is caused by a multi-specie biofilm composed of *Candida* spp. and oral bacteria, such as *Streptococcus*, *Actinomyces*, and *Fusobacterium*, and the non-oral bacteria *S. aureus* and *Escherichia coli*, adhered to the surface of the prosthesis and causing the lesions on the palate described above [59,61,62]. So, the interaction between *C. albicans* and *S. aureus* benefit the growth of both microorganism, besides protecting *S. aureus* against the action of antimicrobial drugs, such a vancomycin [63,64]. *C. albicans* has also positive interactions with other Gram-positive bacteria, such as *S. epidermidis, Streptococcus pyogenes*, and *Streptococcus gordonii* in mixed-species biofilms [64]. The last specie secrets a diffusible signal molecule that enhances filamentation by bypassing the effect of farnesol [61]. On the other hand, *S. mutans* and *S. intermedius* suppress hyphal formation and in the same way the probiotic bacteria *Lactobacillus acidophilus* is able to inhibit *C. albicans* biofilm and filamentation and also attenuates *Candida* infection in invertebrate animal model [64,65]. These effects may be attributed to the production of antifungal substances, hydrogen peroxide, organic acids, and antiadhesive bio surfactants that may interfere with growth and virulence of the fungus, and control the *Candida* population in the intestinal and female reproductive tracts [61,62].

Association between *E. coli* and *C. albicans* is also responsible for increasing of infections of urinary tract, although the interaction is negative for *C. albicans* [62,64]. The same as for *E. coli*, other Gram-negative bacteria have antagonistic effects over *C. albicans* biofilm and filamentation caused by the species *Klebsiella pneumoniae, Serratia marcescens, Enterobacter cloacae*, and *Pseudomonas aeruginosa* [64]. Mixed- biofilms of *C. albicans* and *P. aeruginosa* represents an antagonistic interaction, though this interaction is responsible for ventilator- associated pneumonia and high mortality rate of cystic fibrosis patients [61]. *P. aeruginosa* produces redox- active phenazines that antagonized *C. albicans* biofilm formation by affecting the development of wrinkled colony biofilms, yeast-to-hyphae transition, and biofilm growth progression at mature stage, but they did not act on adhesion step [66,67].

The most prevalent non- *albicans* species *C. krusei, C. glabrata, C. tropicalis*, and *C. parapsilosis* are also able to form more biofilm than *C. albicans*, according to the literature depending on the substrate and growth conditions producing more biomass and biofilm cells [68,69]. In mixed communities, *C. glabrata* and *C. albicans* favors biofilm development by both species, while the combination between *C. albicans* and *C. krusei* or *C. tropicalis* cause the depletion of biofilm formation; however the combination of *C. albicans, C. tropicalis*, and *C. glabrata* produce more biofilm, but at a lower level than the combination of *C. albicans* and *C. glabrata*, likewise the four species together create an antagonistic situation producing less biofilm than the other conditions [68]. The growth of *C. albicans* in mixed- species biofilms is facilitated when *C. albicans* dominates the proportion of cells and colonize the substrate first [70]. In addition, the supernatant of the close related specie *C. dubliniensis* can interfere with *C. albicans* filamentation and the last one can inhibit pseudohyphae formation by *C. dubliniensis*, further the action of the signaling molecules is inter- and intraspecies, however they cannot reduce growth [71]. Thus, the quorum-sensing molecule farnesol produced by *C. albicans* can also affect the growth and morphological transition of *Aspergillus nidulans, A. niger, A. fumigatus, C. parapsilosis*, and *Paracoccidioides brasiliensis* [37].

6. Biofilm drug susceptibility

Given the importance and prevalence of infections caused by *Candida* biofilms, to manage biofilm infections, first, the biofilm formation on oral cavity must be prevented by improving the oral hygiene, having adequate denture cleansing, and using mouthwashes. In the case of denture stomatitis, the prosthesis must be cleaned with anti-candidal solutions and removed overnight [18,58]. It has been suggested the use of lock therapy, which comprehend the instillation of high doses of an antimicrobial agent into the catheter, may be effective against biofilm infections and useful to preserve the catheter, but in some cases the removal and the substitution of the contaminated device is required [18,64]. As an opportunistic pathogen, one of the set of actions is also the identification and correction of the predisposing factors that favor the change from commensal form to parasitic life-style [72].

The treatment of oral candidosis with conventional antifungal drugs is indicated for individuals with underlying diseases (e.g. leukemia and AIDS) which is usually prescribed the antifungals of the class of polyenes and azoles, by topic or systemic administration [18]. Both antifungal classes act on plasma membrane forming pores or inhibiting synthesis of ergosterol with fungicidal or fungistatic effects, respectively. But unfortunately, over the past decade resistance to these antifungal drugs has been observed in strains isolated from individuals with risk factors for oral candidosis and recurrent episodes [4,73]. Biofilm formation by itself is difficult to eliminate, because the biofilm structure offers protection against the antifungal agents and immune response, which keep itself 5 to 8 more metabolically active and 30 to 2000 times more resistant to antifungal drugs far from the minimum inhibitory concentration (MIC) values than the planktonic counterparts [74].

Biofilms formed by *C. albicans* are intrinsically resistant to fluconazole and sensitive to high concentrations of amphotericin B. It has also been demonstrated low susceptibility to the antimicrobials voriconazole, chlorhexidine, nystatin, terbinafine, ravuconazole in relation to the planktonic cells, but the new class of antifungal echinocandin that inhibits β-glucan synthesis has demonstrated encouraging results for activity against *in vitro* and *in vivo* biofilm models [75-77].

The low susceptibility is attributed to multifactorial events mediated by high cell density, overexpression of drug targets, heterogeneous population of cells with different growth rates, genetic alterations, expression of efflux pump genes, extracellular matrix, and persisters cells. The overexpression of efflux pumps, *CDR1, CDR2*, and *MDR1*, occurs at early stage of biofilm formation, whereas the resistance of mature biofilm is conferred by extracellular matrix that sequesters azole and polyene antifungals preventing their access to biofilm. *C. albicans* is able to produce persisters cells in biofilm that are a subpopulation of phenotypic variants of the wild type strain attached to the substratum. They are very tolerant cells encased within extracellular matrix that protects them from host defenses and antifungal drugs. It has been suggested persisters cells are not programmed to apoptosis, favoring tolerance to antimicrobial agents and starvation and after the challenge they are able to repopulate the biofilm [26,32,78-80].

The failure of treatments with conventional antifungal drugs has called attention to develop new strategies of biofilm control in order to disrupt the biofilm structure and kill the cells. Photodynamic antimicrobial chemotherapy (PACT) is a new approach that has been successfully used against *C. albicans* biofilms. This therapy is mediated by a dye called photosensitizer that is excited by a light source, e.g. laser, light emitting- diode (LED), and white light, producing reactive oxygen species (ROS) and free radicals [81,82]. The great advantage of this therapy is that the microbial cells have no resistance mechanisms against the PACT- produced products [82]. Moreover, there are other promising alternative therapies with different mechanisms of action that include phytotherapy, disruptors of extracellular matrix (enzymes), signaling molecules, antimicrobials, combined therapies, and so on [83-89]. The alternative strategies of anti-biofilm effect have been recently proposed are detailed in Table 1. Although, the methods used to design the biofilm experiments can influence the results, these alternative strategies bring new insights over novel mechanisms of action that may contribute in the future to treat infections caused by *Candida* biofilm.

Table 1 New alternative antifungal strategies against *Candida* biofilms.

Alternative strategies	Experiment design	Anti- *Candida* biofilm effect
Photodynamic antimicrobial chemotherapy (PACT)	(I) Methylene blue photosensitizer (PS) and InGaAlP laser on *in vitro* multi-species biofilm [90] (II) Toluidine blue PS and red light-emitting diode (LED) on *in vitro C. albicans* biofilm [91] (III) Photogem® and blue led on disinfection of patients dentures [88] (IV) Erythrosine and green LED on experimental oral candidosis and adherence virulence factor [82]	(I) Reduction of over 1 log of *C. albicans*, *S. aureus*, and *S. mutans* cells and antimicrobial effect on the external layers [90] (II) Decrease of growth and biofilm formation caused by ROS and increase of cell permeability [81] (III) Elimination of over 90% of microorganisms, *Candida* spp., *S. aureus*, and streptococci [91] (IV) Reduction of yeast cells and *C. albicans* adherence to buccal epithelial cells without damaging adjacent tissues [82]
Herbal medicine	(I) Essential oil of *Melaleuca alternifolia* on *in vitro* and *in vivo* biofilms [83] (II) Purpurin (pigment extracted from madder root (*Rubia tinctorum* L.) [84]	(I) Eradication of *in vitro C. albicans* biofilm and reduction of yeast cells and lesions of experimental oral candidosis [83] (II) Suppress hyphae formation and affects biofilm structure [84]
Disruptor of extracellular matrix (Enzymes)	(I) DNase [85,86] (II) β-1,3 glucanase [51]	(I) Decreases biomass accumulation without affecting cell viability and have synergic effects of combined treatment with amphotericin B and fluconazole [85-86]. (II) Desegregates biofilm cells, reduces matrix biomass, and has synergic effects with fluconazole and amphotericin B [53,84]
Silver nanoparticle	Nanosilver- based inorganic antibacterial agents (NSBIAA) [92]	Reduction of metabolic activity and biomass by over 95% of *Candida albicans* biofilm [92]
Signaling molecule	Farnesol on *in vitro* and *in vivo* biofilm [88,89]	Prevents filamentation at the first step of biofilm formation and reduces metabolic activity of *in vitro* mature biofilm and supresses hyphae growth on the tongue of mice model [88,89]

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