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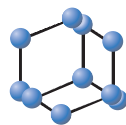
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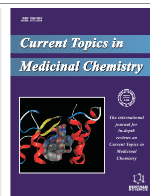

Molecules and Metabolites from Natural Products as Inhibitors of Biofilm in *Candida* spp. pathogens

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Abstract: ***Background*:** Biofilm is a critical virulence factor associated with the strains of *Candida* spp. pathogens as it confers significant resistance to the pathogen against antifungal drugs.

***Methods*:** A systematic review of the literature was undertaken by focusing on natural products, which have been reported to inhibit biofilms produced by *Candida* spp. The databases explored were from PubMed and Google Scholar. The abstracts and full text of the manuscripts from the literature were analyzed and included if found significant.

***Results*:** Medicinal plants from the order Lamiales, Apiales, Asterales, Myrtales, Sapindales, Acorales, Poales and Laurales were reported to inhibit the biofilms formed by *Candida* spp. From the microbiological sources, lactobacilli, *Streptomyces chrestomyceticus* and *Streptococcus thermophilus* B had shown the strong biofilm inhibition potential. Further, the diverse nature of the compounds from classes like terpenoids, phenylpropanoid, alkaloids, flavonoids, polyphenol, naphthoquinone and saponin was found to be significant in inhibiting the biofilm of *Candida* spp.

***Conclusion*:** Natural products from both plant and microbial origins have proven themselves as a goldmine for isolating the potential biofilm inhibitors with a specific or multi-locus mechanism of action. Structural and functional characterization of the bioactive molecules from active extracts should be the next line of approach along with the thorough exploration of the mechanism of action for the already identified bioactive molecules.

Keywords: Biofilm inhibitors, *Candida*-biofilm, Virulence factor, Anti-*Candida* metabolites, Natural products, Antifungal drugs.

1. INTRODUCTION

Candida species are the opportunistic human pathogen, who resides as a part of human microbiota in the innocuous stage. It becomes virulent generally when the patient gets exposed to broad-spectrum antibiotics for a longer duration, chemotherapies as well as dominantly in the immune-compromised patients [1-5]. *C. albicans* is responsible for 15-20% of the vaginitis cases, which is a very common health issue in women [6, 7]. It has also been recorded that out of all *Candida* spp., *C. albicans* accounted for more than 42% fungal infections worldwide [8-10]. Biofilm forming capability in the *Candida* spp. contributes significantly towards resistance against currently available antifungal drugs, making it difficult to eradicate the infection [11]. In the case of implants like pacemakers, stents, intravascular catheters, etc, *Candida* spp. biofilm was considered as one of the important causes of clinical repercussions and they serve as inseminating reservoirs for further yeast infections [12, 13]. Literature reported that the biofilm of *C. albicans* contained 55% of proteins and their glycosylated counterparts, 25% of carbohydrates, 15% of lipids, and 5% of noncoding DNA [14, 15]. Among the carbohydrates, the mannan-glucan complex is composed of α-1,2-branched α-1,6-mannans and unbranched α-1,6-glucans [14, 15].

Becherelli and co-workers had studied that the correlation in expression of heat shock proteins, hsp90, hsp70 and hsp104, in the *C. albicans* ATCC SC5314 and ATCC 24433 with their biofilm-forming capability. Their results indicated that heat shock proteins are required in the early phase of biofilm development [16]. Six master transcriptional regulators are required for normal biofilm development, which are Efg1, Tec1, Bcr1, Ndt80, Brg1 and Rob1 [17].

Cateau and co-workers had shown very interesting results like the supernatant, recovered from the 24 hours old *C. albicans* ATCC 3153 biofilm, has the capability to inhibit the new biofilm growth in the same strain or other strains of *C. albicans* with no effect on the preformed biofilm. This antagonistic molecule was found to be hydrophilic in nature

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with a mass of less than <3000 Da [18]. Bruzual and co-workers had shown that fluconazole could inhibit the growth of biofilm in the fluconazole-resistant *C. albicans* [19].

Caspofungin is an azo derivative of pneumocancidin B0, which was actually a fermented product of the fungus, *Glarea lozoyensis* [20, 21]. Bachmann and co-workers [22] reported 97% reduction in the metabolic activity of sessile cells of the biofilm of *C. albicans* 3153A when treated with 0.125 µg/ml caspofungin (marketed by Merck & Co. as Cancidas), a first of its class of glucan synthase inhibitor in 2001 [23]. Though complete sterilization of biofilm had not been observed.

Natural products from plant and microbial sources are often regarded as gold-mine for the exploration of molecules for therapeutic applications [24-26]. In the present review article, we have focused on the natural products (extracts as well as pure compounds), which have the potential to inhibit the biofilm formed by pathogenic *Candida* spp.

2. METHODOLOGY

Using the keywords 'Natural Products', '*Candida*' and 'Biofilm Inhibitors', we have made an initial search on Google Scholar as well as on PubMed. It was further refined, as per the data explored. As a total, around 130 articles had been downloaded. Out of these 130 articles, 75 relevant articles have been cited in the present manuscript.

2.1. Anti-Biofilm Metabolites from Natural Sources

2.1.1. Plant-based Metabolite Extracts

2.1.1.1. Acorus calamus L.

Subha and Gnanamani (2009) studied the effect of active fractions AF1 and AF2, which were obtained from the methanolic extract of rhizomes of *Acorus calamus* L. (Plant Source Location: South India) on the biofilm of *C. albicans* CLCA 0590 (clinical strain from SRMC, Chennai, India) and *C. tropicalis* CLCT 0610 (clinical strain from General Hospital, Chennai, India). Ketoconazole and amphoteracin B were taken as standard drugs. Results indicated that at a concentration of 2 mg/ml, both AF1 and AF2 were able to perfuse through biofilm layers of *Candida* spp. leading to cell death [27]. However, the biofilm inhibitory concentration of the extract in the present case was seen much higher in comparison to other extracts.

2.1.1.2. Peganum harmala

Aboualigalehdari and co-workers studied the effect of extract of *Peganum harmala* against the biofilm of 27 clinical isolates of *C. albicans*. These clinical isolates were collected from the vulvovaginal *candida*cies in women from the west part of Iran. Results indicated that the extract of *P. harmala* was capable of inhibiting the biofilm formation in the strong biofilm-forming *C. albicans* at a concentration of 12 µg/ml, while for the moderate and weak biofilm-forming *C. albicans*, effective concentrations were 10 and 6 µg/ml [6]. In this study, we were not able to identify which extract was used by the authors for their experiments as well as their geographic location.

2.1.1.3. Solidago virgaurea

Chevalier and co-workers [28] collected the aerial parts of two taxa of *Solidago virgaurea* during the flowering period. Two taxa were *S. virgaurea* L. subsp. *virgaurea* (Plant Source Location: Tineevalley, Col Saint Martin, la Colmiane, France) and *S. virgaurea* subsp. *alpestris* (Waldst. & Kit.) Gremli (Plant Source Location: Tinee valley, Piste Roubine, Isola 2000, France). Saponin content in aqueous extract of both subspecies *i.e. S. virgaurea* L. subsp. *virgaurea* and *S. virgaurea* subsp. *alpestris* was found to be 0.7 mg/ml and 0.95 mg/ml, respectively. Both the aqueous extracts did not express anti-*candida* activity against the four strains of *C. albicans*, namely ATCC 10231, IM001, IM003 and IM007, but showed the potential to inhibit the yeast to hyphae transition as well as lead to shorter forms of germ-tubes. After treatment with the 250 µg/ml aqueous extract of *S. virgaurea* L. subsp. *virgaurea*, a highly significant reduction in biofilm formation was noticed, for example, 98.41% in *C. albicans* ATCC 10231, 99.18% in *C. albicans* IM001, 97.32% in *C. albicans* IM003 and 96.49% in *C. albicans* IM007. After treatment with the 250 µg/ml aqueous extract of *S. virgaurea* subsp. *alpestris*, the reduction of biofilm formation was found to be 95.86% in *C. albicans* ATCC 10231, 96.00% in *C. albicans* IM001, 99.46% in *C. albicans* IM003 and 95.14% in *C. albicans* IM007. Both the extracts had strongly inhibited the pre-formed *Candida*-biofilms (18 hours old). Saponin content does not seem to have a dose-dependent correlation with the anti-biofilm activity [28].

2.1.1.4. Thymbra capitata

Palmeira-de-Oliveira *et al.* [29] prepared the essential oil from the aerial parts of *Thymbra capitata* (flowering stage; Plant Source Location: Algarve, South of Portugal) using hydrodistillation technique. The essential oil was characterized by its chemical nature using the GC-MS technique. Antifungal property of this oil was assessed in planktonic cells as well as in 24-hr old biofilms of 15 *Candida* strains: *C. albicans* (*C. albicans* ATCC 10231 and *C. albicans* ATCC 90028 as well as 3 clinical strains); *C. glabrata* (4 clinical strains); *C. tropicalis* (2 clinical strains); *C. parapsilosis* (3 clinical strains) and 1 clinical strain of *C. guilliermondii*. Fluconazole and amphotericin B were taken as reference compounds. Clinical isolates were collected from patients having mucocutaneous infections and all strains expressed a variable degree of resistance towards fluconazole and amphotericin B when tested *in vitro*. GC-MS analysis indicated that the probable compounds which could present this essential oil were: carvacrol (75%), γ-terpinene (5.1%), p-cymene (5%), linalool (2%) and myrecene (1.9%). Against all the tested *Candida* spp, the MIC and MFC value are the same for this essential oil and it is 0.32 µg/ml. At the concentration of 0.64 µg/ml (MIC x 2) of essential oil, the percent reduction in biofilm biomass in *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. parapsilosis* and *C. guillermondii* was 71.96, 39.43, 67.82, 64.50 and 78.50, respectively, while the percent reduction in biofilm metabolism in these strains was 62.71, 85.68, 97.47, 96.05 and 98.48, respectively. These results showed strong biofilm inhibitory potential of essential oil preparation from *T. capitata* against albicans and non-albicans strains of *Candida* spp. [29].

2.1.1.5. Coriandrum sativum

Freires and co-workers [8] had prepared the essential oil from the leaves of *Coriandrum sativum* (Plant Source Location: Germoplsam Bank, University of Campinas, Brazil) by using the hydrodistillation technique and evaluated for its antifungal activity against the *Candida* spp. The essential oil obtained in this case was actually obtained from the aqueous phase which was treated with dichloromethane to separate essential oil in the organic phase. Authors had also taken a previously studied fraction *i.e.* F8-F10 in this work. GC-MS analysis indicated that the probable compounds which may be majorly present in this essential oil were decanal (19.09%), trans-2-decenal (17.54%), 2-decen-1-ol (12.33%), cyclodecane (12.15%), dodecanal (4.10%), cis-2-dodecenal (10.72%) and dodecan-1-ol (3.13%). The probable compounds in the active fractions (F8-F10) were suggested to be decanal (4.91%), trans-2-decenal (24.11%), 2-decen-1-ol (16.57%), cyclodecane (16.62%) and cis-2-dodecenal (15.81%). The MIC/MFC value (µg/ml) of the essential oil against *C. albicans* CBS 562, *C. tropicalis* CBS 94, *C. krusei* CBS 573, *C. dubliniensis* CBS 7987 and *C. rugosa* CBS 12 was 15.6/31.2, 31.2/62.5, 15.6/31.2, 31.2/62.5 and 15.6/31.2, respectively while that of F8-F10 against these strains were 250/1000, 250/500, 125/250, 31.2/125 and 62.5/125, respectively. Nystatin and amphotericin B were taken as reference compounds. At 62.5 µg/ml of essential oil, the % of inhibition in biofilm adherence in case of *C. albicans* CBS 562, *C. tropicalis* CBS 94, *C. krusei* CBS 573, *C. dubliniensis* CBS 7987 and *C. rugosa* CBS 12 was 53.43, 85.80, 42.13, 61.51 and 68.03, respectively while that of nystatin, at same concentration against these strains were 41.92, 86.16, 46.06, 71.81 and 73.71, respectively.

It had been observed that there is a strong synergism among the anti-*Candida* compounds present in the essential oil since the antifungal activity decreased upon fractionation. Other mechanistic assays indicated that the essential oil may act by binding with membrane ergosterol, by increasing ionic permeability and causing the membrane damage which could lead to cell death [8].

2.1.1.6. Cinnamomum zeylanicum

Pires and co-workers [30] had assessed essential oil preparation from *Cinnamomum zeylanicum* (Procurement Source Location: The local Body and Mind Beautiful Com. De Cosmeticos Ltda., Franca, SP, Brazil) for the antifungal activity against *C. parapsilosis* ATCC 90018, *C. orthopsilosis* ATCC 96141, *C. metapsilosis* ATCC 96143, *C. krusei* ATCC 6258, *C. parapsilosis* ATCC22019 and *C. albicans* biofilm reference strain SC 5314. Results indicated that *C. zeylanicum* expressed MIC value of 250 and 500 µg/ml against *C. orthopsilosis* ATCC 96141 and *C. parapsilosis* ATCC 22019 respectively, while the MFC against these strains were 500 and 1,000 µg/ml. Further studies to assess the effect of cinnamon oil on biofilm inhibition of these two test strains suggested that minimum biofilm reduction concentration (MBRC) was at 1,000 and 2,000 µg/ml. In addition, when used in combination with amphotericin B, no synergistic affect was noticed [30].

2.1.1.7. Essential Oils of Cymbopogan citrate and Syzygium aromaticum

Khan and Ahmad [31] had studied the inhibitory effect of essential oil of *Cymbopogan citratus* (Procurement Source location: Aroma Sales Corporation, New Delhi, India) and *Syzygium aromaticum* (Procurement Source location: Dabur India Limited, New Delhi, India) on the biofilms of 23 drug-resistant *Candida* strains of clinical origin (*C. albicans*, CA01-18; *C. tropicalis*, CT01, 02; C. *glabrata*, CG01, 02; *C. krusei*, CK01) and 4 reference strains (*C. albicans* NRRLY12983, *C. albicans* MTCC183, *C. glabrata* MTCC3019, *C. albicans* SC5314). Susceptibility analysis of planktonic (PMIC: MIC in case of planktonic cells) and sessile (SMIC: MIC in case of sessile cells) cells in *C. albicans* 04 indicated that PMIC/SMIC (µg/ml) for *C. citratus* was 360/360; for *S. aromaticum* 200/400; for amphotericin B 1/16 and for fluconazole 256/>1024 while in case of *C. albicans* SC5314, PMIC/SMIC (µg/ml) for *C. citratus* was 180/180; for *S. aromaticum* 100/200; for amphotericin B 0.12/64 and for fluconazole 0.25/512. At 0.5 x SMIC, *C. citratus* was capable to inhibit the biofilm formation of approximately 88% in *C. albicans* 04 and 82% in *C. albicans* SC5314 whereas, at the same concentration, *S. aromaticum* inhibited 52% and 57% biofilm in above-mentioned test strains, respectively [31].

2.1.2. Microbe-based Metabolite Extracts

2.1.2.1. Streptococcus thermophilus B

Streptococcus thermophilus B was isolated from the heat exchanger plates present in the downward section of a pasteurizer in the Netherlands by Dr. A.H. Weerkamp [32], who provided this isolate to Dr. Busscher for further studies. Busscher and co-workers examined *S. thermophilus* B and its biosurfactant for their potential to inhibit the adhesion of two strains of *C. albicans* and two strains of *C. tropicalis* on silicone rubber. It was reported that 1 to 4% of the coating of *S. thermophilus* B on the surface of silicone rubber substrate greatly reduced the adhesion of these yeasts. The biosurfactants released from this strain was a mixture, which included the glycolipid like component, as the most surface-active agents. Pre-adsorption of the silicone rubber with this biosurfactant was quite effective to reduce the adhesion of *C. albicans*, but not effective against *C. tropicalis* [33].

2.1.2.2. Lactobacillus rhamnosus, L. casei and L. acidophilus

Matsubara and co-workers had studied the effect of metabolites produced by *Lactobacillus rhamnosus* LR32*, L. casei* L324m and *L. acidophilus* NCFM in their planktonic stage and supernatant form on the biofilm formation and yeast-hyphae transition in *C. albicans* ATCC SC5314 and *C. albicans* 75 (clinical isolate). It was observed that all the tested *Lactobacillus* spp. were capable of inhibiting the early stage and mature biofilms of *C. albicans* SC5314 even at the lowest concentration of tested cells *i.e.* 1.25 x 10^6 and 6.25 x 10^5 cells, but the effect is not dose-dependent. In the case of *C. albicans* 75, the affect of the *Lactobacillus* spp. was dose-dependent and only at high density *i.e.* 1x 10^7 cells, reduction in biofilm was observed. Biofilm viable cell reduction

assay (initial colonization/maturation) suggested that at 1 x 10^7 cells, *L. rhamnosus* LR32 resulted in 32.0 %/61.8% and 31.8%/39.8% reduction in the biofilm viable cell in *C. albicans* ATCC SC5314 and 75 respectively; *L. casei* L324m resulted in 59.0 %/54.7% and 27.7%/36.6% reduction in the biofilm viable cell in *C. albicans* ATCC SC5314 and 75 respectively; *L. acidophilus* NCFM resulted in 56.3 %/34.2% and 27.4%/25.5% reduction in the biofilm viable cell in *C. albicans* ATCC SC5314 and 75 respectively. Planktonic cells were more effective than supernatant. Further studies revealed that all lactobacilli negatively impacted the yeast to hyphae transition in the *Candida* strains [34].

2.1.2.3. *Streptomyces chrestomyceticus*

Srivastava and Dubey [35] had characterized the anti-*candida* property of *Streptomyces chrestomyceticus* strain ADP4 (Microbe Source Location: Soil of the desert region of Jhunjhunu, India). Various *Candida* spp. used in the study were *C. albicans* ATCC 10231, *C. albicans* ATCC 90028, *C. albicans* ATCC 36082, *C. albicans* ATCC Y0119, *C. albicans* ATCC 1239, *C. krusei* ATCC 6258, *C. krusei* ATCC 766.1, *C. tropicalis* ATCC 750 and *C. parapsilosis* ATCC 22019. Results indicated that the MIC_{90} value (µg/ml) for the ADP4 strain was in the range of 3.70 to 51.70 when tested against these test strains with maximum susceptibility of *C. albicans* ATCC 36082. Further, biofilm studies suggested that if the test strains were preincubated with the secondary metabolite extract, then 90% biofilm reduction had been observed at 4-8 µg/ml. However, for a 90% reduction of biofilm in the case of the preformed biofilm of 2 hours, the concentration of ADP4 metabolite increased to 8-16 µg/ml. SEM studies revealed the membrane disruption and leakage of cellular materials as the treatment effect of ADP4 metabolites on *Candida* cells [35]. Srivastava and co-workers [36] had further prepared the ME_{SDB} extract from the ADP4 strain and its fractionation by silica gel chromatography. ME_{SDB} extract and its fractions (PPM 1 to 8) were assessed for their anti-*candida* activity and biofilm inhibition potential against the same test strains of *Candida*. Results indicated that for ADP4's ME_{SDB} extract, MIC_{90} (µg/ml) against these test strains were found to be in the range of 31.25 to 125 while MFC(µg/ml) was $2xMIC_{90}$. It was reduced when the fractionation was done which indicated a synergistic effect in the ME_{SDB} extract. Further, 90% inhibition of biofilm of *C. albicans* (ATCC 10231, ATCC90028, 1239 clinical isolate, ATCC 36082 and Y0119) was observed upon treatment with15.62, 62.5, 250 and 250 µ g/ml of ME_{SDB} extract, PPM3, PPM1 and PPM5, respectively. GC-MS and docking studies predicted that some of the probable compounds, diketopiperazine alkaloids and fatty acid esters have an affinity towards the aspartic acid residue of Sap3 enzyme in *C. albicans* [36].

2.1.3. Other Natural Products

2.1.3.1. Jujube Honey

Ansari and co-workers [37] had studied the effect of natural jujube honey (Source: Beekeeper's Association of Al-Baha, Saudi Arabia) on the growth and biofilm formation of *C. albicans* ATCC 10231. MIC value for jujube honey against this biofilm-forming *C. albicans* was 40% w/v while the MFC value was 50% w/v. Different concentrations of honey *i.e.* 5, 10, 20, 40 and 80% were tested to study its inhibitory effect on biofilm formation and results indicated that at 40% (w/v) and 80% (w/v), there was no significant biofilm biomass. The minimal effective dose to affect the biofilm formation was 10% (w/v). In the case of 24 hours matured biofilm, a significant decrease in biofilm biomass was observed at the jujube honey concentration of 40% (w/v) following a treatment period of 24 hours. Scanning electron microscopy (SEM) and atomic force microscopy suggested that jujube honey affected the cellular morphology of *Candida* cells as well as decrease the thickness of biofilms [37]. Literature suggested that H_2O_2, flavonoids, methylglyoxal, etc. may be responsible for the antimicrobial activity of honey [37-40].

2.2. Anti-Biofilm Molecules of Natural Origin

The structure of various anti-fungal molecules, reported for their anti-biofilm properties has been illustrated in Fig. (**1**).

2.2.1. Carvacrol, Eugenol, Thymol and Geraniol

Carvacrol, a phenolic monoterpene, can be isolated from various plants like *Origanum dictamnus* L. [41], *O. vulgare* [42] and *Thymus vulgaris* L. [43]. Eugenol, a phenylpropanoid, can be isolated from the plant like *Syzygium aromaticum* [44], *Eugenia caryophyllata* [45], and *Ocimum gratissimum* L. [46]. Thymol, a phenolic monoterpenoid, can be isolated from various natural sources like *T. vulgaris* L. [43], *O. dictamnus* L. [41] and *Arnica sachalinensis* [47]. Geraniol, acyclic alcohol monoterpene, can be isolated from various sources like *Rosa damascene* [48], *Cymbopogon winterianus* [49], *etc.*

Dalleau *et al.* [50] had studied the effect of carvacrol, geraniol and thymol at a varying concentration (0.030%, 0.060% and 0.125%) on the inhibition of biofilm development in *C. albicans* ATCC 66396, *C. glabrata* IHEM 9556 and *C. parapsilosis* ATCC 22019. The results are very interesting. Carvacrol, geraniol and thymol at 0.03% were capable to inhibit 24 hours of matured biofilm in *C. albicans* ATCC 66396 by 88%, 84% and 87% respectively. Carvacrol (0.06%), geraniol (0.06%) and thymol (0.03%) were capable to inhibit 24 hours matured biofilm in *C. glabrata* IHEM 9556 by 94%, 94% and 92%, respectively. Carvacrol (0.06%), geraniol (0.06%) and thymol (0.03%) were capable to inhibit 24 hours matured biofilm in *C. parapsilosis* ATCC 22019 by 85%, 87% and 83%, respectively [50]. The effect of eugenol on preformed biofilms, adherent cells and subsequent biofilm formation in *C. albicans* ATCC 10231 had also been investigated. The results indicated that eugenol has an MIC_{50} value of 500 µg/ml for the sessile *Candida* cells in the biofilm. Inhibition by eugenol was dependent upon the adherence time as well as on concentration of the eugenol. SEM studies revealed that eugenol could inhibit filamentous growth in yeast cells [51].

Further, Doke and co-workers had studied the synergistic effect of carvacrol, eugenol and thymol while tested in a combination of fluconazole against *C. albicans* at the planktonic, biofilm development stage and matured biofilm stage. It was reported that carvacrol and eugenol, at sub-inhibitory concentrations, were capable of sensitizing the cells towards

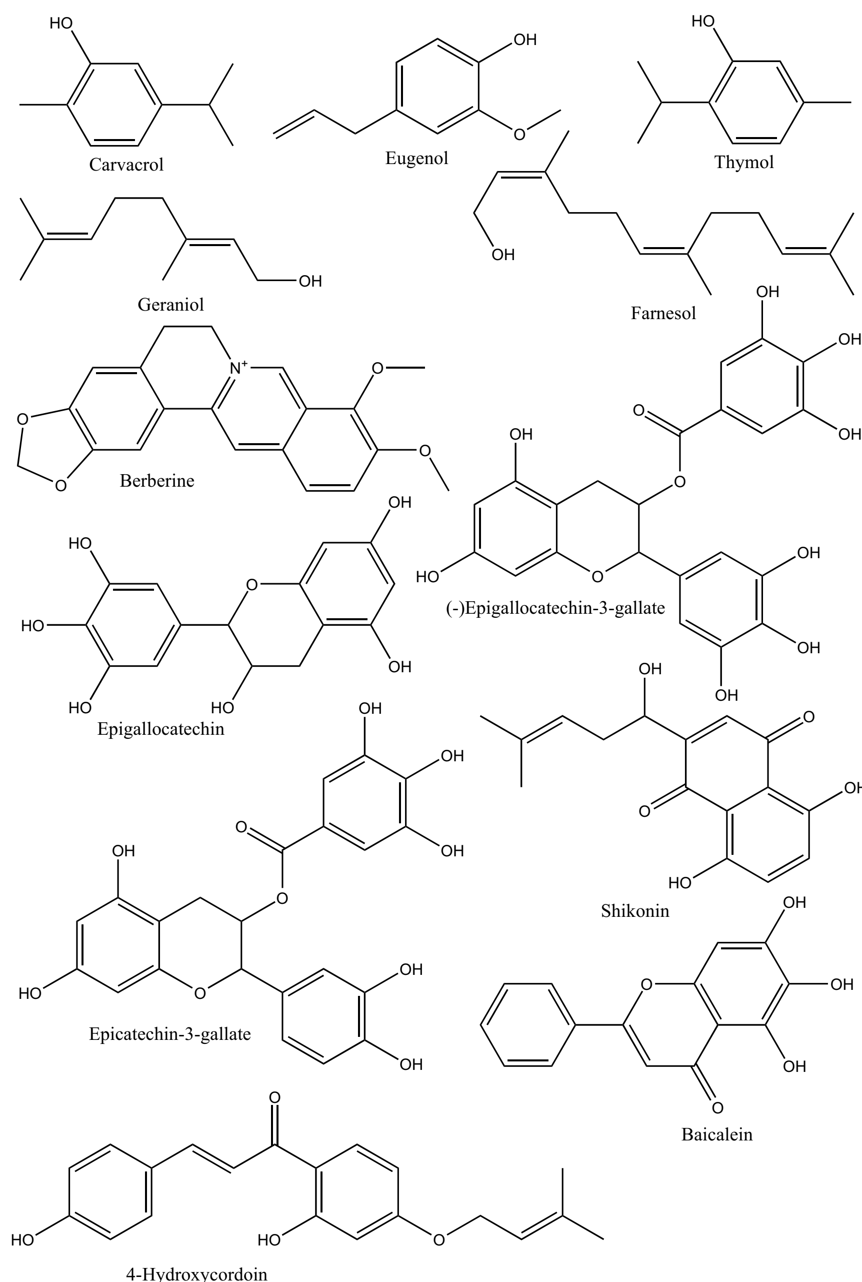

Fig. (1). Structure of natural products reported for their potential to inhibit biofilm in *Candida* spp.

fluconazole which led to the prevention of biofilm formation. Thymol had not expressed any synergistic potential with fluconazole at any stage of *C. albicans*. Only carvacrol at 0.5 mg/ml was capable of inhibiting the mature biofilms at a potentially low concentration *i.e.* 0.032 mg/ml of fluconazole [52].

2.2.2. Farnesol

Farnesol, sesquiterpene alcohol, can be isolated from many plants like *Chamaedora tepejilote* [53], *Phytophthora cactorum* [54], *Achillea millefolium* L. ssp. Millefolium [55], *etc.* Ramage and co-workers had studied the farnesol, a quorum-sensing molecule, for its potential to inhibit the biofilm formation in *C. albicans*. It was reported that pre-incubation with 300 μ M farnesol completely inhibited biofilm formation. Further RNA analysis indicated that in the farnesol treated biofilms, the expression level of the HWP1 gene decreased which encodes for the hyphae-specific wall protein [56]. Cao and co-workers had extended this study by performing cDNA microarray analysis. Results indicated that after the *Candida* biofilm exposed to farnesol, around 104 genes were unregulated and 170 genes were down-regulated. Specifically, the cell surface hydrophobicity associated genes were down-regulated, which was further confirmed by the water-hydrocarbon two-phase assay that indicated the decrease in the cell surface hydrophobicity in the farnesol-treated group when compared with the control group [57].

2.2.3. Berberine

Berberine is an isoquinoline type of alkaloid, which can be isolated from the roots, rhizomes and stem barks of various medicinal plants like *Berberis aquifolium*, *B. vulgaris*, *B.*

aristata, *Hydrastis canadensis*, and *Phellodendron amurense* [58]. da Silva and co-workers had studied the berberine for its potential antifungal activity against clinical (blood source; LABEL/FF/UFC) and ATCC strains of *Candida* spp. (*C. tropicalis* 1, *C. tropicalis* 2, *C. albicans* 1, *C. albicans* 2, *C. albicans* 3, *C. parapsilosis* 1, *C. parapsilosis* 2, *C. krusei* ATCC 6258, *C. parapsilosis* ATCC 22019) as well as against the biofilm formation. Results indicated that the berberine had a MIC value of 8 µg/ml against these fluconazole-resistant *Candida* strains with an exception of *C. krusei* ATCC 6258, *C. parapsilosis* ATCC 22019 where the MIC were 4 and 16 µ g/ml, respectively. Further, the cytometric analysis indicated that treatment with berberine leads to the alterations to the integrity of the plasma and mitochondrial membranes as well as DNA damage, which cohesively lead to cell death, probably by apoptosis. At a concentration of 37.5 µg/ml of berberine, a 50% reduction in the metabolic activity of growing and mature biofilms of *C. tropicalis* had been observed while in the case of fluconazole, same observations were recorded at 512 µg/ml [58].

2.2.4. Polyphenon 60 and Tea Polyphenols

Evensen and Braun had studied polyphenon 60 (a green tea extract which contains mixture of polyphenolic compounds) as well as three catechins (Fig. **1**), (-)epigallocatechin-3-gallate (EGCG), epigallocatechin (EGC) and epicatechin-3-gallate (ECG) for their efficacy to inhibit the growth of *C. albicans* 4918 and *C. albicans* ATCC 10231. At 2 mg/ml concentration of polyphenon 60, 40% reduction in the growth rate constant, K was observed. When tested at 2.5 µM, EGCG was found to be more potent than EGC and ECG in inhibiting the biofilm formation up to around 75%. Preformed biofilms of 48 hours were also treated with EGCG, EGC and ECG at concentrations between 1.0 to 3.0 µM. There was around 60% disruption in *Candida* biofilms. It was also determined by these studies that EGCG, EGC and ECG inhibited *C. albicans* proteasomal chymotrypsin-like and peptidyl-glutamyl peptide hydrolyzing (PGPH) activities *in vivo* [59]. EGCG, ECG and EGC can be isolated from green tea, *Camellia sinensis* [60].

2.2.5. 4-Hydroxycordoin

4-Hydroxycordoin, an isopentenyloxychalcone, can be isolated from a natural source like *Ligularia nelumbifolia* [61]. Messier and co-workers had studied 4-hydroxycordoin for its effect on the growth, biofilm formation and yeast to hyphae transition in two the strains of *C. albicans,* LAM-1 and ATCC 28366. It was found that 4-hydroxycordoin at 20 µg/ml was capable enough to reduce 96.7% of biofilm formation in the case of LAM-1 while it was 87.5% in the case of ATCC 28366. Even at a concentration of 2 µg/ml, there was a reduction of 30% biofilm formation. At a concentration of 50 and 100 µg/ml, 4-hydroxycordoin had inhibited 85% and 100 % of the yeast to hyphae transitions respectively in both the strains [62].

2.2.6. Polybia-MPI

A cationic peptide, Polybia-MPI can be isolated from the venom of social wasp *Polybia paulista*. Wang and co-workers had synthesized this Polybia-MPI by adopting a stepwise solid-phase method using N-9-fluorenylmethoxycarbonyl (F-MOC) chemistry [63]. Souza and co-workers had initially characterized this polybia-MPI and the sequence was found as IDWKKLLDAAKQILa where 'a' represented an amide [64]. It was observed that the MIC (µM) values for polybia-MPI against *C. glabrata* ATCC 2001 and *C. albicans* ATCC 14053 were 8 and 16 respectively, whereas the MFC (µM) against these strains were 32 for both [63]. Further studies suggested the involvement of membrane disruption mechanisms in the anti-*Candida* activity. Biofilm inhibition studies indicated that polybia-MPI inhibited the biofilm formation in *C. glabrata* ATCC 2001 in a dose-dependent manner, with effective concentration ranging from 2 x MIC to 8 x MIC [63].

2.2.7. Shikonin

Shikonin, a naphthoquinone can be isolated from a natural source like *Lithospermum erythrorhizon* [65]. Yan and co-workers had studied the effect of shikonin (Procurement Source Location: National Institutes for Food and Drug Control, Peking, China) against the biofilms of *C. albicans* SC5314 and clinical isolates (Source for clinical isolates was Changhai Hospital of Shanghai, China). Results indicated that shikonin at 4 µg/ml could inhibit more than 60% of the biofilms with approximately 50% inhibition of mature biofilms at the same concentration. Further, the results from *in vivo* mouse vulvovaginal candidiasis model reflected that shikonin treatment significantly reduced the fungal burden. Real-time reverse transcription PCR analysis indicated that shikonin treatment resulted in the downregulation of genes that included ECE1, HWP1, EFG1, CPH1, RAS1, ALS1, ALS3 and CSH1; and upregulation of the genes: TUP1, NRG1 and BCR1 [66].

2.2.8. Baicalein

Baicalein, 5,6,7-trihydroxyflavone can be isolated from various plant sources like *Scutellaria baicalensis* [67, 68], *S. lateriflora* [69], *S. violacea* [70], *Oroxylum indicum* [71], *Thymus vulgaris* [72], *etc.* Cao and co-workers had studied the effect of baicalein (Procurement Source Location: Sigma–Aldrich, St Louis, MO) against the biofilm formation as well as on the mature biofilm of *C. albicans* SC5314. Results indicated that baicalein inhibited biofilm in a dose-dependent manner with more than 70% inhibition at 4 µg/ml. In case, when baicalein was added at 0 hour and 24 hour of incubation, 89% and 52% inhibition in the biofilm was observed. Results from real-time reverse transcriptase-polymerase chain reaction indicated that baicalein treatment leads to the lower expression of *CSH1* mRNA [73].

2.2.9. AginosideA16 and BarrigenolA19

Coleman and co-workers [74] had studied the effect of Aginoside A16 (saponin) and barrigenol A19 (saponin) on the biofilm inhibition in the *C. albicans* DAY185 and compared the findings with the standard drug, caspofungin. Results indicated that aginoside A16 (10 µg/ml), barrigenol A19 (20 µg/ml) and caspofungin (2 µg/ml) led to significant decrease in the biofilm biomass with restriction to < 1 mg, ~ 1mg and < 2mg respectively [74].

2.2.10. Solasodine-3-O-β-D-Glucopyranoside

Li and co-workers had isolated a steroidal alkaloid glycoside, solasodine-3-O-β-D-glucopyranoside from *Solanum nigrum* L. and assessed its effect to inhibit the biofilm for-

mation as well as on the mature biofilms of *C. albicans* (Wild type strain YEM30). At 16 µg/ml, solasodine-3-O-β-D-glucopyranoside could restrict the biofilm growth to 25-30% and it could be reversed by the addition of db-cAMP. This suggested that solasodine-3-O-β-D-glucopyranoside could act by retarding the synthesis of cAMP [75].

On the basis of literature covered in this study for the articles published on the natural products as inhibitors of biofilm in *Candida* spp., it had been observed that significant research work on the subject was pursued primarily by researchers from China, USA, India, Brazil and France. Scientists from other countries like Portugal, Netherlands, Australia, Saudi Arabia, Canada and Iran were also contributing to the knowledge in this field (Fig. **2**).

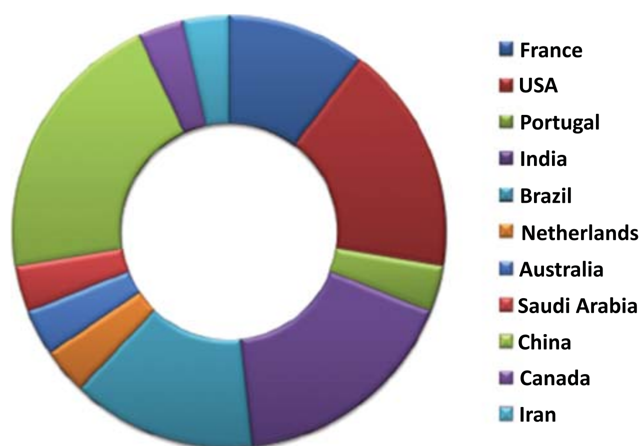

Fig. (2). Representation of the geographical distribution among the people working on natural inhibitors for the biofilm in *Candida* spp.

The taxonomic classification chain for all the medicinal plants covered in this manuscript, which demonstrated the potential to inhibit the biofilm formed by *Candida* spp. has been presented in Fig. (**3**). It was found that all these medicinal plants belong to clade Angiosperms, followed by sub-clade Eudicots, Monocots and Mangoliids. From eudicots, sub-clades that covered this therapeutic target zone were asterids and rosids. Out of all, eudicots are a very important clade in Kingdom plantae, followed by its sub-clade asterids and rosids, which covered most of the reported medicinal plants for this specific therapeutic target (Fig. **3**).

The phytochemical classification tree of the reported natural products (Fig. **4**) showed that there is a diverse class of compounds which were reported as biofilm inhibitors. These include terpenoids, phenylpropanoid, alkaloids, flavonoids, polyphenol, naphthoquinone and saponins. Out of all of these, terpenoids, alkaloids, flavonoids and saponins covered the major section of reported compounds and it could be assumed that different classes of biofilm inhibitors might elicit different mechanisms for their inhibitory action.

3. MODE OF ACTION OF ANTI-BIOFILM MOLECULES

Some of the scientific reports as cited above had mentioned the possible mechanisms for the inhibition of *Candida*-biofilm by the natural products and their molecules (Fig. **5**). For instance, Freires and co-workers [8] had suggested that essential oil of *C. sativum* acts *via* binding with membrane ergosterol, which leads to an increase in its ionic permeability. Chevalier *et al.* suggested that the aqueous extract of *S. virgaurea* acts by inhibiting the yeast-hyphal transition as well as lead to shorter forms of germ-tubes [28]. Matsubara *et al.* and He *et al.* also suggested the same mechanism for the Lactobacilli and eugenol, respectively

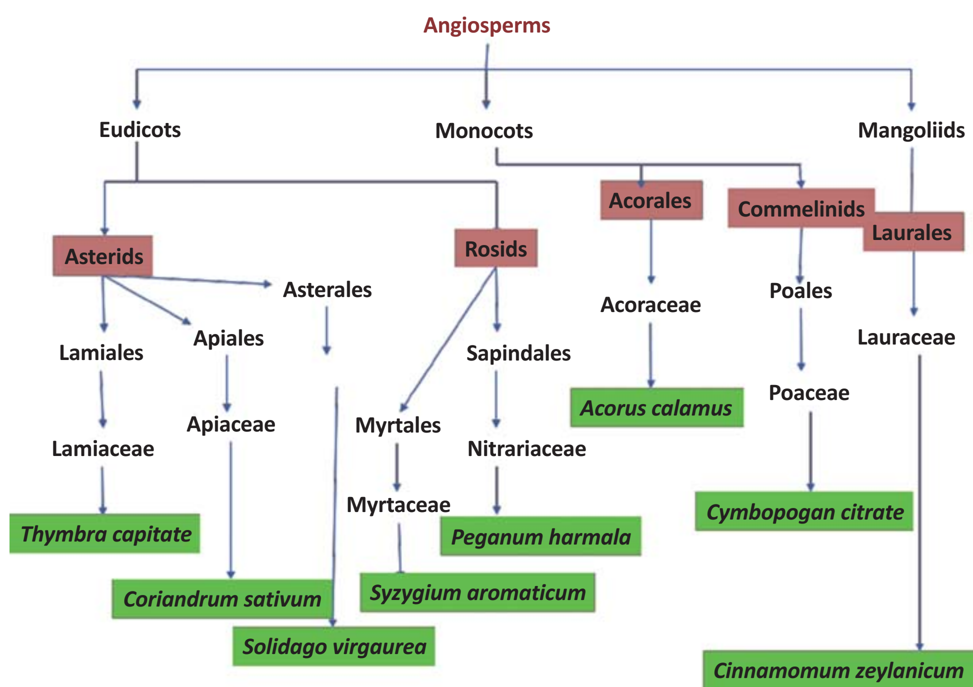

Fig. (3). Taxonomic tree of the medicinal plants, reported to produce inhibitors of *Candida* biofilm.

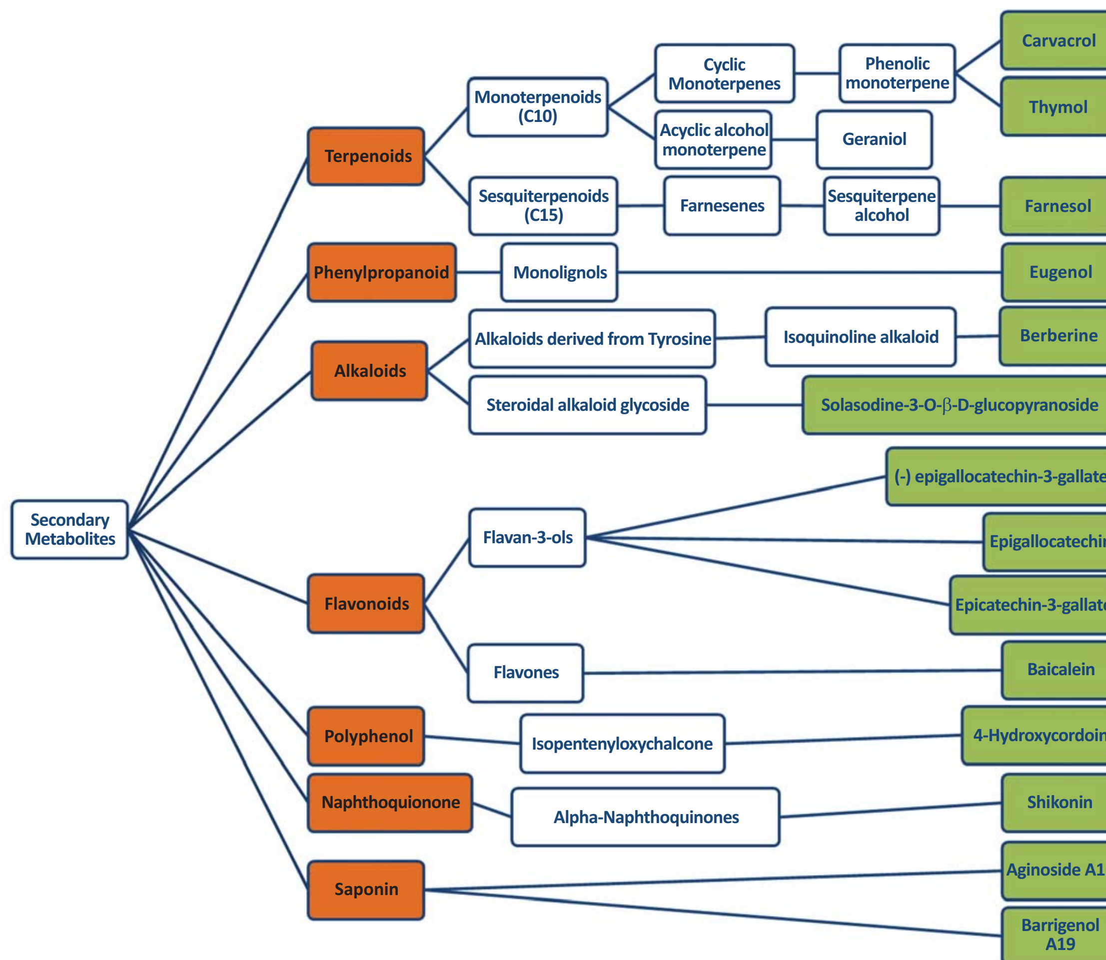

Fig. (4). Phytochemical classification of the natural products reported for their potential to inhibit the *Candida*-biofilm.

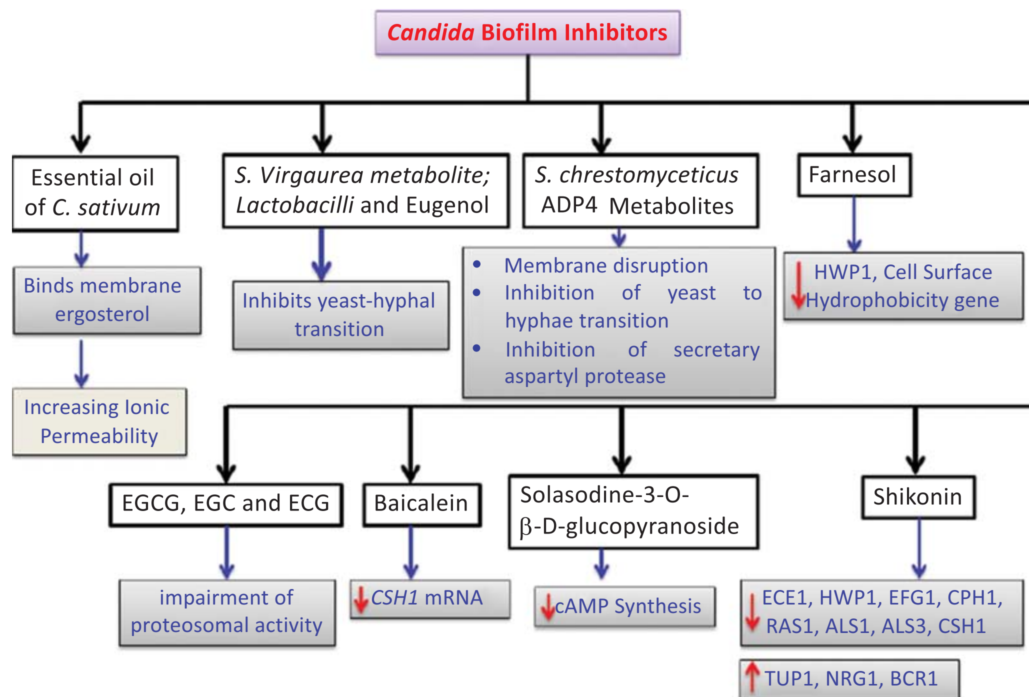

Fig. (5). Mode of action of various *Candida*-biofilm inhibitors from natural sources. The arrows indicate up (↑) - and down (↓) - regulation of gene expression.

[34, 51]. Srivastav and Dubey had reported membrane disruption of C. albicans by the metabolites of *S. chrestomyceticus* ADP4 [35]. Srivastava and co-workers had suggested that the probable molecules, diketopiperazine alkaloids and fatty acid esters in *S. chrestomyceticus* ADP4 possessed affinity towards the aspartic acid residue of Sap3 enzyme in *C. albicans* when checked through docking studies and also inhibition of yeast to hyphae transition was noted [36]. Cao and co-workers had done the molecular level mechanistic analysis in farnesol and suggested that farnesol decreased the expression level of the HWP1 gene which was responsible for the synthesis of hyphae specific wall protein as well as downregulated the cell surface hydrophobicity associated gene [57]. Evensen and Braun suggested that impairment of proteosomal activity, which was caused by EGCG, EGC and ECG, to be responsible for the mechanism [59]. Yan and co-workers suggested that shikonin treatment resulted in the downregulation of ECE1, HWP1, EFG1, CPH1, RAS1, ALS1, ALS3, CSH1 genes and upregulation of TUP1, NRG1, BCR1 genes [66]. Cao and co-workers had also reported the lower expression of *CSH1* mRNA when the biofilm treated with baicalein [73]. Li and co-workers suggested that solasodine-3-O-β-D-glucopyranoside could act by retarding the synthesis of cAMP [75].

CONCLUSION AND FUTURE PROSPECTS

Isolation of bioactive molecules from *T. capitate*, *C. sativum*, *S. virgaurea*, *P. harmala*, *A. calamus*, *C. citrate* and *C. zeylanicum*, which have the strong potential to inhibit the biofilm formation in *Candida* spp. pathogens, should be the next line of research towards finding an effective remedy for biofilm-associated candidiasis. It has also been observed that the mechanism of action for the molecules like carvacrol, thymol, geraniol, berberine, 4-hydroxycordoin, aginoside A16 and barrigenol A19 is missing. Exploration of their mechanism of action might offer some new insights for targeting hard to treat *Candida* infections and their biofilms. Geraniol, thymol and carvacrol may have different mechanisms than farnesol as the former belong to the monoterpenoids class and the later belongs to the sesquiterpenoids class.

From the work compiled in the present article, it is certain that the natural products have a very strong prospect to deliver effective chemotherapeutics for the treatment for candidiasis involving biofilm. In most cases, the studies are not complete. The bioactive molecules present in the extracts are often not identified and not characterized by their mechanism of action. *In silico* studies might prove useful in gaining mechanistic insights into the molecular mechanism of action of the identified molecules with anti-*Candida* and anti-biofilm properties [36].

CONSENT FOR PUBLICATION

Not applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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