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Review on Combinatorial Approach for Inhibiting *Candida albicans* Biofilm

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Abstract

C. albicans has the ability to form biofilms on biotic and abiotic surfaces. Biofilms are highly resistant to various antifungal drugs and there are complex mechanisms underlying biofilm resistance. Biofilm associated infections are urgently needed successful treatment. It has become essential to develop alternative strategies in order to overcome the limitations of current antifungal therapy against biofilm associated fungal infections. Developing new therapeutic approaches for anti-biofilm activities, there is a need to find novel drugs from natural sources. Combination therapies and exploring natural compounds and immune therapies are good approach to overcome this problem. In addition to the discovery of new antifungal drugs, vaccines, antibiotics, natural products, small molecules and synthesized compound are the important alternatives to be used alone or in combination with antifungal drugs. Combination of molecules with different drugs is an excellent strategy due to advantages of combination therapy like reduced side effects and reducing the concentration of dosages of individual drugs and reduced toxicity.

Introduction

The polymorphic fungus, *Candida albicans* is a commensal organism found in the digestive, oral and urinogenital areas of healthy humans [1]. It is not pathogenic in healthy individuals but may become pathogenic in immune compromised patients [2]. It is the most common hospital-acquired infectious agent. *C. albicans* may cause superficial and serious systemic mycosis. The diseases caused by *Candida* are called as candidiasis [1]. A patient with low number of neutrophils might have a chance of Candidemia i.e. infection related with blood streams. Vaginitis in women and oral pharyngeal thrush in AIDS patients are the most common infections caused by *C. albicans* [2,3]. Patients suffering from diseases like AIDS and those under chemotherapy and organ transplantation have most chances of life threatening infections of candidiasis [3].

Vulva vaginal Candidiasis may affect up to 75% of women. *Candida* infections are the fourth most common hospital-acquired infection in India and second most in the United States of America [4]. *Candida albicans* can exist in various morphological forms like yeast, hyphae and psedohyphae and chlamydospores [5]. Hyphae are the filamentous tube like structures without constriction at the septal junctions consider as virulence factor of *C. albicans*. Hyphae have invasive property that can promote tissue penetration [5,6]. The use of antifungal agents has increased from many years for the treatment of a variety of diseases caused by fungi. There are eight different targets for antifungal therapy like chitin synthesis, ergosterol synthesis, glucan synthesis, squalene epoxidase, nucleic acid synthesis, protein synthesis, and microtubules synthesis [7]. Azole antifungals are widely used to treat *candida* infections. It consists of imidazole and triazole derivatives such as Ketoconazole, Fluconazole, voriconazole etc., that block the synthesis of ergosterol in the cytoplasmic membrane [8]. Fluconazole is effective against oropharyngeal and vaginal candidiasis and is effective at very low concentrations and shows very less side effects [9]. Morpholins and allylamines inhibit the conversion of lanosterol to ergosterol, while Echinocandins are glucan synthesis inhibitors. Flucytosine was first developed as an anticancer agent but later on it was developed as an antifungal agent and today it is used in adjunctive therapy with Amphotericin B [10]. Polyene antifungal agents such as amphotericin B and nystatin are having broad spectrum of action and shows fungicidal activity. Echinocandins and their analogue and has excellent activity towards fluconazole resistant candida strains [8,11]. Antifungals like fluconazole, pyrimidine analogues and allylamines are less useful against biofilms of *C. albicans*. So, commercial antifungal agents including fluconazole and amphotericin are widely prescribed but they are not very effective in clinical situations [12]. Pathogenicity of *C. albicans* increases because of resistance activity of virulence factors like biofilm formation, yeast to hyphae transition [2,13].

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Table 1: List of Anti-fungal agents.

No	Antifungal Drug	Mode of action	Mechanism of resistance	References
1	**Azoles**	Fungistatic, broad spectrum antifungal drug. Inhibits fungal cytochrome P450 14α-lanosterol demethylase.	Drug efflux carried out due to decreased affinity in Erg11 protein by mutations up regulation of multidrug transporter genes. Alteration of specific steps in the ergosterol biosynthetic pathway.	[7,8,9]
2	**Polynes (Amphotericin B)**	Fungicidal, broad spectrum antifungal drug which is used in liposomal form having reduced toxicity. It binds to ergosterol major sterol of fungal membrane.	Due to binding ergosterol content is decreased. Absence of ergosterol alters the specific steps in biosynthetic pathway.	[7,8,9]
3	**5-flurocytosine**	Inhibition of nucleic acid synthesis by formation of fluorinated pyrimidine metabolites.	Lack of enzyme essential in the metabolism of 5-Fc. Deregulation of the pyrimidine biosynthetic pathway. Defects in cytosine deaminase.	[7,8,9]
4	**Echinocandins**	Inhibition of cell wall synthesis enzyme β-1, 3 glucan synthase.	Alteration of affinity of echinocandins for β-1,3 glucan synthase.	[7]
	Caspofungin			
	Micafungin			
5	**Allylamines**	Fungicidal Poorly active against *Candida* species. Inhibition of squaleneepoxidase (ERG1)	Alteration in the gene ERG1	[8]
	Terbinafine			
6	**Morpholines**	Inhibition of sterol reductase and isomerase	Unknown	[9]

Biofilms are microbial communities containing a dense network of yeast and filaments embedded inside an exo-polymeric matrix which makes it resistant against chemotherapeutic agents [14]. Biofilms are not just a mixture of yeast and filaments but it shows different developmental phases differing from planktonic growth mode. Specific gene programme and metabolic processes are activated during biofilm formation [15,16]. *Candida* biofilm shows increased resistance against most the antifungal agents [17].

Biofilms formed by *C. albicans* are more pathogenic than the budding yeast [2]. Biofilms are responsible for broad spectrum infections in the human host [2]. Implanted devices in human host like inside wall of intravenous catheters, indwelling bladder catheters, joint prosthesis, eye lenses and mechanical heart valves are common site for biofilm growth [18,19]. Biofilms have the ability to efflux out the drugs outside the cell. Biofilms are made up of high density cells and persister cells. Changes in gene expression take place during formation of biofilms [14]. At high concentration of repeated doses, some antifungal agents showed toxicities [19]. Overall, increased cost and drug resistance has put limitations on the use of antifungal drugs. *Candida albicans* biofilms are difficult to eradicate [14]. So there is a need to find better drug agents to cure life threatening infections associated with biofilms of *C. albicans*.

Combination therapy is considered as an effective approach to improve the efficacy of therapy in the treatment of invasive infections [20]. Combination therapy is very useful and effective since they may increase both the rate and degree of microbial killing [21]. Another important reason for using combined drugs is that each drug may have different mechanism of action. Two drugs may act on different targets resulting in multi targeting. Due to multi targeting approach, development of drug resistance can be slowed down [22]. Some antifungal drugs like fluconazole affect liver. Toxicity and intolerance of the drug could be avoided with the help of two or more combined drugs [22,23].

Studies on Drug Combinations against *C. albicans in vitro*

Non-steroidal anti-inflammatory drugs are used for the treatment of pain and inflammation. It is demonstrated that COX inhibitors decreased biofilm production by *C. albicans*. These drugs block the biosynthesis of mammalian prostaglandin by acting on one or both of the cyclooxygenase isoenzymes, COX-I, COX-II. Cyclooxygenase enzymes are involved in the synthesis of mammalian prostaglandins. Prostaglandins are lipid molecules and have diverse biological roles like regulation of blood pressure, inflammation, reproduction, renal function etc [2]. Prostaglandins are also produced by pathogenic fungi. Non-steroidal anti-inflammatory drugs like Aspirin, Diclofenac sodium, Ibuprofen, Ketoprofen, Piroxicam block prostaglandin synthesis by inhibiting COX enzyme [24]. Among NSAIDs, Aspirin, Diclofenac and Ibuprofen exhibit anti biofilm activity. Ibuprofen combined with fluconazole show synergistic interaction against fluconazole resistant *C. albicans*. Ibuprofen has both fungicidal and fungistatic activity depending upon its doses. Ibuprofen at high doses can kill Candida by causing damage to cell membrane under *in vitro* condition. Combination of Ibuprofen with Fluconazole increases the sensitization of *C. albicans* [23]. The non-steroidal anti-inflammatory drug Diclofenac, a COX-I and COX-II inhibitor, when combined with Fluconazole or Ketoconazole shows synergistic effect against cell viability of *C. albicans* [25]. The mechanisms of action for synergistic interaction between NSAIDs with antifungal drugs might be that they inhibit the activity of phospholipase A and B in *C. albicans*. Phospholipase A and B have the ability to cleave the fatty acid side chains of phospholipids. Biofilm and yeast cells of fungi synthesized fungal prostaglandins which are very sensitive to the COX inhibitors [26].

Caspofungin, an Echinocandin has the ability to interfere with fungal cell wall biosynthesis by inhibiting beta-1, 3-D-glucan synthase. Caspofungin is effective against resistant strains of *C. albicans*. Caspofungin is used in the treatment of invasive candidiasis [7]. To increase the efficacy of caspofungin towards biofilm of *C. albicans*, caspofungin is combined with Diclofenac. Diclofenac is an anti-inflammatory drug which inhibits hyphae development and biofilm formation. Susceptibility of *C. albicans* could be increased by combined interaction of caspofungin towards biofilm by Diclofenac pretreatment. This combination can be used against *C. albicans* biofilm associated infections [27]. Caspofungin combined with posaconazole, resulted synergistic interaction *in vitro* and *in vivo* against clinical isolates of *C. albicans* [28].

Curcumin, a polyphenol found in turmeric, is known to have antifungal activity. Curcumin when combined with azoles like fluconazole, miconazole, ketoconazole, nystatin, and amphotericin B *in vitro* resulted in synergistic interaction against *C. albicans* [29]. Curcumin increases the level of ROS and regulation of expression

Table 2: Combination of Chemo-sensitizing agents with Antifungals against *C.albicans in vitro*.

Number	Combination	Interaction	Reference
1	Latex of *Euphorbia characias* + Ketoconazole	Synergistic	[88]
2	Caspofungin+ Amphotericin B	Synergistic	[89]
3	Estragole + AmB	Antagonistic	[90]
4	Amphotericin B + FC, echinocandins, TBF	Antagonism	[23]
5	Berberine + amphotericin B	Synergistic	[23]
6	Ibuprofen + FLC, KTC, ITR	Synergistic	[23]
7	Cinnmaldehyde + AmB	Synergistic	[91]
8	Berberine + fluconazole	Synergistic	[58]
9	Cyclosporine A, FK506 + FLC	Synergistic	[23]
10	Amiodarone + FLC, ITR, VOR	Synergistic	[61]
11	Baicalein + Fluconazole	Synergistic	[31]
12	Retigeric acid B + FLC, ITR, KTC	Synergistic	[39]
13	Allicin + FLC, AmB	Synergistic	[55]
14	Thymol + FLC, AmB, ITR	Synergistic	[61]
15	Curcumin + FLC, VOR, ITR, AmB, miconazole, nystatin	Synergistic	[29]
16	Linalool, benzyl benzoate, eugenol + FLC	Synergistic	[46]
17	Lectoferrin + FLC	Synergistic	[52]
18	Cinnmaldehyde, Eugenol + FLC	Synergistic	[44]
19	Eugenol, Cinnmaldehyde + Amphotericin B	Indifference	[44]
20	Thymol + Fluconazole	Synergistic	[43]
21	Cyclosporine A + FLC, VOR, ITR, AmB, nystatin	Synergistic	[67]
22	Chloroquine + FLC, VOR;	Synergistic;	[68]
	Chloroquine + Caspofungin, amphotericin B	No Interaction	
23	Diclofenac + caspofungin	Synergistic	[27]
24	Posoconazole + Caspofungin	Synergistic	[75]
25	Geldanamycin + Fluconazole	Synergistic	[60]
26	Diketopiperazine + AmphotericinB	Synergistic	[38]
27	Tyrocidines + Amphotericin B and Caspofungin	Synergistic	[36]
28	Glabridin + Fluconazole	Synergistic	[70]
29	Stillbines + Fluconazole	Synergistic	[38]
30	Econazole- Nitrate + chelerythrine	Synergistic	[28]
31	Tetrandrine + Fluconazole	Synergistic	[76]
32	Fluoxetine + Fluconazole	Synergistic	[71]
33	Farnesol + Fluconazole, Amphotericin B or micafungin	Synergistic	[65]
34	Thymol + Nystatin	Synergistic	[45]
35	Eugenol-tosylate + Fluconazole	Synergistic	[49]
36	Diorcinol D + fluconazole	Synergistic	[72]
37	Plant Defensin HsAFP1 + Caspofungin	Synergistic	[74]
38	Thionin-like peptide + fluconazole	Synergistic	[53]
39	Calcium channel blockers (amlodipine, nifedipine, bendipine, flunarizine) + fluconazole	Synergistic	[73]
40	Budesonide + fluconazole	Synergistic	[77]
41	Essential oil and its major component + fluconazole	Synergistic	[48]
42	Synthetic chalcones + fluconazole	Synergistic	[34]
43	Extract of *Rchingii* + Fluconazole	Synergistic	[79]
44	PVP-coated AG-NPs + Fluconazole	Synergistic	[78]
45	Quercetin + Fluconazole	Synergistic	[80]

46	Farnesol + Fluconazole	Synergistic	[66]
47	Fluxitine + FLZ, ITR, VOR	Synergistic	[82]
48	Allylisothiocyanate + Fluconazole	Synergistic	[85]
49	Osthole +Fluconazole	Synergistic	[83]
50	Lipopeptide AC7BS + Amphotericin B	Synergistic	[84]
51	Caffic acid phenethyl ester + Caspofungin	Synergistic	[87]
52	Lovastatin + Itraconazole	Synergistic	[86]

Table 3: Combination of chemosensitizing agent with antifungal agent against *C.albicans in vivo.*

Numbers	Combinations	Interaction	Model Organism	Reference
1	Epigallocatechin + AmB in murine model	Synergistic	Mice	[92]
2	Allicin + FLC	Synergistic	Murine	[93]
3	Berberine + AmB	Synergistic	Murine	[58]
4	Caspofungin + Diclofenac	Synergistic	Rat	[27]
5	Tyrocidienes + Caspofungin	Synergistic	*C.elgans*nematode	[36]
6	Amiodarone + fluconazole	Synergistic	Murine	[23]
7	Budesonide + fluconazole	Synergistic	*G.mellonella*	[83]
8	Quercetin + fluconazole	Synergistic	Murine VVC	[80]
9	Farnesol + fluconazole	Indifference	Murine	[66]
10	Fluxetine + FLZ, ITR, VOR	Synergistic	*G.mellonella*	[82]

of several genes associated with fungal oxidative stress, including superoxide dismutase, catalase, and oxydoreductase [30].

A flavonoid, Baicalein, which was originally isolated from the Chinese plant, *Scutellaria baicalens* has anticandidal property. Baicalein is a lipooxygenase inhibitor or efflux pump inhibitor [31]. It shows inhibitory effect against different stages of biofilm development and growth of *Candida*. Baicalein directly act on cell surface hydrophobicity of the biofilm [32]. Baicalein combined with fluconazole shows synergistic interaction against *C. albicans*. As baicalein is an inhibitor of efflux pump, in combination with fluconazole it reduces the ability of cells to efflux out the drugs [31]. Baicalein combined with amphotericin B resulted in apoptosis in *C. albicans*. Caspase activity and reactive oxygen species in *C. albicans* is enhanced in presence of both baicalein and amphotericin B [33].

Chalcones are naturally occurring flavonoids composed of two aromatic rings connected by α, β unsaturated carbonyl group. They have antitumor, antifungal and anti-inflammatory activities. Twenty-four derivatives of chalcones were synthesized by aldol condensation and combined with fluconazole against resistant strains of *C. albicans*. Synthetic chalcones combined with fluconazole show synergistic interaction and might be following similar mechanism like baicaelin [34].

Tyrocidines isolated from *Bacillus* have antibacterial, antimalarial as well as antifungal activities. At low concentrations they show strong inhibitory effect against planktonic growth of *C. albicans*. Tyrocidines are cationic cyclodepeptides and have been used as topical antibiotics for bacterial and parasitic infections [35]. Additionally tyrocidines inhibit biofilm development and maturation by direct attack on cell membrane. Membrane disruptive activity of tyrocidines causes cell death. When tyrocidenes were combined with Amphotericin B and Caspofungin it potentiated the activity of AMB and CAS towards *C. albicans* by inducing reactive oxygen species and disrupted membrane permeability [36].

Small cyclic peptides namely diketopiperazines has different properties like antitumor, antifungal and antibacterial activities [37]. Diketopiperazines increase the efficacy of amphotericin B and clotrimazole against *C. albicans*. Amphotericin B is toxic and hemolytic effect *in vivo*. Diketopiperazines in combination with Amphotericin B reduces the side effects or cytotoxicity of Amphotericin B [38].

Retigeric acid B, a pentacyclictriterpene acid isolated from the lichen, Lobaria kurokawae, possesses antifungal activity [39]. The antifungal mode of action of Retigeric acid B was found to be associated with reactive oxygen species related apoptosis. Retigeric acid b when combined with fluconazole and ketoconazole resulted in synergistic interaction [40]. Retigeric acid B inhibits efflux pump activity and ergosterol biosynthesis pathway in *C. albicans* [39].

Components of essential oils, like eugenol, thymol, carvacrol, geraniol, linalool, cinmaldehyde and menthol possess antifungal activity against biofilm of *C. albicans* [41]. Essential oils have the ability to create pores in the cell membrane and efflux the drugs out of cell and decrease the ergosterol content of the cell membrane. Thymol, eugenol and Carvacrol cause membrane disintegration; ion loss and interference in the TOR pathway [42]. By creating ionic disruption, carvacrol may make cells unable to regulate acidification. Thymol, eugenol, and carvacrol combined with fluconazole show synergistic interaction against biofilm of *C. albicans* [43]. Thymol and eugenol are reported to have synergistic interaction with fluconazole against planktonic form of *C. albicans*. Terpenoids are fungicidal in nature and it increases the sensitivity of *C. albicans* biofilms towards fluconazole by altering the fungistatic activity into fungicidal [44]. Combined effect of thymol with fluconazole and amphotericin B resulted in synergistic interaction between thymol and fluconazole [42]. Some studies have shown that thymol has broad spectrum of biological activities like antiseptic, anti-inflammatory antioxidant and fungicidal effect on Candida. Thymol combined with the synthetic antifungal drug, Nystatin, resulted in synergistic interaction by inhibiting ergosterol formation. Thymol acts on different enzymes

responsible for the synthesis of ergosterol of the fungal plasma membrane [45].

Cinnamaldehyde combined with amphotericin B shows synergistic interaction against *C. albicans* by disrupting the plasma membrane of *Candida*. Other terpenoids like linalool, benzyl benzoate, and eugenol combined with fluconazole resulted in synergistic interaction against planktonic forms of *C. albicans* [46]. The monoterpenes, linalool and phenyl propene methylchavicol combined with fluconazole and ketoconazole shows synergistic interaction against *C. albicans* by acting on membrane permeability [47].

Essential oil from *Ocimum basilicum* var. Maria Bonita and its major components like linalool and geraniol when combined with fluconazole showed increased antifungal activity. Geraniol combined with fluconazole showed synergistic interaction & also in combination of linalool with geraniol showed synergistic interaction against resistant strain of *C. albicans*. Essential oil and linalool have ability to reduce ergosterol content it means they might act on ergosterol biosynthesis pathways and showed synergistic interaction against biofilm of *C. albicans* [48].

Semi-synthetic analogue of eugenol, Eugenol-tosylate and its congeners combined with fluconazole and tested their antifungal activity against Fluconazole-resistant *C. albicans* have showed significant synergistic interaction. Eugenol-tosylate and its congeners from E1 to E6 have ability to down regulate the activity of ERG11 gene. By inhibiting ergosterol biosynthesis pathway eugenol-tosylate acts as an augmenting agent [49].

Polyphenol, epigallocatechin-o-gallate found in the extract of tea possess antifungal activity. Epigallocatechin-o-gallate combined with Amphotericin B shows synergistic interaction against *C. albicans*. Epigallocatechin-o-gallate is reported to be a highly pH dependent compound, and in acidic condition it shows synergism with amphotericin B [50].

Antimicrobial peptides are very important components of immunity against acute infections. They kill rapidly Gram positive and Gram negative bacteria, viruses and fungi. Antimicrobial peptides can alter membrane permeability due to which leakage of cellular ingredients may occur and result into cell death [51]. Lactoferrin an antimicrobial peptide combined with fluconazole shows synergistic interaction against *C. albicans*. Chelating function of lactoferrin target the mitochondrion of Candida, due to which production of reactive oxygen species increase and this is responsible for synergistic interaction between lactoferrin with fluconazole [52].

A plant derived peptide Thionin, named CaThi has strong candidacidal activity and when combined with fluconazole show synergistic interaction by permeabilization of plasma membrane in *C. albicans*. The combined treatment of CaThi with fluconazole is results strong improvement in therapeutic results against resistant strains of *Candida* spp [53].

Allicin, aallyl-sulfur organic compound derived from garlic, have ability to inhibit the yeast to hyphal form transition and inhibit growth of hyphae in *Candida albicans*. Allicin can reduce Glutathione lavel and increase oxidative stress in *C. albicans* [54]. Allicin combination with amphotericin B and flucytosine shows synergistic interaction against *C. albicans*. It is reported that when allicin is combined with flucytosine and amphotericin B the outer membrane of cells completely destroyed. Allicin combined with fluconazole shows synergistic interaction against clinical isolates of *C. albicans* [55,23].

A common alkaloid Berberine is found in many plants like *Coptis chinensis* and *Hydratis canadensis* L. Berberine is reported to have synergistic interaction with fluconazole against clinical isolates of *C. albicans*. In this combination of Berberine with fluconazole, berberine acts as a chemosensitizer of fluconazole [56]. Berberine directly attack on the mitochondrial respiration resulting in the production of reactive oxygen species [57]. Berberine chloride combined with fluconazole exhibits synergistic interaction against clinical isolates of *C. albicans* by inhibiting presence of sterol 24 alpha methyl transferase enzymes in the membrane [58].

Geldanamycin is a benzoquinone ansamycin antibiotic which possesses antifungal activity [59]. Geldanamycin combined with fluconazole shows synergistic interaction against *C. albicans*. Geldanamycin have the ability to inhibit Hsp90 proteins by binding to proteins aminotrminal domain, where ATPs bind. Due to inhibition of Hsp90 protein, its client proteins degraded and cell lysis take place. This mechanism is responsible for the synergistic interaction of geldanamycin with fluconazole [60].

Amiodarone is an antiarrhymic drug which possesses antifungal properties against fungi like Aspergillus, *Cryptococcus* and Candida. Amiodarone is reported to exhibit synergistic interaction with fluconazole, itraconazole and voriconazole against drug resistant strains of *C. albicans* [61]. Amiodarone has the ability to disrupt the cell membrane. Due to alteration in cell membrane, ergosterol lavel can be reduced resulting in cellular ion imbalance. Changes in ergosterol pathway and Calcium stress are responsible for alteration of cellular organization. Amiodarone combined with fluconazole exhibit synergistic activity against *C. albicans* [62].

Farnesol is a quorum sensing molecule which is reported to have synergistic interaction with fluconazole. Farnesol causes apoptosis in *C. albicans* by different mechanisms like production of reactive oxygen species and mitochondrial degradation. Farnesol inhibits yeast to hyphae transition in *C.albicans*. In combination with fluconazole farnesol can accumulate reactive oxygen species and down regulate the ergosterol biosynthetic pathway [63,64]. Farnesol combined with Amphotericin B, micafungin and fluconazole have shown synergistic interaction against biofilm of *C. albicans* by targeting the mitogen activated protein kinase and cAMP- protein kinase pathway. This pathway is regulated by Ras protein. Farnesol inhibits the activity of RAS signals in *C. albicans*. Ras and ROS have important roles in physiological functions and development of *C. albicans* [65]. But combinations of farnesol with fluconazole against sessile *C. albicans* isolates were shown indifference interaction. Synergistic interactions were found against only planktonic forms of strain of *C. albicans* [66].

Cyclosporine in combination with fluconazole, voriconazole and caspofungin show synergistic interaction against *C. albicans*. Cyclosporine A is a calcineurine inhibitor. Calcineurine have properties like hyphae elongation, vegetative growth, cation homeostasis, cell wall synthesis and regulation of cell cycle and also plays role in the regulation of the intracellular Ca^{+} concentration. Calcineurine is a calmodulin activated phosphatase. Phosphatase has a key role in development of *C. albicans*. These drugs suppress the immune system by inhibiting calcineurine [67]. Chloroquine an antiparasitic drug when combined with fluconazole and voriconazole shows synergistic interaction against *C. albicans* biofilm. Chloroquine

sensitizes biofilms of *C. albicans* to fluconazole and voriconazole may be due the activation of drug efflux pump and by inhibiting ergosterol content in the membrane [68].

Glabridin is found in the root extract of the plant *Glycyrrhiza glabra*. Glabridin is associated with properties like anti-oxidant, anti-inflammatory, neuroprotective and skin whitening. Glabridin shows antifungal activity against *C. albicans* [69]. Glabridin exhibit synergistic interaction with fluconazole against resistant strains of *C. albicans*. On the basis of cell membrane permeability test it is reported that after combined treatment of fluconazole with glabridin, changes are found in cell envelop of *C. albicans*. Cell membrane permeability increased by decreasing cell size, therefore cells is very sensitive to the cell wall inhibitors [70]. Vulvovaginal candidiasis is most common infections caused by *Candida* spp. combined dose of a selective serotonin reuptake inhibitor drug, fluoxetine with fluconazole is used in treatment of vulvovaginal candidiasis. This combination showed synergistic interaction against fluconazole resistant strain by increasing the production reactive oxygen species [71].

Diorcinol D is an antifungal compound isolated from the fungus *Aspergillus versicolor*, an endophytic fungus derived from the lichen, *Lobaria quercizan,* when combined with fluconazole synergistically inhibited mature biofilms of *C. albicans.* Diorcinol D inhibited the activity of efflux pump by reducing the expression of Cdr1 in *C. albicans*. It also blocked the biosynthesis of ergosterol; these possibilities are responsible for synergistic interaction with fluconazole [72].

In the treatment of cardiovascular diseases, Calcium Channel Blockers (CCBs) are used. These drugs have antifungal activity against *C. albicans*. Some commonly used CCBs like Amlodipine (AML), Nifedipine (NIF), Bendipine (BEN), Flunarizine (FNZ) show synergistic interaction with fluconazole against *C. albicans*. CCBs have the ability to down regulate the activity of Yvc1protein, the putative vacuolar calcium channel, and plays important role in *C. albicans* infection. Combined treatment of CCBs with fluconazole disturb the calcium concentration by inhibiting the expression of CNA1, CNB1 (encoding calcineurin) and YVC1 (encoding calcium channel protein in vacuole membrane) protein due to which growth of *C. albicans* inhibited [73].

HsAFP1 is a small cysteine-rich peptide molecule isolated from the seeds of coral bells (*Heucherasanguinea*). It is nontoxic to human cells but have antifungal activity. HsAFP1 has inhibitory activity against yeast cells but not against the mature biofilm of the *C. albicans*. rHsAP1 combined with Caspofungin show synergistic interaction against biofilm of *C. albicans.* Plant defensins inhibit growth of fungal cell by the mechanism of increasing production of ROS and by the induction of apoptosis [74].

Chelerythrine (CHT) is a benzopherathridine alkaloid isolated from the roots of *Chelidonium majus*. CHT have strong anti-inflammatory activity. CHT show synergistic interaction with Econazole nitrate (Imidazole) by reducing its toxic side effects. The actual mechanism responsible for this interaction is ECZN inhibits ergosterol biosynthetic pathway and by accumulating toxic sterol in membrane of *C. albicans* causing cell death. Also, CHT inhibited activity of Protein Kinase C (PKC) in *C. albicans*. PKC is a key enzyme in fungi responsible for regulating the cell proliferation, differentiation and survival [75].

Tetradrine (TET) is a major alkaloid used in the treatment of rheumatic arthritis and hepatic fibrosis. When tetradrine is combined with fluconazole, synergistic interaction is found between them against *C. albicans* [76]. Tetradrine inhibits the function of drug efflux in *C. albicans*. The actual mechanism for this synergistic combination is related with respiratory metabolism. Classical respiratory chain is the major source of respiration or O_2 consumption in *C. albicans*. TET affects respiratory energy generation and its conversion in *C. albicans*. TET +FLZ treatment increases the production of ATP by inhibiting respiratory enzyme complex due to which ROS production increases in the cell and cell goes to apoptosis [76].

Budesonide (BUD) is an inhaled corticosteroid in the group of glucocorticoids. Glucocorticoids are used only in the treatment of severe infections due to its strong anti-inflammatory effects. BUD has the ability to increase the antifungal effect of fluconazole against resistant *C. albicans in vitro*. BUD combined with fluconazole show synergistic interaction against *C. albicans in vitro* as well as *in vivo*. This combination inhibits the function of drug transporters, reducing the biofilm formation, promoting apoptosis and inhibiting the activity of extracellular phospholipases. On treatment with combination transporter genes, CDR1, CDR2, MDR1 and FLU1 were down regulated. In presence of BUD and fluconazole expression of biofilm formation genes were decreased. HSP90, CRZ1 and CNA1 were down regulated after treatment with BUD combined with fluconazole indicating that apoptosis plays an important role in synergistic interaction [77].

Silver nanoparticles (AG-NPs) coated with Polyvinyl Pyrroliodone (PVP) is combined with fluconazole and voriconazole against resistant strain of *C. albicans* resulted in synergistic interaction. This combination up regulated the expression of ERG5 and ERG6, and down regulated the expression of CDR1 gene. Also, PVP-coated AG-NPs exhibited tendency to attach to cell membrane and reduce hyphae formation [78].

Rubus chingii is one of the members of the genus *Rubus*. *Rubus* species possess antimicrobial and antifungal activities. The extract of R. chingii combined with fluconazole, synergistically inhibit *C. albicans*. Possible mechanism behind this synergistic interaction might be changes in the cell cycle of *C.albicans* after the treatment with combination of drugs, fluconazole and extract of chingii arrested *C. albicans* in the S phase, Extract of Chingii decreased the efflux of Cdr1 ABC transporter which may be responsible for fluconazole resistance [79].

Quercetin (QCT), a dietary flavonoid combined with fluconazole show synergistic interaction against *C. albicans* biofilm which is isolated from vulvovaginal candidiasis patients. Combined effect of QCT and fluconazole had ability to reduce cell-cell adhesion and CSH. QCT with fluconazole combination affected the expression of genes responsible for biofilm formation, like ALS1, HWP1, SUN41, UME6, PBS2 and PDE2 [80].

Sodium Houttuyfonate (SH) combined with fluconazole showed synergistic interaction against clinical strain of *C. albicans*. Sodium Houttuyfonate (SH) is a chemical compound synthesized from houttuynin and sodium bisulfate. The antifungal mechanism of SH with fluconazole involve the synthesis and transportation of β-1,3-glucan. SH have ability to negatively regulate ZAP1and IFD6 responsible for the accumulation of β-1, 3-glucan [81].

Fluxetine is a Serotonin-Reuptake Inhibitor (SSRI), which is used an antidepressant. Antifungal activity of SSRI is reported. Fluxetine

combined with three azoles like fluconazole, voriconazole and itraconazole show synergistic interaction *C. albicans* biofilm formed. Fluxetine have the ability to down regulate the activity of Secreted Aspertase Protease (SAP) formed. Fluxetine have the ability to down regulate the activity of SAP genes and also inhibit extracellular phospholipase activity [82].

Osthole is an important antifungal o-methylated natural Coumarin derived from *Candida fructus*. *C. fructus* has been widely used in China for the treatment of supportive dermatitis and vaginitis. Osthole exhibit synergistic effect with fluconazole. RAS augmentation might be an important cause of this synergistic interaction [83].

Lipopeptide AC7BS and amphotericin is synergistic against *C. albicans* biofilm. AC7BS have the ability to affect cell membrane. Due to this amphotericin can enter into the cell and show synergistic interaction. AC7BS is a potent inhibitor of *C. albicans* biofilm on medical insertional materials and it is used as coating agent [84]. Allylisothiocyanate (AITC) with fluconazole were shows synergistic interaction against planktonic and biofilm growth forms of *C.albicans* [85]. It hypothesized that AITC might damage cell membrane of the *C. albicans* due to which influx of fluconazole may increase and inhibit the biofilm.

Caffic Acid Phenethyl Ester (CAPE) a polyphenol, having antifungal activity showed synergistic interaction with Caspofungin against *C.albicans* by disrupting iron homeostasis. By RTPCR analysis they were confirmed that iron deprivation induced by CAPE led to down regulation of iron utilization gene and up-regulation of iron uptake and regulation genes, it indicates that disrupting iron homeostasis by CAPE may be the cause of synergism with Caspofungin against *C. albicans* [86]. Zhou et al. proposed that lovastatin is an synergistic antifungal potentiator of Itraconazole against *Candida albicans* planktonic cells and biofilms. By regulating ergosterol biosynthetic pathway lovastatin showed synergistic interaction with itroconazole [87].

Studies on Drug Combinations against *C. albicans in vivo*

The Non steroid anti-inflammatory drug, Diclofenac combined with caspofungin is tested on sub cutaneous biofilm formed on catheters in a rat model. Diclofenac alone had no effect on biofilms on catheters. In combination with fluconazole it was able to eradicate the biofilms and increase the survival of rats. Combined treatment of Diclofenac with caspofungin increased the survival of rats up to seven days [27].

Effect of Tyrocidines and Caspofungin is studied in the Nematode model of *Caenorhabditis elegans*. After five days of infection, *C. elegans* is treated with combined doses of Tyrosidines and Caspofungin. This treatment doubled the survival of *C. elegans* against Candida infection without any toxicity [36].

Epigallocatechin-o-gallate combined with Amphotericin B shows synergistic interaction against *C. albicans*. This combination is studied in mice model against disseminated Candidiasis. Infected mice injected with combined doses of Amphotericin B and EGCG showed increased survival rate than mice injected with Amphotericin B. EGCG itself inhibit hyphal formation and ergosterol synthesis in *C. albicans* [92].

Guo et al., reported that allicin and fluconazole when applied together can reduce infection of kidney in a murine model of Candidiasis and increase the survival. Berberine a common alkaloid combined with amphotericin B increases the survival rate of mouse which infected by *C. albicans* [93]. Berberine plus amphotericin B injection in mouse model increased the survival of mice twice than amphotericin B alone [58]. Berberine can directly attack on the mitochondrial respiration resulting in the production of reactive oxygen species [57].

Amiodarone with fluconazole can reduce infection of kidney in murine model of candidiasis and increase the survival four times. Amiodarone has the ability to disrupt the cell membrane in *C. albicans*. Due to alteration in cell membrane, ergosterol lavel is reduced and cellular ion imbalance takes place. This may be responsible for increasing the survival rate [23].

Galleria mellonella larva, a new fungal infection model, is used to study the effect of combined treatment of Budesonoide (BUD) with fluconazole. Infected *G. mellonella* larvae exposed to a combination of fluconazole and BUD resulted in significant increase in survival. Histopathology study of infected larvae showed that the treatment with fluconazole and BUD resulted in much fewer melanized nodules than the alone treatment. Immuno-modulatory activities of BUD might be responsible for the protection of *G. mellonella* larvae from *C. albicans* infections [77]. Kovacs, et al [66] tested farnesol with fluconazole against *C. albicans* biofilms in a murine vulvovaginitis model. Indifference interaction was showed by this combination.

Quercetin (QCT), a dietary flavonoid combined with fluconazole showed synergistic interaction in a murine vulvovaginal candidiasis model. Following treatment fungal load decreased, hyphal forms disappeared, and the inflammation of mucosal epithelial cells was decreased [80].

Fluxetine combined with three azoles like fluconazole, voriconazole and itraconazole showed synergistic interaction against *C. albicans* biofilm in *G. mellonella* model. Fluoxetine down regulated the expression of SAP genes. Also, phospholipase activity was decreased during treatment with combined drug in the larvae [82].

Future Prospects

Universally, Candidiasis is a major life-threatening disease, due to the increased incidence of drug resistance in *Candida* spp and the limited number of antifungal agents available. Combination therapy might be a valid alternative. The main objective of combination therapy is to achieve synergistic interaction between two compounds, by increasing their activity and lowering the toxic effects of both compounds. The results of this combination review show a promising new strategy for control of fungal diseases. There are many reports available on combination of antifungal drug with synthetic small molecules and with natural compound *in vitro*. Some of them combinations were tried *in vivo*. There is a need to try more these combinations *in vivo*. Promising attributes of chemosensitizing agents like synthetic compound show greater specificity towards *C. albicans* biofilm. There will be greater opportunity to eradicate the biofilm. By using combinatorial approach of chemosensitizing agents with appropriate commercial antifungal agents might be improve efficacy of available antifungal agents.

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