

Effective Combinations Against Efflux Pump Overexpressed on Azole Resistance Candida and Dermatophytes: A Systematic Review

Aishwarya Jayan and Swati Gupta*

Amrita School of Pharmacy, Amrita Institute of Medical Sciences,
Amrita Vishwa Vidyapeetham, AIMS Ponekkara P.O , Cochin, Kerala, India.

*Corresponding Author E-mail: swatigupta@aims.amrita.edu

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Dermatophytes and Candida are the two most common causes of fungal infections worldwide, affecting millions of people annually. The emergence of resistance among these groups of fungi and the limited availability of effective antifungal drugs may become a real challenge in the coming era. Thus, use of a combination of resistant reversion agents along with antifungal drugs is worth investigating. One of the causes of resistance development is the overexpression of efflux pumps and associated genes. Therefore, we examined the scientific literature on antifungal combinations against resistant species of dermatophytes and Candida caused by efflux pump overexpression. A literature search on the subject performed in PubMed and Google scholar resulted from a total of sixteen relevant publications. The inclusion criteria mainly focused on dermatophyte and Candida strains resistant to azoles, as well as publications that combined antifungal medications with natural compounds or other chemicals to combat resistance. Out of sixteen, fourteen articles focused on resistant strains of Candida and two on dermatophytes. Among articles published on resistant strains of Candida, five articles were based on combining azole with other drugs, while nine were with natural compounds like essential oils, curcumin etc. Whereas with resistant strains of dermatophytes, both articles were based on combining azole with natural compounds. It can be concluded that antifungal combinations against resistant strains of Candida and dermatophytes are more effective than single drugs. Combinatorial approaches have gained considerable scientific interest over the years, with promising results. Thus, it is worthwhile to continue research in this area.

Keywords: Antifungal Combination; Candida; Efflux pumps; Dermatophytes; Dermatophyte resistance.

The development of resistance against antifungal therapy is the primary emerging challenge for the coming era. Many fungus species may develop resistance to antifungal drugs after prolonged exposure, prophylaxis therapy and irrational use.¹ Resistance may arise from overexpression of genes encoding efflux pumps, overexpression of efflux pumps, reduced drug

import into fungal cells, and overexpression/ alteration/ reduced affinity of target enzyme [14-alpha demethylase] towards antifungal drugs. Efflux pumps are commonly present on fungal cells for detoxification and extended exposure to antifungal drugs; these efflux pumps and associated genes are over-expressed. Consequently, antifungal drugs are actively flushed from fungal

cells and resistance develops in susceptible fungi. Dermatophytes & yeasts, mostly *Candida* are the common fungi known for overexpression of efflux pumps, ABC (ATP binding cassettes) and MFS (major facilitator super family), and genes which are responsible for coding of these efflux pumps are mainly CDR1, CDR2 (Candida drug resistant gene) MDR1 (Multi drug resistance) FLU1 (Fluconazole resistance).²⁻³

Infection with resistant species is predominantly observed in immunocompetent and immunocompromised patients, where the host's immune status plays a significant role in the outcome. Chronic conditions with considerable morbidity rates can be severe in immunocompromised patients and result in invasive infection.

Candida is an opportunistic fungal pathogen, affecting the nails, skin, and oral and genital mucosa. Certain conditions like diabetes⁴, genetic disorders, and circulatory diseases impact the immune system, and such patients are at high risk of getting fungal infections.

Dermatophytes are a group of related fungi belonging to the three main categories, i.e. *Epidermophyton*, *Trichophyton*, and *Microsporum*. Two new classes i.e. *Lophophyton* and *Nannizia* were included according to the new proposal of phylogenetic taxonomy.⁵ Dermatophytosis is a superficial infection of hair, skin and nail caused by dermatophytes. Most commonly, it is caused by *Trichophyton* genus.⁶ The prevalence of both these infections is high worldwide and, if not treated appropriately, could lead to invasion into the deeper layers of skin and resistance.

Treatment/therapy

Two major classes of antifungal agents active against susceptible strains of *Candida* and dermatophytes uniformly, have been detailed in Table 1. For cutaneous infection, topical therapy is preferred and for extensive infection, systemic treatment.⁷ In general, topical therapy shows fewer side effects. The side effects are dose-related, dependent on the length of treatment and route of administration.

Among azoles, fluconazole (FLC) and itraconazole (ITZ) are the safest drugs. Both are triazoles and inhibit cytochrome P450 14 α lanosterol demethylase. Compared to FLC, ITZ

had a much lower MIC (Minimum Inhibitory concentration) value. On long-term azoles exposure unfortunately, there are an increasing number of resistant strains of *Candida spp.* and dermatophytes.⁸⁻⁹ The strains found to be resistant to FLC include *Trichophyton tonsurans* (*T. tonsurans*), *Microsporum canis*, *Microsporum gyp.* and *Trichophyton rubrum* (*T. rubrum*).⁸ The resistance was mediated by TruMDR2, TruMDR1, and ABC for FLC, and in the case of candida Cdr1p and/or CaMdr1p belonging to the ABC and MFS superfamilies. Among dermatophytes, *T. rubrum* prevalence increased in the past few years. Yamada *et al.*, (2021) proposed that ITZ resistance in *T. rubrum* was mediated by ABC transporter TruMDR2 and, to a small extent, by TruMDR3 genes.⁹

Studies indicate that the Azole class of drugs exhibits resistance by various mechanisms, primarily overexpression of the efflux pump. Azoles bind to cell efflux transporter proteins and are expelled from the cells. Overexpression of these efflux pumps can lead to treatment failure. Another reason might be the mutation of Erg II gene, which is responsible for drug target modification.^{8,10}

The major mechanisms of antifungal drug resistance and possible ways to overcome it are depicted in Figure 1.

The development of new antifungal drugs requires more time and efforts. Researchers are trying to resolve the problem of resistance in several ways; combination therapy is one of them.¹¹⁻¹² For a decade, many compounds, including phytoconstituents combined with standard antifungal drugs, were found to be effective against resistant strains of fungus, but these areas are less explored. Other advantages of combination therapy include reduced toxicity, fewer side effects compared to the single drug prescribed at a high dose, and enhanced antifungal effects.

In combination therapy, one molecule may sensitize fungal species against antifungal drugs primarily by blocking efflux pumps like ABC and MFS in which ABC is most often found to be overexpressed. These are two classes of azole pumps. These proteins carry compounds across the cell membrane with the help of some energy sources. Both have distinct protein domain parts, like the nucleotide-binding domain in the case of

ABC pumps and the transmembrane domain in the case of MFS pumps. The genes encode these pumps are CDR1, CDR2, FLU1, MDR1 and MDR2. In the past decade, much work have been done to resolve the issue of resistance.¹³

Brescini and co-workers (2021) reviewed antifungal combinations against recalcitrant and resistant dermatophytes. These combinations were mainly focused on combination of two antifungal drugs or combination of one antifungal drug with some chemical entity. In this review, the combination of antifungal drugs with plant-based molecules/phytoconstituents and the mechanism of combinatorial effect were less explored.¹²

In another review by Holmes *et al.*, (2016), all the targets for efflux pump against all the species, including *Candida* and dermatophytes were described. It was mainly focused on reversal of resistance by targeting these efflux pumps but the combination used to overcome resistance or for targeting the efflux pumps wasn't discussed.¹⁴

The present review mainly focuses on azoles, since these antifungal drugs play an essential role in treating dermatophytosis and candidiasis, where overexpression of drug efflux is a major cause of resistance. Different strategies like combination therapy suitable for reducing gene expression, blockage of efflux pumps and competitive substrates for efflux pumps proven effective against azole-resistant *Candida* and dermatophytes have been discussed in detail. The studies on the resistant of dermatophytes are less explored as compared to *Candida*. It needs immediate scientific intervention and attention before the disease becomes a pandemic.

MATERIALS AND METHODS

This systematic review was conducted as per the latest PRISMA guidelines [Fig: 2]. The published articles were exhaustively searched on PubMed and Google scholar during 23rd December 2021 to 14th March 2022. Different search strings like “antifungal resistance,” “combination used for overcoming,” etc were used while retrieving the articles. Consensus for all the discrepancies which occurred during the process of inclusion of papers was reached through discussion. The inclusion criteria were papers that focussed mainly on antifungal resistance. Another inclusion criterion was the articles that focussed on the combination of antifungal drugs with phytoconstituent or the combination that focussed primarily in efflux pump blockage. The exclusion criteria were the papers that didn't refer to dermatophyte or *Candida* resistance, unreachable publications and the ones that didn't specify the species name.

RESULTS AND DISCUSSION

A total of 61832 articles were initially identified (Figure 2). The articles published from 2010 to 2022 were selected through further screening. The exclusion was done based on papers that didn't focus on resistance, which didn't refer to dermatophytes or related species. Other exclusions were those papers that were out of the scope of this review. Total of sixteen articles were selected for the review. Out of 16 article 14 includes combinational therapy against resistant strains of *Candida*, only 2 articles were found to be based on

Table 1. Details of commonly used antifungal drugs, their mechanisms and adverse effects

S.	Antifungal agent	Mechanism of action	Adverse effects	Reference
1	Class: Imidazoles Bifonazole, Clotrimazole, Econazole, Ketoconazole, Luliconazole, Miconazole, Sertaconazole, and Tioconazole	Block the synthesis of ergosterol, alter the cell membrane permeability of fungi	Gastrointestinal disturbances like nausea, vomiting, abdominal pain, diarrhea and nephrotoxicity, hepatotoxicity photosensitivity, and neurotoxicity	[7]
2	Class: Triazole Fluconazole, Isavuconazole, Itraconazole, Posaconazole, Ravuconazole, Voriconazole, Luliconazole, and Lanocazole	Interruption of conversion of lanosterol to ergosterol		

combinational therapy against resistant strains of dermatophytes.

Combination against the resistant strain of *Candida isolates*

In recent years, a number of studies have been published on *Candida* as compared to other fungus species. Combination therapy has shown reversal of azole resistance as evidenced from the reduction in MIC values, reduction in gene expression etc. Most of these studies contain *in vitro* study data, thus lacking the studies of adverse effects associated with use of these drugs for antifungal therapies. A comprehensive account of antifungal combinations against azole resistant candida species with over expression of efflux pumps has been presented in Table 2.

Fluconazole and ketorolac

Sayed *et al.*, (2021) conducted the sensitivity and efficacy study of fluconazole and ketorolac combination on resistance species of

Candida albicans (*C. albicans*) obtained from acute myeloid leukemia patients and compared with monotherapy. They also confirmed the expression of efflux pump gene (CDR1, CDR2, MDR1) by real-time PCR on clinical isolates of *C. albicans*, which are responsible for resistance development. They reported a drastic decrease in the minimum inhibitory concentration of fluconazole and ketorolac combination as compared to fluconazole and ketorolac alone as 0.3-1.25 µg/ml, 160 µg/ml and 10 µg/ml respectively. Reported FICI (Fractional inhibitory concentration index) value (0.25) confirms synergism.¹⁵

Fluconazole and *Ginkgo biloba*

Yiman *et al.*, (2020) conducted a study where phytoconstituent like Ginkgolide B was combined with fluconazole and its effect on fluconazole-resistant *C. albicans* was observed. Fluconazole alone had no effect on an Azole-resistant strain of *C. albicans*, as seen by its

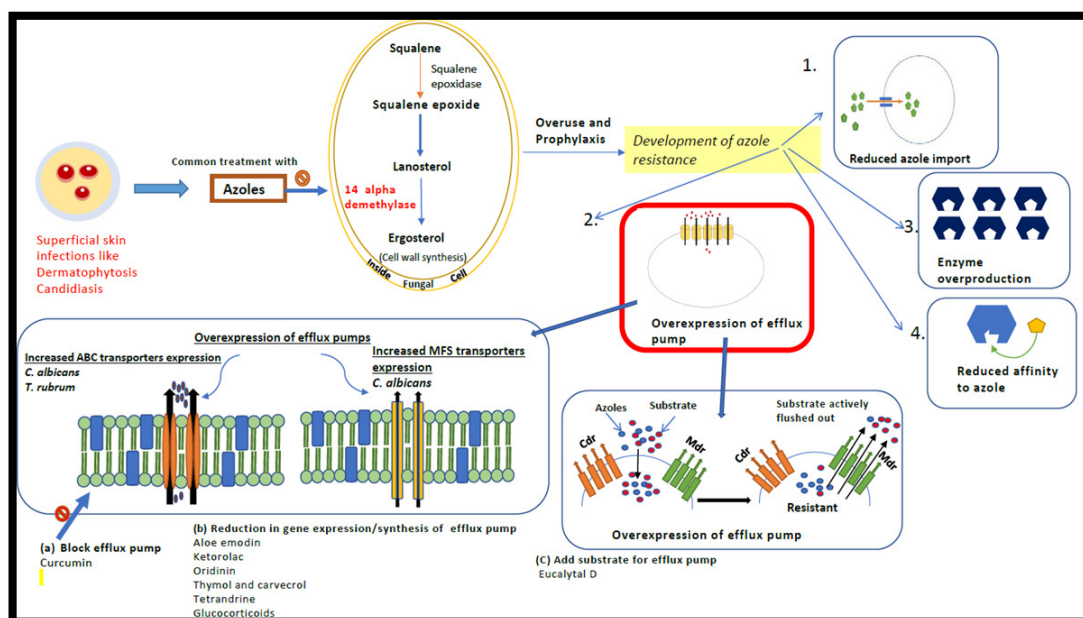


Fig. 1. Mechanism of antifungal drug resistance and possible ways of overcoming efflux-mediated azole drug resistance: Azole antifungals exert their action by inhibiting the enzyme 14-alpha demethylase, in turn inhibiting the process of cell wall synthesis in fungi. Due to irrational and/or prophylactic use of these drugs, fungi develop resistance via multiple mechanisms: (1) Reduced azole import, (2) Overexpression of efflux pump, leading to active flushing of azole drugs, (3) Enzyme overproduction (4) Reduced affinity to azole. These processes lead to a significant reduction in the concentration of azoles inside the cells, which results in failure of desired antifungal action. The consequences of over-expression of efflux pumps can be overcome by combined approach of (a) blocking of efflux pump (Curcumin), (b) reduction of genes synthesis/efflux pumps (aloe emodin, ketorolac, thymol, carvercol, tetrandrine, and ornidine glucocorticoids), (c) adding competitive substrate for efflux pump (Eucalypt D)

MIC value (>256 g/ml). The MIC was lowered from >512 g/ml to 0.25 g/ml in conjunction with *Ginko biloba*, indicating synergism. They also discussed that calcium homeostasis is the basis for the average growth and pathogenicity of *C. albicans*. The intracellular calcium concentration in these strains was altered, which can be the base of synergism. Yet the mechanism is not mentioned correctly. A fluorescent assay was performed, and the result indicated enhanced uptake of fluconazole when given in combination with ginkgolide.¹⁶

Eugenol and methyleugenol with fluconazole

Ahmed *et al.*, (2010) evaluated the efficacy of two bioactive compounds found in essential oil, eugenol and methyleugenol, alone and in combination with fluconazole against fluconazole resistant clinical *Candida* isolates.

The method used to confirm the resistance has not been discussed. Even though both the bioactive compounds were effective against resistant species, methyl eugenol was much more effective than eugenol. The MIC value of eugenol and methyleugenol against resistant strain ranged from 475 – 500 µg/ml and 340-350 µg/ml, while in the combination ranged from 110–120 µg/ml and 70-90 µg/ml, respectively. The high fungicidal effect of these compounds on combination with fluconazole was confirmed using a disc diffusion assay. Proper mechanisms regarding how this combination helps to overcome resistance have not been discussed. But it was reported that the lipophilic character of eugenol and methyleugenol might enable these compounds to enter into the membrane lipid bilayer altering the fluidity and

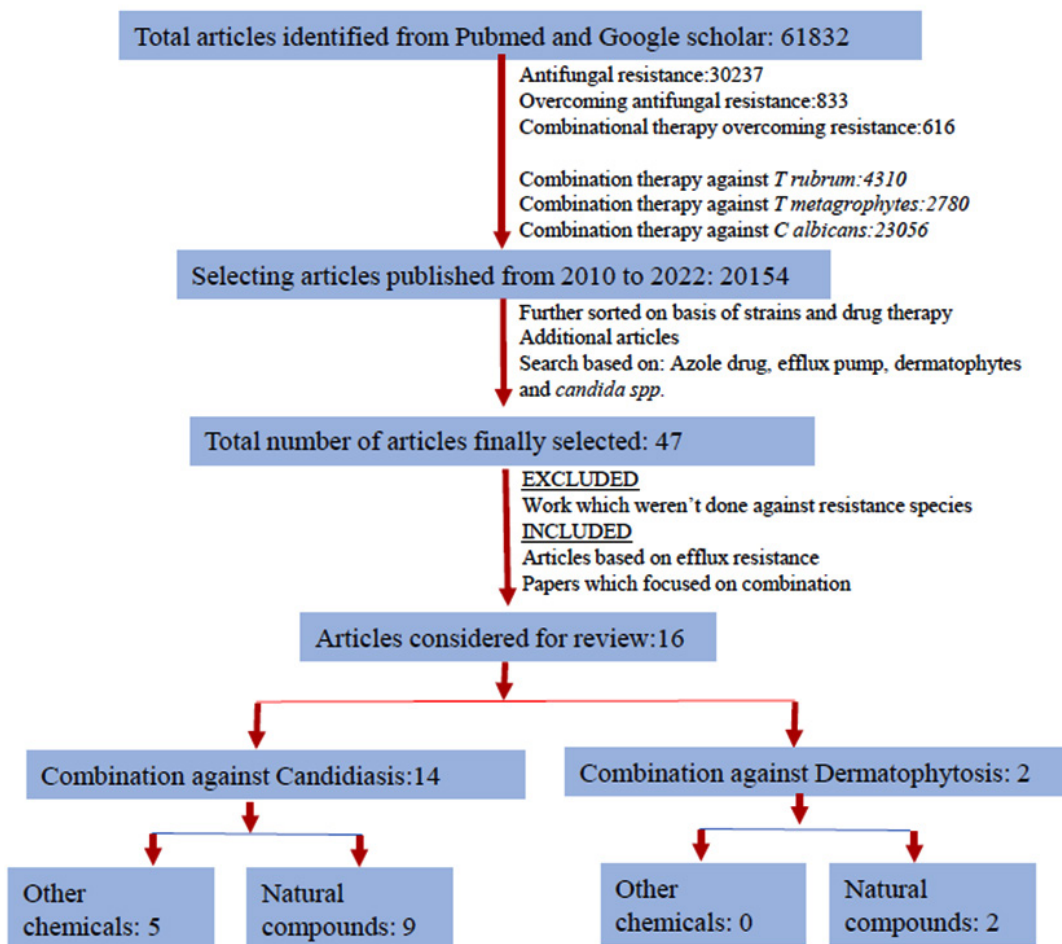


Fig. 2. Flowchart of the different phases of article selection of the review

Table 2. Antifungal combinations against azole resistant *Candida* species with over expression of efflux pumps

S.	Resistance sp.	Source	Therapy	Result	azole	MIC VALUES Phytochemical/ other chemical	combination	Confirmation tests	Possible mechanism against resistant	Reference
1	<i>C. albicans</i>	Clinical isolates from acute myeloid leukemia patients	Fluconazole (FLC) + ketorolac	FICI = 0.25 synergism & reversal of resistant	FLC160 µg/ml	KETER OLAC10 µg/ml	0.3-1.25 µg/ml	RT-PCR	Decrease in genes MDR1 CDR1 CDR2	[15]
2	<i>C. albicans</i>	NA	FLC + ginkgolide (GK)	Decreased MIC & reversal of resistant	FLC>512 µg/ml	GK>256 µg/ml	0.25 µg/ml	Increased uptake of fluconazole confirmed with fluorescent assay	Altered intracellular calcium concentration	[16]
3	<i>Candida</i> isolates	NA	FLC + Eugenol (EUG) / Methyl Eugenol (MEUG)	Decreased MIC & reversal of resistant	FLC >80 µg/ml	EUG 475 - 500 µg/ml MEUG 340-350 µg/ml	EUG+ FLC110-120 µg/ml MEUG+ FLC 70-90 µg/ml	Increased fungicidal effect confirmed with disc diffusion assay	Altering fluidity and permeability of membrane lipid bilayer	[17]
4	<i>Candida</i> isolates	NA	FLC+ thymol (THY) and carvacrol (CARV)	Decrease in MIC and larger inhibition zone & reversal of resistant	FLC2.5-7.5 µg/ml	THY 90-150 µg/ml CARV 50-100 µg/ml	THY + FLC 21-32 µg/ml CARV+ FLC 7-23 µg/ml	RT-PCR, Fluorescent assay	Decrease in MDR and CDR genes Reduction in efflux	[18]
5	<i>Candida</i> isolates	Isolates from vulvo vaginal candidiasis	FLC + Fluoxetine (FLO)	Reduction in MIC, synergism & reversal of resistant	FLC-64-0.25 µg/ml	FLO- 9.8-625 µg/ml	8-0.12 µg/ml	Molecular identification and an API 32C	NA	[19]
6	<i>C. albicans</i>	NA	FLC+ cyclosporine inhibitors (calcineurin inhibitors)	Synergism & reversal of resistance	—	—	FIC 2.0	Chequerboard method, time-killing test and fluorescent study	Inhibits biofilm formation	[20]
7	<i>C. albicans</i>	NA	FLC+ Tetrandrine (TET)	Synergism & reversal of resistance	FLC-0.25-0.5 µg/ml	TET-64-128 µg/ml	FLC 0.063-0.125 µg/ml TET 2-16 µg/ml	—	Inhibition of MDR CDR1 CDR2 genes & Inhibits biofilm formation	[21]
8	<i>C. albicans</i>	NA	FLC+ Dexamethasone (DXM)	Synergism & reversal of resistance	FLC>512 µg/ml	DXM 128 µg/ml	FLC <1 µg/ml DXM 32 µg/ml	RT-PCR	Decreased MDR1 expression and Phospholipase activity	[22]
9	<i>C. albicans</i>	NA	FLC+ Gentamycin (GN)	Synergism & reversal of resistance	FLC >512 µg/ml ITR >16 µg/ml	GM-512 µg/ml	FLC+GM1 µg/ml ITR+GM 0.25 µg/ml	Rhodamine efflux assay	Reduction in efflux & Reduction in phospholipase activity	[23]
10	<i>C. albicans</i>	Clinical isolates from cancer patients	FLC and itraconazole (ITR) + oridinin	Reduction in MIC & reversal of resistance	FLC-512 µg/ml ITR >8 µg/ml	—	FLC <8 µg/ml ITR <0.2 µg/ml	Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) & flow cytometry	Reduction in CDR1 CDR2 resistance genes, inhibition of drug efflux and induction of apoptosis	[24]
11	<i>C. albicans</i>	Clinical isolates	Antimicrobial photodynamic therapy (aPDT) + photosensitizer (PS) (aloe emodin)	Inactivated <i>C. albicans</i> and no dark toxicity.	—	—	—	Confocal laser scanning microscopy (CLSM) Transmission electron microscopy analysis, scanning electron microscopy	Induce damage to the cell wall, cytoplasm, and nucleus	[25]
12	<i>C. albicans</i>	NA	FLC + Curcumin	Reduction in MIC & reversal of resistance	FLC 256 µg/ml	—	<2 µg/ml	Checkerboard experiment & Nile red accumulation assay	Reduction in efflux activity confirmed	[26]
13	<i>Candida</i> isolates	Isolates from oral candidiasis patients	FLC + Citral (CT) (monoterpene)	Reduce the biofilm formation and exhibit synergism & reversal of resistant	FLC >16 µg/ml	CT >256 µg/ml	FLC + CT <1 µg/ml /mFICI <1	—	Efflux pump reduction encoded by MDR1	[27]
14	<i>C. albicans</i> (different strains)	Clinical isolates and ATCC-USA.	FLC + ITZ + Eucalyptol D	Synergism & reversal of resistant	>128 µg/ml (FLC) >8 (ITZ)	>128 µg/ml	2-8 µg/ml	Rhodamine efflux assay	Reduction in Cdr1p and Cdr2p gene expression, Reduction in efflux activity	[28]

Table 3. Antifungal combinations against azole resistant dermatophytes with over expression of efflux pumps

S.	Resistance sp.	Source	Therapy	Result	azole	MIC VALUES Phytochemical/ other chemical	combination	Confirmation tests	Possible mechanism against resistant	Reference
1	<i>T. rubrum</i> and <i>A. fumigatus</i>	NA	Essential oils like geraniol, Cinnamaldehyde + FLC	Synergism confirmed with FICI	FLC>64 µg/ml	—	<10 µg/ml	TEM analysis	Cell lysis and disorganisation of cytoplasmic content	[29]
2	<i>Trubrium</i> and <i>T. metagrophytes</i>	NA	Protocatechuic acid 3,4 diacetoxylbenzoc acid and its 14 alkyl derivative + FLC	Additive and synergistic effect	FLC-2 µg/ml	—	<0.5 µg/ml	-	Proper mechanism not been discussed	[30]

permeability, thus interfering with enzyme activity which helps in cell wall synthesis.¹⁷

Monoterpenes with fluconazole

Ahmed *et al.*, (2012) conducted a study where the efficacy of the combination of thymol and carvacrol with fluconazole was tested against fluconazole-resistant clinical *Candida* isolates. MIC values of thymol, carvacrol, and fluconazole for fluconazole-sensitive and fluconazole-resistant strains of *C. albicans* were 90-150µg/ml, 50-100µg/ml, and 2.5-7.5µg/ml, respectively. Using combination MIC was reduced to 21-32µg/ml, 7-23µg/ml, 0.5-2µg/ml respectively. MIC values and wider inhibition zones revealed that all of the isolates were sensitive to monoterpenes and their combination. The sensitivity index was comparatively larger for the carvacrol fluconazole combination [3.2+0.05]. A reduction in the efflux activity was confirmed by using a fluorescent assay. RT PCR (Real time polymerase chain reaction) was used to confirm the decrease in the quantity of CDR and MDR genes that encode for efflux pumps and are responsible for fluconazole resistance. *C. albicans* resistant strains overexpress just CDR1 and not MDR1, whereas *Candida glabrata* and *Candida krusei* overexpress both CDR1 and MDR1.¹⁸

Fluoxetine with fluconazole

Oliveira *et al.*, (2014) investigated the efficacy of fluoxetine alone and in combination with fluconazole against *Candida* strains isolated from patients with vulvo vaginal candidiasis. The combination resulted in a reduction in MIC values (decreased up to 64 fold). Among the tested strains, six of the candida species showed a synergistic effect, among which 4 of them were resistant to fluconazole whereas the remaining showed an indifferent effect which was evident from fractional inhibitory index values between 0.15-0.31 and 0.63-1, respectively.¹⁹

Calcineurin inhibitors with fluconazole

Kaya *et al.*, 2021 reported the calcineurin mutants exhibited increased susceptibility towards antifungal drugs, including azoles. Calcineurin inhibitors such as cyclosporin A and tacrolimus demonstrated synergistic activity with fluconazole against various fungi, including *C. albicans*. The interaction of cyclosporin A with azoles such as fluconazole and itraconazole against azole susceptible and resistant *C. albicans* was studied

using the checkerboard method. Based on a fluorescent study, this combination inhibited *C. albicans* growth and hyphal formation, which in turn inhibits biofilm formation. Similarly, tacrolimus in combination with fluconazole could reverse drug resistance. The synergistic activity was studied with the checkerboard method and time-killing test.²⁰

Tetrandrine with fluconazole

Keyal *et al.*, 2017 evaluated that Tetrandrine (ancient Chinese medicine), a dibenzylisoquinoline alkaloid increased the sensitivity of *C. albicans* towards fluconazole. Both compounds had synergistic interactions. Tetrandrine could inhibit the expression of genes like MDR1, FLU1, CDR1, and CDR2 reducing the drug efflux and increasing intracellular azole accumulation. It also had antibiofilm activity and targeted sterol biosynthesis altering fluidity and permeability of cell membrane.²¹

Glucocorticoid with fluconazole

Wenwen *et al.*, 2017 combined glucocorticoids like dexamethasone and budesonide along with fluconazole. They exhibited synergistic activity against *Candida spp.* which was evident by FICI index of less than 0.5 but had no antifungal action when used alone. RTPCR results confirmed the reduction in expressions of drug-resistant genes like CDR1, CDR2, and MDR1, which indicates synergism and extracellular phospholipase activity. A critical virulence factor was also measured using the egg yolk agar method showed a reduction in virulence.²²

Gentamycin with fluconazole

Lu *et al.*, 2017 combined gentamycin with fluconazole against resistant *C. albicans* species. The combination reduced the MIC and FICI values by 0.13-0.14, confirming synergism. The rhodamine efflux assay was used to verify the reduction in efflux activity dose-dependently. A decrease in phospholipase activity also showed a reduction in the pathogenicity of the strains.²³

Oridonin with azole

Chen *et al.*, 2020 used a combination of azole drugs (Itraconazole, Fluconazole) with oridonin against resistant *C. albicans* strain isolated from cancer patients. A reduction in MIC was observed. MIC of fluconazole and itraconazole decreased from >512 µg/ml and >8 µg/ml to <8 and <0.2 µg/ml, respectively. The resistance reversal

mechanisms, namely inhibition of drug efflux and induction of apoptosis, were investigated by flow cytometry. Expression levels of the efflux pump-related genes CDR1 and CDR2 were assessed by RT-qPCR.²⁴

Antimicrobial photodynamic therapy with aloe-emodin

Leu *et al.*, 2019 evaluated the photodynamic effect of Aloe-emodin on drug-resistant *C. albicans*. Aloe-emodin could effectively inactivate *C. albicans* in a concentration-dependent manner in the presence of light. The uptake of aloe-emodin by cells was studied using confocal laser scanning microscopy. SEM and TEM analysis showed that therapy could induce the damage of the fungal cell wall, cytoplasm and nucleus.²⁵

Curcumin with fluconazole

Gomez *et al.*, 2012 evaluated the capability of curcumin to sensitize the clinical fluconazole-resistant isolate of *C. albicans*. The MIC was reduced from 256 µg/l to <2 µg/l. Synergistic interaction was assessed using a checkerboard experiment. Efflux pump activity was evaluated using Nile red accumulation assay. 11 µM of curcumin was able to restore Nile red accumulation indicating inhibition of efflux activity.²⁶

Fluconazole with citral

In an investigation by Cadena *et al.*, 2022, citral, a phytochemical constituent of lemongrass oil, was found effective in combating *Candida* infections when combined with fluconazole. Citral reduced the biofilm formation and the combination exhibited synergism against most of the strains tested with FICI 0.5. RNA analysis further revealed the efflux pump reduction encoded by MDR1. However, the results weren't conclusive about the expression of ERG11 gene. The expression of ERG11 gene weren't significant even though a slight upregulation was observed.²⁷

Eucalyptal D with Fluconazole

Xu *et al.*, (2019) reported that Eucalyptal D (ED), significantly enhance the anticandidal activity of fluconazole (FLC) in treating FLC resistant *C. albicans*. They performed, checkerboard microdilution assay, rhodamine 6G (R6G) efflux assay, and reverse transcription PCR analysis and reported that the combination shows a synergistic effect. It was hypothesized that ED was a substrate of efflux pump (Cdr1p and Cdr2p), actively

flushed out in place of fluconazole in resistant *C. Albicans*.²⁸

Combination against resistant dermatophytes

A few studies have been published on antifungal combinations against azole resistant dermatophytes with over-expression of efflux pumps.²⁹⁻³⁰ These are detailed in the Table 3. Both the studies were conducted *in vitro* and hence lacking in studies/data on adverse effects.

Essential oils with fluconazole

In a study Khan *et al.*, 2011 analyzed several essential oils and their active compounds like S. Aromaticum, C. Verum, C. Martini, Geraniol, Cinnamaldehyde etc. The strains *A. ompounds* and *T. rubrum* were tested for azole drug resistance and fluconazole showed higher resistance level than itraconazole. Most of the essential oils showed intense activity against tested fungi, among which cinnamaldehyde showed the highest zone of inhibition against *T. rubrum*. When combined with fluconazole, all the tested oils showed a significant synergistic interaction against *T. Rubrum* and *A. ompounds*, evident from FICI values. Among all the tested ones, cinnamaldehyde showed the highest degree of synergism. Its effect on ultrastructure was investigated further using TEM, and it was discovered that hyphal specimen treatment with cinnamaldehyde resulted in cell lysis as well as cytoplasmic disorganization.²⁹

Protocatechuates with fluconazole

Luciana *et al.*, (2014) investigated the antifungal activity of protocatechuic acid 3,4 diacetoxybenzoic acid and its 14 alkyl derivative against clinical strains of *T. rubrum* and *T. metagrophytes* alone and in combination with fluconazole. Both these compounds are considered to have low antidermatophytic activity because of their very high MIC values (125mg/ml and above). But addition of methyl groups to these compounds showed reduction in MIC values and increase in antifungal activity. The best MIC values were shown by pentyl, hexyl, heptyl, octyl, nonyl and decyl protocatechuate compounds ranging from 1.95 to 7.8mg/ml. It was reported that carbon chains have a crucial role in antifungal and antioxidant activity, but the actual mechanism has not been discussed in the paper. FIC index of 0.49 showed additive and synergistic action of all the compounds in combination with fluconazole.³⁰

CONCLUSIONS

The persistence and frequent recurrence of dermatophyte infections necessitate prolonged treatment. This leads to the risk of severe adverse effects and development of drug resistance. It has already been witnessed in systemic fungal infections caused by *Candida* and the emergence of drug resistant strains among *T. rubrum*. Most of the *in vitro* studies have investigated the combination of azole antifungal agents with several other chemical compounds and phytochemicals. The association between an antifungal drug and plant extract, including essential oils, seems to evoke a particular interest. The reciprocal potentiation of the molecules upon combination makes these approaches particularly appealing against resistant strains. Although the intrinsic mechanisms of antifungal activity of these natural products have not been thoroughly investigated, several cell targets are simultaneously involved, thereby chances of occurrence of resistance remains minimal. Anti-fungal studies indicate that an association of antifungal agents is influential, and it might be helpful in speeding up the microbiological healing of superficial infections. One of the major limitations with published studies is lack of sufficient evidences in support of combination therapy over treatment with single drug as a viable approach in minimizing the adverse effects associated with irrational use of individual drugs. In summary, antifungal combinations against dermatophytes and *Candida* have gained considerable scientific interest over the years. To establish this approach as a reliable treatment option in clinical settings, additional studies are warranted.

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