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Synergistic antifungal effect of cyclized chalcone derivatives and fluconazole against *Candida albicans*

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The occurrence of invasive fungal diseases, particularly in immunocompromised patients, is life-threatening and increases the economic burden. The rising problem of multi-drug resistance is becoming a major concern for clinicians. In addition, a repertoire of antifungal agents is far less in number than antibacterial drugs. To combat these problems, combination therapy has gained a lot of interest. We previously reported the synergistic interaction of some mono- and bis-dihydropyrimidinone and thione derivatives with fluconazole and amphotericin B for combination antifungal therapy. In this study we used the same approach and synthesized different azole and non-azole derivatives of mono-(M) and bis-(B) chalcones and evaluated their antifungal activity profile alone and in combination with the most commonly used antifungal drug – fluconazole (FLC) – against seven FLC susceptible and three FLC resistant clinically isolated *Candida albicans* strains. Based on the minimum inhibitory concentration results, the bis-derivatives showed lower MIC values compared to their mono-analogues. Both fractional inhibitory concentration index and isobologram results revealed mostly synergistic, additive or indifferent interactions between the tested compounds and FLC against different *Candida* isolates. None of the tested compounds showed any effect on energy dependent R6G efflux, revealing that they do not reverse the mechanism of drug efflux. However, surprisingly, these compounds profoundly decreased ergosterol biosynthesis and showed down regulation of *ERG11* gene expression, which is the possible mechanism of reversal of azole drug resistance by these compounds. These results provide a platform for further research to develop pyrimidinone/thione ring containing compounds as promising new antifungal agents, which could be used in antifungal combination therapy.

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1. Introduction

Invasive fungal infections, mostly caused by *Candida*, are responsible for high morbidity and mortality worldwide, and

drug resistance exacerbates them.¹ Currently available antifungal drugs approved by the FDA belong to different chemical classes such as polyenes, pyrimidines, azoles, and echinocandins.² *Candida* is now becoming increasingly resistant to first-line and second-line antifungal medications, namely, fluconazole and echinocandins.^{3,4} Polyenes (amphotericin B and nystatin) cause serious host toxicity whereas azoles are fungistatic and their prolonged use contributes to the development of drug resistance in *C. albicans* and other species.^{4–6} Among azoles, fluconazole shows good antifungal activity with relatively low toxicity and is preferred in first-line antifungal therapy. However, fluconazole is not effective against various invasive pathogens and is prone to severe drug resistance.^{7,8} Unfortunately, our repertoire of antifungal agents is limited, and no new drug has been discovered for quite a long time.^{2,9} At this point new therapeutic strategies are urgently needed to combat this problem. As an efficient strategy to improve the antifungal spectrum, combination therapy offers potential benefits like a broad

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spectrum of efficacy, greater potency than either of the drugs used in monotherapy, improved safety and tolerability, and reduction in the number of resistant organisms.^{10–12} Drug combinations have been used for the treatment of diseases like cancer, HIV or cardiovascular diseases and it is believed that drug combinations are better at controlling complex diseases with minimal resistance.^{13–15} Combination antifungal therapy for invasive fungal infections is receiving growing attention.^{10,11,16,17} and considering our previous work^{18–22} it is our premise that combining a new antifungal agent with a known antifungal drug, with a different or similar mechanism of action, would represent a novel therapeutic approach which could circumvent the multi-drug resistance problem.

In a previous study we observed that a bis-DHPM derivative (ethyl 4-[5-(ethoxycarbonyl)-6-methyl-2-sulfanylidene-1,2,3,4-tetrahydropyrimidin-4-yl]phenyl}-6-methyl-2-sulfanylidene-1,2,3,4-tetrahydropyrimidine-5-carboxylate), as a flucytosine mimic showed better synergy compared to its mono-DHPM analogue.¹⁸ In this study we synthesized mono and bis-chalcone and their cyclized analogues for combination studies with fluconazole. Chalcone is an important scaffold which can be synthetically manipulated into different heterocyclic compounds of immense biological importance.²³ Our interest was to cyclize chalcones into their corresponding azole (pyrazoline, oxazoline) and non-azole (pyrimidinone/thione) derivatives to find the most suitable structure which could show synergy with fluconazole.

Azole drug resistance in *Candida* is often related to drug efflux pumps and over expression of genes involved in the ergosterol biosynthesis pathway.^{24,25} Therefore targeting the efflux pumps and/or ergosterol biosynthesis pathway may reverse the azole drug resistance in these pathogens. We performed rhodamine 6G (R6G) assays on the efflux pumps

to investigate whether the synergistic interaction of FLC and the tested compounds could reverse FLC resistance in tested *Candida* isolates. We also studied the effect of the test compounds on ergosterol biosynthesis by quantifying the total intracellular sterols and checked the effect of these compounds on the expression of one of the striking genes of the ergosterol biosynthesis (ERG11) pathway by qRT-PCR.

Since none of the current antifungal agents have all the characteristics of an ideal antifungal agent²⁶ and the discovery rate of new antifungals is declining, we believe that this approach has the potential to discover new antifungal agents as potentiators to be used in combination therapy treatment of invasive fungal diseases.

2. Results and discussion

2.1. Chemistry

The α,β -unsaturated ketones **1M** and **1B** were synthesized by a base catalyzed Claisen–Schmidt condensation reaction of acetophenone with benzaldehyde and 1,4-dicarboxaldehyde, respectively. The cyclized derivatives were obtained by following the synthetic routes outlined in Schemes 1 and 2. The structures of both the chalcones **1M** and **1B** were confirmed by their spectral data revealing that they are geometrically pure with *trans*-configuration as indicated by the coupling constant ($J_{\text{H}\alpha\text{--H}\beta} = 16\text{--}15\text{ Hz}$) in their ^1H NMR spectra. In general two *trans* protons corresponding to the chalcone moiety appeared as two doublets. The proton at the β -position to the carbonyl group resonates more downfield at δ 7.45–7.37 while the α -proton resonates at δ 7.25–7.14. The ^1H NMR spectra of **3M** and **3B** showed the characteristic ABX pattern of three protons of pyrazoline including two at C4 and one at C5. A similar pattern was shown by the oxazoline ring

Scheme 1 Synthesis of chalcone (**1M**) and its cyclized analogues (**2M–6M**). Reagents and conditions: (a) hydrazine hydrate/EtOH/reflux; (b) thiosemicarbazide/AcOH/dioxane/reflux; (c) hydroxylamine hydrochloride/EtOH/reflux. (d) Urea/KOH/EtOH/reflux; (e) thiourea/KOH/EtOH/reflux.

Scheme 2 Synthesis of bis-chalcone (**1B**) and its cyclised analogues (**2B–6B**). Reagents and conditions: (a) hydrazine hydrate/EtOH/reflux; (b) thiosemicarbazide/AcOH/dioxane/reflux; (c) hydroxylamine hydrochloride/EtOH/reflux. (d) Urea/KOH/EtOH/reflux; (e) thiourea/KOH/EtOH/reflux.

containing compounds **4M** and **4B**. The benzylic proton CH (H-4) of **5M**, **6M**, **5B**, and **6B** appeared as a doublet of doublets around 7.20–7.03 ppm due to its coupling with the adjacent NH (H-3) and CH (H-5) proton of the pyrimidinone/thione ring. However, in all cases, the NH (H-3) proton appeared as a broad singlet around 10.99–9.55 ppm due to poor resolution. Another NH (H-1) also appeared as a singlet in all spectra. Similarly distinctive peaks characteristic of the structures of the compounds were observed in the ^{13}C NMR spectra. FTIR spectra also showed characteristic vibrational bands for all the functional groups and unsaturation in the compounds. ESI⁺ MS data of all the compounds showed expected molecular ion peaks corresponding to the molecular masses of the compounds. The detailed physical and spectroscopic data are given in the Experimental section.

2.2. Microbiological analysis

2.2.1. Comparative antifungal activity of compounds. The MIC of fluconazole against susceptible and resistant *C. albicans* strains ranged from 4 to 31 $\mu\text{g mL}^{-1}$ and 250 to 500 $\mu\text{g mL}^{-1}$, respectively (Table 1). All the tested compounds showed potent antifungal activities against both fluconazole susceptible and resistant *C. albicans* strains. However, fluconazole resistant strains achieved slightly higher MIC values. The highest antifungal activity was shown by **6B** against both the fluconazole susceptible (median MIC of 0.5 $\mu\text{g mL}^{-1}$) and resistant strains (median MIC of 2 $\mu\text{g mL}^{-1}$). On the basis of the median MIC values against fluconazole susceptible strains, the order of potency of the tested compounds was **6B**

$> \mathbf{5B} > \mathbf{6M} \geq \mathbf{5M} > \mathbf{4B} > \mathbf{2B} \geq \mathbf{1B} > \mathbf{4M} \geq \mathbf{2M} > \mathbf{1M} > \mathbf{3B} > \mathbf{3M}$. Comparatively bis-chalcone derivatives showed higher antifungal activity against all the strains tested than their respective mono-analogues. These results are congruent with our previous findings where bis-pyrimidinone/thione derivatives were found to be more efficacious than their corresponding mono-analogues.¹⁸ The results from this study also suggest that the other cyclized analogues such as pyrazoles, pyrazolines and oxazolines are not suitable structures for further optimization because of their poor MIC values. Nevertheless, studies have shown that pyrazoles and pyrazolines can

Table 1 Minimum inhibitory concentrations ($\mu\text{g mL}^{-1}$) of mono- (M) and bis- (B) chalcone derivatives, and fluconazole (FLC), against fluconazole susceptible and resistant *C. albicans* strains

Compounds	Median (range) MIC in $\mu\text{g mL}^{-1}$ against <i>C. albicans</i>	
	Fluconazole susceptible strains including ATCC90028 ($n = 7$)	Fluconazole resistant strains ($n = 3$)
Fluconazole	4 (2–8)	250 (125–500)
1M	31 (31–62)	62 (31–62)
1B	8 (2–16)	16 (16–31)
2M	16 (16–62)	125 (62–125)
2B	8 (2–16)	31 (31–125)
3M	500 (125–500)	500 (500)
3B	62 (8–125)	125 (125–500)
4M	16 (4–31)	31 (16–31)
4B	4 (2–8)	8 (8–16)
5M	2 (1–8)	8 (8–16)
5B	1 (0.25–2)	4 (4–8)
6M	2 (0.5–4)	8 (8–16)
6B	0.5 (0.125–1)	2 (2)

have antiviral, antibacterial and antifungal activity against filamentous fungi.^{27–29} Oxazolines are also known to have some antifungal activity against some unicellular fungi.^{30,31} In another study, pyrazole carboxylic and dicarboxylic acid derivatives have shown poor anti-*Candida* activity, which has been related to the positions of the electronegative atoms in the pyrazole substituents and the amount of associated charges on these electronegative atoms play an important role in regulating the antifungal activity for the *C. albicans* strains.³²

5B and **6B** are bis-pyrimidithione derivatives, whereas **5M** and **6M** contain a single pyrimidithione ring in their skeleton. The activity of the bis-analogues is further governed by the presence of sulphur as a thioamide group in **6B** in place of oxygen in **5B**, where **6B** showed enhanced activity compared to **5B**. However, in any case the bis-analogues were more active than their corresponding mono-analogues, which led us to say that the presence of similar bioactive rings in a single molecular scaffold enhances its biological profile. This enhancement could also be due to the strong interactions (hydrogen bonding, van der Waals or hydrophobic) of the bis-analogue with the target, which results in the enhancement of the activity. The presence of thione sulphur (C=S) in **6B** in place of the keto oxygen (C=O) in **5B** may also be a reason for its higher activity possibly due to the higher nucleophilic character of sulphur, due to its large size, which makes it readily polarizable and its lone pairs of electrons readily accessible.

2.2.2. Combination interaction with fluconazole. To investigate the combination interaction of all the synthesized derivatives with FLC in 1 : 1 ratios, fractional inhibitory concentration indices (FICI) were determined. The mean FICI values for all the fluconazole susceptible strains ranged from 0.26 to 3.34, whereas for fluconazole resistant strains mean FICI values were 0.12 to 1.55 (Table 2). Out of the 84 combinations for all the compounds against seven fluconazole susceptible strains, 29% ($n = 24$) showed synergy while 38% ($n = 32$) showed additive, 27% ($n = 23$) showed indifferent and 6% (n

= 5) showed antagonistic interactions. These figures for the three FLC resistant strains for 36 combinations are 53% ($n = 19$) for synergy, 28% ($n = 10$) for additive, and 19% ($n = 7$) for indifferent; however, no antagonistic interaction was observed for any of the derivative. Of all the tested compounds, **5B** and **6B** showed synergism against fluconazole susceptible strains and resistant strains which suggests that if these compounds are developed into therapeutic agents, they will be effective against fluconazole resistant and susceptible strains of *C. albicans*. In addition **5M**, **6M** and **1B** also showed a synergistic effect against fluconazole resistant strains only, which does not have clinical implications because newly developed drugs should be effective for many strains of *C. albicans*, regardless of their resistance profile.

Some possible mechanisms for the synergy have been proposed.³³ Firstly, one compound may act on its own and the second compound can attach to the target site, which would facilitate and enhance the attachment of the first compound to the target site. Secondly, two drugs can target different sites or biological pathways and exert a collective effect. Finally, two drugs can act on the same biological pathway at two different stages enhancing the activity. These mechanisms can improve the pharmacokinetics of the drugs, slow down metabolism and elimination, lower the doses and hence toxicity and improve efficacy.

2.2.3. Varied ratio combinations. As reported earlier it is impossible to obtain better formulations in 1 : 1 ratio combinations,³⁴ and therefore we further investigated nine different ratio combinations of the test compounds and FLC for one susceptible and one resistant synergy combinations. Based on the low FICI values in a 1 : 1 ratio, combinations of **6B** with FLC against *C. albicans* 0079gr (FICI = 0.127) and **5B** with FLC against *C. albicans* 167–2 (FICI = 0.031) were selected for in-depth varied ratio combinations. From the FIC values, isobolograms were constructed as detailed in the Materials and methods section and from the isobolograms, it is evident that most of the combinations, irrespective of the

Table 2 Fractional inhibitory concentration index of the six mono- (M) and bis- (B) chalcone derivatives with fluconazole

Combination 1 : 1	Fractional inhibitory concentration index (FICI) for <i>C. albicans</i>			
	Fluconazole susceptible strains including ATCC90028 ($n = 7$)		Fluconazole resistant strains ($n = 3$)	
	Mean $\pm$ SD (range)	Interpretation	Mean $\pm$ SD (range)	Interpretation
1M + FLC	1.99 $\pm$ 1.28 (0.75–4.38)	IND	0.58 $\pm$ 0.03 (0.562–0.62)	ADD
1B + FLC	0.78 $\pm$ 0.42 (0.31–1.5)	ADD	0.27 $\pm$ 0.00 (0.266–0.27)	SYN
2M + FLC	1.5 $\pm$ 0.73 (0.63–2.5)	IND	1.55 $\pm$ 0.95 (0.625–1.5)	IND
2B + FLC	0.98 $\pm$ 0.31 (0.63–1.5)	ADD	1.35 $\pm$ 0.87 (0.531–2.27)	IND
3M + FLC	3.34 $\pm$ 1.29 (1.97–4.28)	IND	1.25 $\pm$ 0.35 (0.750–1.5)	IND
3B + FLC	1.00 $\pm$ 0.56 (0.53–2.19)	IND	0.79 $\pm$ 0.62 (0.372–1.5)	ADD
4M + FLC	1.21 $\pm$ 0.42 (0.75–2)	IND	0.54 $\pm$ 0.02 (0.531–0.56)	ADD
4B + FLC	0.61 $\pm$ 0.13 (0.37–0.75)	ADD	0.56 $\pm$ 0.44 (0.532–1.02)	ADD
5M + FLC	0.75 $\pm$ 0.46 (0.31–1.5)	ADD	0.22 $\pm$ 0.08 (0.129–0.27)	SYN
5B + FLC	0.27 $\pm$ 0.07 (0.13–0.38)	SYN	0.12 $\pm$ 0.12 (0.031–0.25)	SYN
6M + FLC	0.63 $\pm$ 0.28 (0.37–1.13)	ADD	0.26 $\pm$ 0.01 (0.254–0.27)	SYN
6B + FLC	0.26 $\pm$ 0.059 (0.13–0.31)	SYN	0.25 $\pm$ 0.00 (0.251–0.25)	SYN

SYN: synergy; ADD: additive; IND: indifferent.

ratios combined, were either within the triangle of synergism (<0.5) or between 0.5 to 1 values representing additive interactions (Fig. 1). None of the combinations fall in the indifferent or antagonistic periphery. The best ratio showing significant synergistic interaction in both isobolograms is 6:3 of the tested compounds and FLC, respectively.

2.2.4. Effect on drug efflux pumps. To elucidate the possible mechanism of action, we investigated the effect of the synergistic combination of the tested compounds and fluconazole on the reversal of fluconazole resistance in *Candida* cells, by measuring the efflux of R6G by efflux pumps. Rhodamine 6G is a fluorescent dye and can be used as a substrate for MDR efflux pumps to investigate their functional activity.^{35,36} Rhodamine 6G passively enters into the cells and is pumped out by the ATP-dependent efflux pumps of the ABC superfamily in FLC-resistant *Candida* cells.³⁷ Both FLC susceptible and FLC resistant *Candida* cells showed prominent R6G uptake when suspended in glucose free medium (Fig. 2). After 25 minutes of incubation, the uptake reached equilibrium for both susceptible and resistant cells. No significant differences were observed between fluconazole susceptible (7.03 ± 0.12 nmol 10^{-8} cells) and fluconazole resistant cells (6.94 ± 0.17 nmol 10^{-8} cells). However with the introduction of 0.1 M glucose, FLC resistant cells started pumping out R6G (2.40 ± 0.27 nmol 10^{-8} cells) to the extracellular media while as for susceptible cells the least quantity of extracellular R6G was quantified (0.53 ± 0.04 nmol 10^{-8} cells). The inhibition of energy dependent R6G efflux against susceptible and resistant strains is less than 20% in all the tested compounds except 6B, which inhibited R6G efflux by 28%. From these results, it is evident that the resistant cells with the help of membrane efflux pumps showed energy dependent R6G efflux. In the case of both mono- and bis-analogues treated *Candida* cells, no effect on the energy dependent R6G efflux was observed (Fig. 2) which suggests that none of the test compounds reverse the mechanism of drug efflux. Therefore, the synergistic antifungal interactions of fluconazole

with these derivatives are related to some other mechanisms extraneous to the efflux pumps.

2.2.5. Effect on synthesis of ergosterol. Next we set out to study the effect of the synthesized compounds on ergosterol biosynthesis by quantifying the total intracellular sterols in control and treated cells. Fig. 3A and B, respectively, presented the effect of mono- and bis-chalcone and their derivatives on FLC susceptible *C. albicans* ATCC90028 and FLC resistant *C. albicans* A71 at MIC and $\frac{1}{2}$ MIC values. The results showed a 66–99% decrease in ergosterol content when FLC susceptible cells were treated with MIC values of bis-chalcone derivatives. These figures range from 49–94% when FLC resistant cells were treated with MIC values of bis-chalcone derivatives. As expected, FLC at a concentration of $8 \mu\text{g mL}^{-1}$ showed inhibition of ergosterol biosynthesis by 100% and 11% in FLC susceptible and FLC resistant cells, respectively. At sub-MIC values of some, more than 50% ergosterol biosynthesis inhibition was observed which indicates that these *C. albicans* isolates belong to the ergosterol tolerant class. These results are in congruence with our previous findings where *C. albicans* were reported to belong to the ergosterol tolerant class.³⁸ The ergosterol tolerant class is a group of fungi which are tolerant to ergosterol biosynthesis inhibition. A decrease in the ergosterol level affects the membrane permeability and fluidity, which could be a responsible mechanism for these compounds irrespective of their structure to reverse the drug resistance of FLC-resistant isolates.

2.2.6. Effect on *ERG11* expression. Real time PCR was performed to study the transcriptional levels of the *ERG11* gene, which is one of the most important genes in the ergosterol biosynthesis pathway and its upregulation plays a critical role in azole drug resistance.³⁹ The *ERG11* gene transcribes to lanosterol 14α -demethylase, which is an important protein of the pathway.³⁹ As the test compounds decreased sterol content, we hypothesized their role in changing the corresponding gene expression levels. We, therefore, tested the effect of the test compounds at their MIC values for the

Fig. 1 Representative isobolograms for the synergistic interactions in nine different ratios of 6B with fluconazole (A) against fluconazole susceptible *C. albicans* 0079gr and 5B with fluconazole against fluconazole resistant *C. albicans* 167-2.

Fig. 2 Energy dependent R6G efflux in fluconazole susceptible *C. albicans* ATCC90028 cells (open symbols) and in fluconazole-resistant *C. albicans* A71 (filled symbols), incubated either with R6G (10 μM) alone (squares) or with R6G plus mono- and bis-chalcone derivatives. All mono-analogues are represented by circles while all bis-analogues are represented by triangles.

compounds caused reductions of *ERG11* gene expression by 1.5–13 and 1.25–10 fold for FLC susceptible and FLC resistant *Candida* isolates, respectively. Fluconazole as expected down-regulates the *ERG11* gene in FLC susceptible cells while in comparison upregulation of this gene was observed in FLC

resistant cells. Upregulation of the *ERG11* gene in resistant cells results in an increase in the level of ERG11p; this has a critical role in azole resistance. These results are congruent with the decrease in ergosterol biosynthesis as observed in sterol quantification assay. It has already been reported that a point mutation or alteration of genes involved in the ergosterol biosynthesis pathway plays a role in azole drug resistance and therefore a decrease in ergosterol content and downregulation of the *ERG11* gene suggest that these compounds reverse azole drug resistance.

Another theoretically possible mechanism of action of these active pyrimidinone/thione analogues could be similar to one of the proposed mechanism of action of other pyrimidine antifungals, where they act as potent inhibitors of thymidylate synthetase, which is a key enzyme in the biosynthesis of DNA. Since thymidylate synthetase is a crucial source of thymidine, its deficiency consequently leads to fungal DNA synthesis inhibition.⁴⁰ These propositions however demand further studies which are currently underway in our laboratories.

3. Conclusion

In conclusion, mono- and bis-chalcones were cyclized into their corresponding azole and non-azole analogues which showed varied degrees of antifungal properties, of which **5B** and **6B** showed prominent antifungal activity against both the fluconazole susceptible and fluconazole resistant *C. albicans* strains. In addition, both these compounds showed good synergistic antifungal effect with fluconazole. Despite significant synergistic interactions, none of the combinations targeted the efflux pumps to reverse the drug resistance in fluconazole resistant *C. albicans*. However, investigation of the mechanism of action indicated that the deficiency of ergosterol and the downregulation of the *ERG11* gene contributed to the synergistic action and reversal of azole drug resistance by these newly synthesized derivatives. Further research is required to optimize the structure of the pyrimidinone/thione analogues to improve the efficacy and pharmacokinetic profile, and to overcome efflux pump mediated drug resistance, which could provide alternative clinical applications to treat drug resistant fungal species.

4. Materials and methods

All the required solvents and organic reagents were purchased from Sigma Aldrich and Merck. Melting points (mp) were determined using a Mel-temp instrument, and the results were uncorrected. Elemental analyses were performed on a Heraeus Vario EL-III analyzer. The results were within 0.4% of the theoretical values. FT-IR spectra were recorded using a Thermo Nicolet 380 instrument equipped with a Smart Orbit ATR attachment. ¹H NMR and ¹³C NMR spectra were recorded on a Bruker AVANCE 300 (300.13) MHz spectrometer using DMSO-*d*₆/CDCl₃ as solvent with TMS as internal standard. Splitting patterns are designated as fol-

lows; s, singlet; d, doublet; dd, doublet of doublets; t, triplet; q, quartet; and m, multiplet. Chemical shift values are given in ppm. MS (ESI) was performed on a MICROMASS QUATTRO II triple quadrupole mass spectrometer. Reactions were monitored using thin-layer chromatography (TLC) using commercially available precoated plates (Merck Kieselgel 60 F254 silica). Visualization was achieved with UV light at 254 nm or I₂ vapor staining.

4.1. Synthesis of cyclized mono- and bis-chalcone derivatives

4.1.1. Synthesis of 1,3-diphenyl-propenone (1M). To a solution of ketone (acetophenone, 5 mmol) in 5 mL of methanol in an ice bath, freshly prepared 2 N methanolic NaOH solution (30 mL) was added and stirred for 10 min. To this 5 mmol of benzaldehyde was added and the reaction mixture was stirred at room temperature for 10 h. The reaction mixture was cooled in an ice bath and neutralized with dilute hydrochloric acid. The appearing precipitate was separated by filtration and washed three times with 50 mL distilled water to give the crude product. The as-obtained product was recrystallized from methanol.

Yield: 85%; m.p. 55–58 °C; *R*_f = 0.48 (DCM–MeOH 7:3); C₁₅H₁₂O; CHN analysis: calc. C 86.51, H 5.81%; found: C 67.64, H 7.42%; IR $\nu_{\max}$ cm⁻¹: 3030 (C–H), 1664 (C=O), 1598 (C=C); ¹H NMR (DMSO-*d*₆, 400 MHz) δ (ppm): 8.04–7.50 (m, 10H, Ar–H), 7.45 (d, 1H, *J* = 15 Hz, H- β), 7.25 (d, 1H, *J* = 15 Hz, H- α); ¹³C NMR (CDCl₃) δ (ppm): 190.59 (C=O), 144.89 (C- β), 136.90, 132.84, 130.59, 128.67, 127.49, 122.09 (C- α); MS (ESI⁺) *m/z* [M + H]⁺ 209.31.

4.1.2. Synthesis of 3,5-diphenyl-1H-pyrazole (2M). To a solution of chalcone (**1B**) (5 mmol) in 10 mL of methanol and 5% NaOH, hydrazine hydrate (5 mmol, 1.24 g) was added and the reaction mixture was refluxed for 8 h. The excess solvent was removed under reduced pressure and the reaction mixture was cooled in an ice bath. The product precipitated out at low temperature, was washed five times with 50 mL distilled water, reconstituted in a minimum amount of methanol and dried under reduced pressure.

Yield: 68%; m.p. 180–183 °C; *R*_f = 0.42 (DCM–MeOH 7:3); C₁₅H₁₂N₂; CHN analysis: calc. C 81.79, H 5.49, N 12.72%; found: C 81.65, H 5.45, N 12.67%; IR $\nu_{\max}$ cm⁻¹: 3375 (NH), 3035 (Ar–H), 1683 (C=O), 1592 (C=N), 1446, (C=C); ¹H NMR (CDCl₃) δ (ppm): 9.45 (bs, 1H, NH), 7.55–7.18 (m, 10H, Ar–H), 5.94 (s, 1H, CH), ¹³C NMR (CDCl₃) δ (ppm): 154.12 (C=N), 149.83 (C=C), 141.35, 130.2, 128.63, 127.68, 114.18; ESI-MS *m/z*: [M + H]⁺ = 221.13.

4.1.3. Synthesis of 3,5-diphenyl-4,5-dihydro-pyrazole-1-carbothioic acid amide (3M). To a solution of chalcone (**1B**) (5 mmol) in 10 mL of methanol and 5% NaOH, thiosemicarbazide (5 mmol, 1.24 g) was added and the reaction mixture was refluxed for 8 h. The excess solvent was removed under reduced pressure and the reaction mixture was cooled in an ice bath. The products precipitated out at low temperature, were washed five times with 50 mL distilled water, reconstituted in a minimum amount of methanol and dried under reduced pressure.

Yield: 63%; m.p. 185–187 °C; R_f = 0.56 (DCM–MeOH 7 : 3); $C_{15}H_{16}N_3S$; CHN analysis: calc. C 68.30, H 5.37, N 14.93%; found: C 68.45, H 5.40, N 14.90%; IR $\nu_{\max}$ cm^{-1} : 3420 (C=S–NH₂), 3030 (Ar–H), 1683 (C=O), 1598 (C=N), 1486, (C=C); ¹H NMR (CDCl₃) δ (ppm): 7.32–7.10 (m, 10H, Ar–H), 6.73 (s, 2H, NH₂), 5.58 [dd, 1H, J = 11.5, 4.8 Hz, H_x (pyrazoline ring)], 3.86 [dd, 1H, J = 18.2, 4.8 Hz, H_a (pyrazoline ring)], 3.42 [dd, 1H, J = 18.0, 11.7 Hz, H_b (pyrazoline ring)], ¹³C NMR (CDCl₃) δ (ppm): 169.45 (C=O), 154.12 (C=N), 152.85 (C-3, pyrazoline ring), (149.83, 141.35, 132.65, 128.63, 127.68, 127.76, 123.33, 120.34, 114.18, phenyl ring), 55.45 (C-5, pyrazoline ring), 42.54 (C-4, pyrazoline ring); ESI-MS m/z : [M + H]⁺ = 282.53.

4.1.4. Synthesis of 3,5-diphenyl-4,5-dihydro-isoxazole (4M).

To a solution of chalcone (1B) (5 mmol) in ethanol and anhydrous sodium acetate (10 mmol) dissolved in a minimum amount of acetic acid, a solution of hydroxylamine hydrochloride (5 mmol) in ethanol was added. The reaction mixture was refluxed for 10 h. After completion of the reaction, the solution was cooled to get the products, which were purified by recrystallization from ethanol.

Yield: 60%; m.p. 196–198 °C; R_f = 0.45 (DCM–MeOH 7 : 3); IR $\nu_{\max}$ cm^{-1} : $C_{15}H_{13}NO$; CHN analysis: calc. C 80.69, H 5.87, N 6.27%; found: C 80.75, H 5.78, N 6.50%; IR $\nu_{\max}$ cm^{-1} : 2938 (C–H), 3075 (C–H in Ar–H), 1630 (C=N–O), 1453 (C=C), 1108 (C–O stretch); ¹H NMR (CDCl₃) δ (ppm): 7.13–7.25 (m, 10H, Ar–H), 5.58 [dd, 1H, J = 11.5, 4.8 Hz, H_x (oxazole ring)], 3.86 [dd, 1H, J = 18.2, 5.0 Hz, H_a (oxazole ring)], 3.42 [dd, 1H, J = 18.2, 11.7 Hz, H_b (oxazole ring)], ¹³C NMR (CDCl₃) δ (ppm): 162.10 (C=N), 142.55, 132.30, 130.65, 129.65, 128.50, 127.76, 126.30, 126.22, 74.10, 32.54; ESI-MS m/z : [M + H]⁺ = 224.13.

4.1.5. Synthesis of 4,6-diphenyl-3,4-dihydro-1H-pyrimidin-2-one (5M). To a solution of chalcone (1B) (5 mmol) in 10 mL of ethanol and 5% KOH, (5 mmol, 1.24 g), urea (5 mmol) was added and the reaction mixture was refluxed for 8 h. The excess solvent was removed under reduced pressure and the reaction mixture was cooled in an ice bath. The products precipitated out at low temperature, were washed five times with 50 mL distilled water, reconstituted in a minimum amount of ethanol and dried under reduced pressure.

Yield: 85%; m.p. 148–150 °C; R_f = 0.54 (DCM–MeOH 7 : 3); $C_{16}H_{14}N_2O$; CHN analysis: calc. C 76.78, H 5.64, N 11.19%; found: C 76.85, H 5.55, N 11.27%; IR $\nu_{\max}$ cm^{-1} : 3030 (C–H aromatic), 1664 (C=O), 1598 (C=C) 3300 (NH stretch), 2978 (CH stretch), 1661 (C=O), 1568 (C=O, amide), 1450 (C=C, Ph); 1108 (C–O stretch); ¹H NMR (DMSO-*d*₆, 400 MHz) δ (ppm): 10.25 (bs, 1H, NH, H-3), 9.45 (bs, 1H, NH, H-1), 7.95–7.25 (m, 10H, Ar–H), 7.20–7.13 (dd, 1H, H-4, J = 8.4 Hz; J = 4.0 Hz), 5.10 (d, 1H, H-5, J = 6.4 Hz); ¹³C NMR (DMSO-*d*₆, 75 MHz) δ (ppm): 154.11 (C=O), 143.15, 138.10, 136.25, 129.50, 128.60, 128.10, 127.30, 126.50, 97.25, 55.20; MS (ESI⁺) m/z [M + H]⁺ 209.31.

4.1.6. Synthesis of 4,6-diphenyl-3,4-dihydro-1H-pyrimidine-2-thione (6M). To a solution of chalcone (1B) (5 mmol) in 10 mL of ethanol and 5% KOH, (5 mmol, 1.24 g), thiourea (5

mmol) was added and the reaction mixture was refluxed for 8 h. The excess solvent was removed under reduced pressure and the reaction mixture was cooled in an ice bath. The products precipitated out at low temperature, were washed five times with 50 mL distilled water, reconstituted in a minimum amount of ethanol and dried under reduced pressure.

Yield: 85%; m.p. 156–158 °C; R_f = 0.48 (DCM–MeOH 7 : 3); $C_{16}H_{14}N_2S$; CHN analysis: calc. C 72.15, H 5.30, N 10.52%; found: C 72.10, H 5.40, N 10.47%; IR $\nu_{\max}$ cm^{-1} : 3125 (C–H aromatic), 1585 (C=C) 3310 (NH stretch), 2975 (CH stretch), 1565, 1385, 1125 (C=S, thioamide), 1465 (C=C, Ph); 1120 (C–O stretch); ¹H NMR (DMSO-*d*₆, 400 MHz) δ (ppm): 9.85 (bs, 1H, NH, H-3), 9.25 (bs, 1H, NH, H-1), 7.98–7.32 (m, 10H, Ar–H), 7.11–7.03 (dd, 1H, H-4, J = 8.0 Hz, J = 4.2 Hz), 5.43 (d, 1H, H-5, J = 6.8 Hz); ¹³C NMR (DMSO-*d*₆, 75 MHz) δ (ppm): 172.10 (C=O), 146.13, 138.12, 136.25, 129.33, 128.45, 128.00, 127.45, 126.50, 99.10, 62.50; MS (ESI⁺) m/z [M+H]⁺ 267.15.

4.1.7. Synthesis of 3,3'-(1,4-phenylene)-bis-1-phenylprop-2-en-1-one (1B). 1B was synthesized, following the same synthetic procedure as that of 1M except for the use of 2.5 mmol of 1,4-dicarboxaldehyde in place of 5 mmol of benzaldehyde.

Yield: 85%; m.p. 55–58 °C; R_f = 0.48 (DCM–MeOH 7 : 3); $C_{24}H_{18}O_2$; CHN analysis: calc. C 85.18, H 5.36%; found: C 85.24, H 5.45%; IR $\nu_{\max}$ cm^{-1} : 3036 (C–H), 1665 (C=O), 1597 (C=C); ¹H NMR (DMSO-*d*₆, 400 MHz) δ (ppm): 8.01–7.45 (m, 14H, Ar–H), 7.37 (d, 2H, J = 16 Hz, H- β), 7.14 (d, 2H, J = 16 Hz, H- α); ¹³C NMR (CDCl₃) δ (ppm): 190.59 (C=O), 144.89 (C- β), 136.90, 132.84, 130.59, 128.67, 127.49, 122.09 (C- α); MS (ESI⁺) m/z [M + H]⁺ 339.25.

4.1.8. Synthesis of 1,4-bis(3-phenyl-1H-pyrazol-5-yl)benzene (2B). 2B was synthesized, following the same synthetic procedure as that of 2M except for the use of 2.5 mmol of 1B in place of 5 mmol of 1M used in the synthesis of 2M.

Yield: 68%; m.p. 255–257 °C; R_f = 0.42 (DCM–MeOH 7 : 3); $C_{24}H_{18}N_4$; CHN analysis: calc. C 79.54, H 5.01, N 15.46%; found: C 79.65, H 5.15, N 15.60%; IR $\nu_{\max}$ cm^{-1} : 3375 (NH), 3033 (Ar–H), 1677 (C=O), 1587 (C=N), 1438, (C=C); ¹H NMR (CDCl₃) δ (ppm): 10.32 (bs, 2H, NH), 8.30 (s, 4H, Ar–H), 8.05–7.45 (m, 10H, Ar–H), 6.45 (s, 2H, CH), ¹³C NMR (CDCl₃) δ (ppm): 150.15 (C=N), 135.40 (C–NH), 130.5, 128.25, 127.50, 101.10; ESI-MS m/z : [M + H] = 363.10.

4.1.9. Synthesis of 3-(4-(1-carbamothioyl-3-phenyl-4,5-dihydro-1H-pyrazol-5-yl)phenyl)-5-phenyl-4,5-dihydro-1H-pyrazole-1-carbothioamide (3B). 3B was synthesized, following the same synthetic procedure as that of 3M except for the use of 2.5 mmol of 1B in place of 5 mmol of 1M used in the synthesis of 3M.

Yield: 68%; m.p. 225–228 °C; R_f = 0.48 (DCM–MeOH 7 : 3); $C_{26}H_{24}N_6S_2$; CHN analysis: calc. C 64.45, H 4.99, N 17.34%; found: C 64.42, H 5.05, N 17.50%; IR $\nu_{\max}$ cm^{-1} : 3416 (C=S–NH₂), 3022 (Ar–H), 1685 (C=O), 1590 (C=N), 1480, (C=C); ¹H NMR (CDCl₃) δ (ppm): 7.87–7.25 (m, 14H, Ar–H), 4.98 (s, 4H, NH₂), 5.87 [dd, 1H, J = 11.2, 4.8 Hz, H_x (pyrazoline ring)], 5.67 [dd, 1H, J = 11.5, 4.8 Hz, H_x (pyrazoline ring)], 3.86 [dd, 1H, J = 18.5, 5.4 Hz, H_a (pyrazoline ring)], 3.65 [dd, 1H, J =

18.2, 5.0 Hz, Ha (pyrazoline ring)], 3.42 [dd, 1H, $J = 18.5, 11.2$ Hz, Hb (pyrazoline ring)], 3.33 [dd, 1H, $J = 18.0, 11.7$ Hz, Hb (pyrazoline ring)], ^{13}C NMR (CDCl_3) δ (ppm): 172.50 (C=S), 152.10 (C=N), 151.01 (C-3, pyrazoline ring), (136.60, 131.10, 128.85, 128.54, 128.20, 127.60, 126.50, 123.35, phenyl ring), 100.30, (C-5, pyrazoline ring), 75.10, (C-4, pyrazoline ring); ESI-MS m/z : $[\text{M} + \text{H}] = 485.25$.

4.1.10. Synthesis of 1,4-bis(3-phenyl-4,5-dihydroisoxazol-5-yl)benzene (4B). 4B was synthesized, following the same synthetic procedure as that of 4M except for the use of 2.5 mmol of 1B in place of 5 mmol of 1M used in the synthesis of 4M.

Yield: 60%; m.p. 245–247 °C; $R_f = 0.45$ (DCM–MeOH 7 : 3); IR ν_{max} cm^{-1} : $\text{C}_{24}\text{H}_{20}\text{N}_2\text{O}_2$; CHN analysis: calc. C 78.24, H 5.47, N 7.60%; found: C 78.12, H 5.45, N 7.80%; IR ν_{max} cm^{-1} : 2930 (C–H), 3084 (C–H in Ar–H), 1645 (C=N–O), 1450 (C=C), 1122 (C–O stretch); ^1H NMR (CDCl_3) δ (ppm): 7.95–7.55 (m, 10H, Ar–H), 7.45 (s, 4H, Ar–H), 5.92 [dd, 1H, $J = 11.5, 4.8$ Hz, Hx (oxazole ring)], 3.85 [dd, 1H, $J = 18.2, 5.0$ Hz, Ha (oxazole ring)], 3.45 [dd, 1H, $J = 18.0, 11.0$ Hz, Hb (oxazole ring)], ^{13}C NMR (CDCl_3) δ (ppm): 160.12 (C=N), 142.55, 133.10, 131.50, 129.35, 128.30, 126.20, 85.10, 45.50, ESI-MS m/z : $[\text{M} + \text{H}]^+ = 369.15$.

4.1.11. Synthesis of 4,4'-(1,4-phenylene)bis(6-phenyl-3,4-dihydropyrimidin-2(1H)-one) (5B). 5B was synthesized, following the same synthetic procedure as that of 5M except for the use of 2.5 mmol of 1B in place of 5 mmol of 1M used in the synthesis of 5M.

Yield: 85%; m.p. 225–227 °C; $R_f = 0.54$ (DCM–MeOH 7 : 3); $\text{C}_{26}\text{H}_{22}\text{N}_4\text{O}_2$; CHN analysis: calc. C 73.92, H 5.25, N 13.26%; found: C 74.10, H 5.15, N 13.25%; IR ν_{max} cm^{-1} : 3038 (C–H aromatic), 1667 (C=O), 1587 (C=C) 3311 (NH stretch), 2972 (CH stretch), 1664 (C=O), 1562 (C=O, amide), 1455 (C=C, Ph); 1123 (C–O stretch); ^1H NMR ($\text{DMSO}-d_6$, 400 MHz) δ (ppm): 9.55 (bs, 2H, NH, H-3), 8.94 (bs, 2H, NH, H-1), 7.85–7.43 (m, 10H, Ar–H), 7.30 (s, 4H, Ar–H), 7.19–7.10 (dd, 2H, H-4, $J = 8.6$ Hz, $J = 4.6$ Hz), 5.25 (d, 2H, H-5, $J = 6.8$ Hz); ^{13}C NMR ($\text{DMSO}-d_6$, 75 MHz) δ (ppm): 152.10 (C=O), 142.11, 138.14, 136.55, 129.10, 128.64, 128.15, 126.50, 97.20, 57.26; MS (ESI^+) m/z $[\text{M} + \text{H}]^+ 423.22$.

4.1.12. Synthesis of 4,4'-(1,4-phenylene)bis(6-phenyl-3,4-dihydropyrimidine-2(1H)-thione) (6B). 6B was synthesized, following the same synthetic procedure as that of 6M except for the use of 2.5 mmol of 1B in place of 5 mmol of 1M used in the synthesis of 6M.

Yield: 85%; m.p. 230–232 °C; $R_f = 0.48$ (DCM–MeOH 7 : 3); $\text{C}_{26}\text{H}_{22}\text{N}_4\text{S}_2$; CHN analysis: calc. C 68.69, H 4.88, N 12.32%; found: C 68.75, H 4.90, N 12.40%; IR ν_{max} cm^{-1} : 3122 (C–H aromatic), 1580 (C=C) 3305 (NH stretch), 2972 (CH stretch), 1560, 1380, 1125 (C=S, thioamide), 1460 (C=C, Ph); 1125 (C–O stretch); ^1H NMR ($\text{DMSO}-d_6$, 400 MHz) δ (ppm): 10.99 (bs, 2H, NH, H-3), 10.75 (bs, 2H, NH, H-1), 7.65–7.30 (m, 10H, Ar–H), 7.22 (s, 4H, Ar–H), 7.15–7.02 (dd, 2H, H-4, $J = 8.2$ Hz, $J = 4.2$), 5.32 (d, 2H, H-5, $J = 6.8$ Hz); ^{13}C NMR ($\text{DMSO}-d_6$, 75 MHz) δ (ppm): 174.10 (C=S), 149.15, 142.10, 138.12, 129.11, 128.95, 128.11, 127.25, 97.15, 60.50; MS (ESI^+) m/z $[\text{M} + \text{H}]^+ 455.18$.

4.2. Microbiological analysis

4.2.1. Strains, media and chemicals. Six fluconazole susceptible (002B1, 003gr, 004gr, 004B1, 0079gr, 0072gr) and three fluconazole resistant (A171, 167-1, 167-2) clinical strains of *C. albicans* were used. A control strain (ATCC90028) was also included. All clinical strains were isolated from either HIV positive patients or patients with other immunocompromised conditions that were attending clinics at the Charlotte Maxeke Johannesburg Academic Hospital, Johannesburg, South Africa. Ethical clearance was obtained from the Human Research Ethics Committee, University of the Witwatersrand. All experiments and procedures were performed in compliance with the relevant laws and institutional guidelines obtained from the Human Research Ethics Committee, University of the Witwatersrand. Informed consent was obtained for any experimentation with human subjects. The strains obtained were sub-cultured at least twice on Tryptone Soya Agar (TSA) plates at 37 °C for 48 hours. Fluconazole was purchased from Sigma Fluke (USA) and a stock solution of 2000 $\mu\text{g mL}^{-1}$ was prepared in sterile distilled water. All other chemicals and media were purchased from Oxoid, England.

4.2.2. Antifungal activity. The minimum inhibitory concentrations (MIC) of all the synthesized compounds against test *C. albicans* were determined using the Clinical and Laboratory Standards Institute (CLSI) guideline's recommended broth microdilution method M27-A3,²¹ and for comparison fluconazole was used as a positive control. For sample preparation, all the test compounds were dissolved using DMSO to obtain a concentration of 2000 $\mu\text{g mL}^{-1}$. In each experiment, a positive control, culture, media and a negative vehicle control were also included. All experiments were carried out in duplicate and all results were expressed in $\mu\text{g mL}^{-1}$.

4.2.3. Combinational interaction with fluconazole. The combinational interaction of all the tested twelve compounds with fluconazole was investigated following the methods described previously.²² All the combinations were determined in a 1:1 ratio of tested compounds and fluconazole in the concentration range 2–4 fold lower than the MIC values calculated alone. Interactions were assessed on the basis of zero-interaction theory of Loewe additivity and FICI values were calculated as follows:

$$\text{FICI} = \text{FICa} + \text{FICb} = \frac{\text{MICa in combination}}{\text{MICa tested alone}} + \frac{\text{MICb in combination}}{\text{MICb tested alone}}$$

where MICa is the MIC of the chalcone derivatives and MICb is the MIC of fluconazole. Interpretations of the FICI values were performed as synergy for ≤ 0.5 , additive between 0.5 and 1.0, indifferent between 1.0 and 4.0 and antagonistic for > 4.0 .²³

4.2.4. Synergies in varied ratio combinations and isobologram constructions. Following all the synergistic interactions between the chalcone derivatives and fluconazole in 1:1 ratios, nine different ratio combinations were also

Table 3 Primers and annealing temperatures used for RT-PCR for genes associated with resistance

Gene	Forward primer (5'–3')	Reverse primer (5'–3')	Annealing temp (°C)
<i>ERG11</i>	ATTGGTATTCTTATGGGTGGTCAACATAC	CCCAATACATCTATGTCTACCACCACC	45
<i>ACT1</i>	TTTAAGAATTGATTTGGCT	GAAGATTGAGAAGAAGTTT	45

investigated to achieve the best synergistic combination. For all the combinations MICs were determined along with the MIC of the tested compounds showing synergy and fluconazole alone. To further explain these different ratio combinations, isobolograms were constructed using GraphPad Prism version 5-software as described previously.¹⁵ All the data points were examined and interpreted as synergistic, additive or indifferent. The data points below or on the 0.5:0.5 lines in the isobologram were interpreted as synergistic.

4.2.5. Rhodamine 6G efflux assay. To investigate the role of chalcone derivatives on the efflux pump function, the activity of drug efflux pumps was tested following the methodology as described previously.¹⁹ Briefly, *Candida albicans* cells, grown in TSB media at 37 °C for 8 h, were harvested by centrifuging, washed and resuspended in glucose free phosphate-buffered saline and adjusted to 1×10^8 cells (w/v). All the cells were then de-energized for 45 min by adding 5 mM deoxyglucose and 5 mM dinitrophenol. After 45 min the cells were centrifuged and resuspended again in PBS buffer without glucose to which 10 mM R6G solution was added at a final concentration of 10 μ M, and incubated for 40 min at 37 °C. All the R6G equilibrated cells were then washed and resuspended in glucose free PBS and aliquots of 500 μ L were taken at specific time intervals (5, 10, 15, 20, 25 min) and the absorbance was measured at 527 nm using a BioRad spectrophotometer. To measure the energy dependent efflux, 0.1 M glucose was added to the cells resuspended in glucose free PBS buffer after 25 min. For competition assays, the test compounds at MIC values were added to the de-energized cells 5 min before the addition of R6G and allowed to equilibrate. In every set of experiments, glucose-free controls, Rh6G-free, and Rh6G-alone groups were included. The concentration of R6G was calculated using a standard concentration curve of R6G.

4.2.6. Effect on ergosterol biosynthesis. To determine the effect of chalcone derivatives on ergosterol biosynthesis, the total intracellular sterols were quantified using an alcoholic KOH method. Briefly, one FLC susceptible *C. albicans* ATCC90028 and one FLC resistant *C. albicans* A71 were grown in 50 ml of TSB with MIC and $\frac{1}{2}$ MIC of mono- and bis-chalcone derivatives. Untreated and 8 μ g mL⁻¹ FLC treated cells were also used as negative and positive controls, respectively. After incubation, cells were harvested and the sterols were extracted and scanned as detailed previously.³⁸ The ergosterol content was calculated by the following equation:

$$\% \text{ ergosterol} + \% 24(28) \text{ DHE} = [(A_{281.5}/290) \times F]/\text{pellet weight}$$

$$\% 24(28) \text{ DHE} = [(A_{230}/518) \times F]/\text{pellet weight}$$

$$\% \text{ ergosterol} = [\% \text{ ergosterol} + \% 24(28) \text{ DHE}] - \% 24(28) \text{ DHE}$$

where *F* is the dilution factor and 290 and 518 are the *E* values (in percentages per centimeter) determined for crystalline ergosterol and 24(28) DHE, respectively. The experiment was performed in triplicate and the results are represented as mean $\pm$ SD.

4.2.7. qRT-PCR analysis of *ERG11* gene expression. To quantify the effect of bis-chalcone derivatives on the expression of the *ERG11* gene, qRT-PCR was performed on one FLC susceptible *C. albicans* ATCC90028 and one FLC resistant *C. albicans* A71. Cells were grown and exposed to MIC concentrations of all the test compounds for 2 h. Following incubation, the total RNA was isolated using a total RNeasy mini kit (Qiagen) and cDNA was synthesized using a First Strand cDNA synthesis kit (Sigma Aldrich), following the manufacturer's instruction. qPCR was performed with an ABI 7500 Real-Time PCR. The primer sequences (detailed in Table 3), reaction systems and conditions were set as described previously.²⁰ The housekeeping gene *ACT1* was taken as the endogenous control and the results were analyzed using ABI LightCycler Software 4.0. All samples were taken from triplicate independent experiments and the results are shown as mean $\pm$ standard deviation.

Live subject statement

This study was performed in strict accordance with the World Medical Association's "Declaration of Helsinki" as a statement of ethical principles (18th WMA General Assembly, Helsinki, Finland, June 1964) and was approved by the Human Research Ethics Committee of the University of the Witwatersrand (Johannesburg, South Africa). *Candida albicans* used in this study were isolated from patients under the ethical clearance number M10102.

Conflicts of interest

The authors declare no conflicts of interest.

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