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ORIGINAL PAPER

Insights into the mode of action of anticandidal herbal monoterpenoid geraniol reveal disruption of multiple MDR mechanisms and virulence attributes in *Candida albicans*

Shweta Singh[1] · Zeeshan Fatima[1] · Saif Hameed[1]

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Abstract The anticandidal potential of Geraniol (Ger) against *Candida albicans* has already been established. The present study reveals deeper insights into the mechanisms of action of Ger. We observed that the repertoire of antifungal activity was not only limited to *C. albicans* and its clinical isolates but also against non-*albicans* species of *Candida.* The membrane tampering effect was visualized through transmission electron micrographs, depleted ergosterol levels and altered plasma membrane ATPase activity. Ger also affects cell wall as revealed by spot assays with cell wall-perturbing agents and scanning electron micrographs. Functional calcineurin pathway seems to be indispensable for the antifungal effect of Ger as calcineurin signaling mutant was hypersensitive to Ger while calcineurin overexpressing strain remained resistant. Ger also causes mitochondrial dysfunction, impaired iron homeostasis and genotoxicity. Furthermore, Ger inhibits both virulence attributes of hyphal morphogenesis and biofilm formation. Taken together, our results suggest that Ger is potential antifungal agent that warrants further investigation in clinical applications so that it could be competently employed in therapeutic strategies to treat *Candida* infections.

Keywords *Candida* · Geraniol · MDR · Calcineurin signaling · Cell wall · Cell membrane, biofilm

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Shweta Singh and Zeeshan Fatima have contributed equally to this work.

✉ Saif Hameed
saifhameed@yahoo.co.in; shameed@ggn.amity.edu

[1] Amity Institute of Biotechnology, Amity University Haryana, Manesar, Gurgaon 122413, India

Introduction

Candida albicans is an opportunistic human fungal pathogen which under wide ranges of immunocompromised conditions makes patients susceptible to superficial as well as systemic infections (Singh et al. 2015). The problem has become severe over the last decade considering the fact that *Candida* infections are fourth leading cause of mortality in patients infected with nosocomial infections (Wisplinghoff et al. 2004). Despite the availability of drugs for candidiasis such as azoles, polyenes, allylamines and echinocandins with distinct mode of actions, the persistent and prolonged use of these antibiotics resulted in the drug resistance which is termed as multidrug resistance (MDR) (Tanwar et al. 2014). Moreover, the collaborative effect of use of broad spectrum antibiotics, development of MDR and weakened immunity facilitates the *Candida* infections. Thus, there is constant need to find new and effective antifungals with novel targets. In the present scenario, given the limited number of effective antifungals, natural compounds from herbal origin can be a promising option as they have lesser toxicity and cost effectiveness. Plants are rich source of bioactive molecules which can work against pathogenic microorganisms (Cowan 1999; Saibabu et al. 2015). Many essential oils have been reported to possess anticandidal activity as they have biological and pharmaceutical properties (Khan and Ahmad 2012). Recently, our group has also established the antifungal potential of sesamol, a phenolic compound, against *C. albicans* (Ansari et al. 2014).

Terpenoids are among the major class of natural compounds that have gain considerable attention during the past decades. Terpeniods are component of plant essential oils which have been found to act as anticancer, antiparasitic, antiviral, antiallergic and have antimicrobial properties (Zore et al. 2011). Geraniol (Ger) is a monoterpene alcohol constituting about 20 % as a main component of geranium oil has been traditionally used in aromatherapy for vaginal candidiasis (Maruyama et al. 2008). The other terpenes such as carvacrol, eugenol, linalool and terpinen-4-ol have already been shown to be effective antifungal compounds (Marcos-Arias et al. 2011). It has been reported in the literature that Ger exhibits various biochemical and pharmacological properties and can work potentially as insecticide along with repellent effects, besides antihelmintic, antibacterial, antioxidant, anticancer and anti-inflammatory activities (Singh et al. 2012; Zhu et al. 2014; Abdel-Rahman et al. 2013; Misra et al. 2013).

The previous study (Leite et al. 2015) has reported the antifungal activity of Ger; however, the precise mechanisms of action of Ger were still elusive. The present study was done with the aim to add to the existing literature about the broad antifungal potential of Ger not only against the *C. albicans* but non-*albicans* species as well. The effect of Ger was linked with the calcineurin signaling pathway leading to disruption in the cell membrane and cell wall homeostasis. We also showed that Ger possibly affects mitochondrial functioning, iron homeostasis and genotoxicity in *C. albicans.* The study also observed the inhibition of yeast to hyphal transition which can be linked to antibiofilm activity of Ger against *C. albicans.*

Materials and methods

All media chemicals yeast extract peptone dextrose (YEPD), agar, horse serum, ascorbic acid (AA), yeast nitrogen base w/o amino acid and ammonium sulfate, ammonium sulfate, nutrient broth and ethidium bromide (EtBr) were purchased from HiMedia (Mumbai, India). Sodium chloride (NaCl), calcium chloride ($CaCl_2$), lithium chloride (LiCl), potassium chloride (KCl), mannitol, disodium hydrogen orthophosphate, potassium dihydrogen orthophosphate, dipotassium hydrogen orthophosphate, sodium hydroxide, D-glucose, and sodium dodecyl sulfate (SDS), dimethyl sulfoxide (DMSO), mannitol, dipotassium phosphate (K_2HPO_4), glucose were obtained from Fischer Scientific. Calcofluor-white (CFW), Congo red (CR), caspofungin (CAS), fluconazole (FLU) and geraniol (Ger), bathophenanthroline disulfonic acid disodium salt hydrate (BPS) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). *N*-heptane was obtained from Central Drug House Pvt. Ltd., New Delhi. Thiazolyl blue (MTT) was obtained from Sisco Research Laboratories Pvt. Ltd., New Delhi.

Growth media and strains used

The reference strain of *C. albicans* used in this study was SC5314. The details of all the other *Candida* strains used in the study are given in Table 1. All the strains of *C. albicans* were cultured in YEPD broth with the composition of yeast extract 1 % (w/v), peptone 2 % (w/v) and dextrose 2 % (w/v). For agar plates, 2 % (w/v) agar (HiMedia, Mumbai, India) was added to the media. All *Candida* strains were stored in 30 % (v/v) glycerol stock at −80 °C. The cells were freshly revived on YEPD broth and transferred to agar plate before each study to ensure the revival of the strains. For biochemical assays, Ger (dissolved in DMSO) was used at its subinhibitory concentration determined by the growth curve experiments which was partially inhibiting and did not have any marked effect on growth of *C. albicans* cells (data not shown).

Drug susceptibility assays

Drug susceptibility was tested using minimal inhibitory concentration (MIC), spot assay and filter disk assay as described below.

Minimum inhibitory concentration (MIC)

MIC was determined by broth microdilution method as described in method M27-A3 from the Clinical and Laboratory Standards Institute (CLSI) formerly NCCLS (National Committee for Clinical and Laboratory Standards 2008). Briefly, 100 µl of media was placed at each well of the 96-well plate following the addition of the drug with the remaining media and then was serially diluted. 100 µl of cell suspension (in normal saline to an OD_{600} 0.1) was added to each well of the plate, and OD_{600} was measured after 48 h at 30 °C. The MIC_{80} was defined as the concentration at which at least 80 % of the growth was inhibited.

Spot assay

Spot assays were performed using a method as described elsewhere (Ansari et al. 2014; Prasad et al. 2006). Briefly, for the spot assay 5 µl of fivefold serial dilutions of each yeast culture (each with cells suspended in normal saline to an OD_{600} nm of 0.1) was spotted onto YEPD plates in the absence (control) and presence of the drugs indicated in figure legends. Growth was not affected by the presence of solvent (DMSO) used in the examination (data not shown). Growth difference was measured after incubation at 30 °C for 48 h.

Table 1 List of strains used in the study

Strain	Genotype	References
C. albicans SC5314	Reference strain	Cruz et al. (2002)
DAY185 (WT)	ura3::imm434/ura3::imm434 his1::hisG::HIS1/his1::hisG arg4::hisG::ARG4-URA3/arg4::hisG	Davis et al. (2000)
JRB64(Δcnb1/Δcnb1)	ura3Δ::λimm434/ura3Δ::λimm434 his1::hisG::HIS1/his1::hisG arg4::hisG/arg4::hisG cnb1::UAU1/cnb1::ARG4	Blankenship et al. (2003)
YAG237(CNB1-1/CNB1)	CNB1 mutant having hyperactive allele	Cruz et al. (2002)
OCC1.1 (Δcrz1/Δcrz1)	ura3Δ::λimm434/ura3Δ::λimm434 his1::hisG::HIS1/his1::hisG arg4::hisG/arg4::hisG crz1::UAU1/crz1::ARG4	Chen et al. (2011)
D1	Clinical isolate[a]	Safdarjung Hospital, New Delhi
D2	Clinical isolate[a]	Safdarjung Hospital, New Delhi
D4	Clinical isolate[a]	Safdarjung Hospital, New Delhi
D7	Clinical isolate[a]	Safdarjung Hospital, New Delhi
D15	Clinical isolate[a]	Safdarjung Hospital, New Delhi
D18	Clinical isolate[a]	Safdarjung Hospital, New Delhi
D20	Clinical isolate[a]	Safdarjung Hospital, New Delhi
Non-*C. albicans*		
Candida glabrata	Clinical isolate[a]	Safdarjung Hospital, New Delhi
Candida tropicalis	Clinical isolate[a]	Safdarjung Hospital, New Delhi
Candida parapsilosis	Clinical isolate[a]	Safdarjung Hospital, New Delhi
Candida krusei	Clinical isolate[a]	Safdarjung Hospital, New Delhi

[a] All the clinical isolates were isolated from diabetic patients suffering from oral candidiasis

Filter disk assay

The filter disk assay was performed as described elsewhere (Prasad et al. 2006, 2010; Mukhopadhyay et al. 2002). The drugs were spotted in a volume of 5–10 µl at the indicated amount, and the diameters of the respective zones of inhibition were measured after incubation of the plates for 48 h at 30 °C.

Phenotypic susceptibility assays

Phenotypic susceptibilities were measured using spot assays as described above. The following stock solutions were used (the solvents used are given in parenthesis): SDS, 10 % w/v (water), NaCl 5 M (water), LiCl 5 M (water), $CaCl_2$ 5 M (water), DTT 1 M (water), ascorbic acid 1 M (water), BPS 100 mM (water), EtBr 5 mg/ml (water), CAS 0.2 mg/ml (water) and FLU 2 mg/ml (water). Cells were spotted on YEPD plates in the absence (control) and presence of the Ger at its subinhibitory concentration (135 µg/ml) in the presence of the chemicals at the concentrations specified in figure legends. Growth differences were recorded following incubation of the plates for 48 h at 30 °C.

Ergosterol quantification

Sterols were extracted by the alcoholic KOH method, and the percentage of ergosterol was calculated as described previously (Arthington-Skaggs et al. 1999; Breivik and Owades 1957). Briefly, a single *C. albicans* colony from an overnight YEPD agar plate culture was used to inoculate 50 ml of YEPD in the presence and absence of Ger (135 µg/ml). The cultures were incubated for 16 h with shaking at 30 °C. The stationary-phase cells were harvested by centrifugation at 2700 rpm for 5 min and washed once with sterile distilled water. The net wet weight of the cell pellet was determined to which 3 ml of freshly prepared 25 % alcoholic potassium hydroxide solution (25 g of KOH and 35 ml of sterile distilled water, brought to 100 ml with 100 % ethanol) was added to each pellet and vortex mixed for 1 min. Cell suspensions were transferred to sterile borosilicate glass screw-cap tubes and were incubated in an 85 °C water bath for 1 h. Following incubation, tubes were allowed to cool to room temperature. Sterols were then extracted by addition of a mixture of 1 ml of sterile distilled water and 3 ml of *n*-heptane followed by vigorous vortex mixing for 3 min. The heptane layer was transferred to a clean borosilicate glass screw-cap tube and stored at −20 °C. Both ergosterol and 24 (28)-DHE absorb at 281.5 nm, whereas only 24(28)-DHE absorbs at 230 nm. Ergosterol content is determined by subtracting the amount of 24 (28)-DHE (calculated from the OD_{230}) from the total ergosterol plus 24 (28)-DHE content (calculated from the $OD_{281.5}$). Ergosterol content was calculated as a percentage of the wet weight of the cells with the following equations:

% ergosterol + % 24(28)-DHE = [(A281.5/290) × *F*]/pellet weight; % 24(28)-DHE = [(A230/518) × *F*]/pellet weight and % ergosterol = [% ergosterol + % 24(28) DHE] − % 24(28) DHE, where *F* is the factor for dilution in petroleum ether and 290 and 518 are the *E* values (in percent per centimeter) determined for crystalline ergosterol and 24(28)-DHE, respectively.

Mitochondrial activity

The effect of Ger on mitochondrial activity was estimated by MTT assay as previously reported (Qilin et al. 2014). Overnight cultured *C. albicans* cells were diluted with fresh YEPD medium to an initial OD_{600} of 0.1 and grown at 30 °C for 5 h. The cultures were then treated with Ger at subinhibitory concentration of 135 µg/ml for 3 h. 500 µl of cells was harvested, washed twice with YEPD medium, mixed with 500 µl of MTT and statically incubated for a further 2 h. Cells were harvested and washed twice with YEPD medium. The pellets were then suspended in 1 ml of DMSO and incubated at 30 °C with shaking for 5 min. The suspensions were centrifuged, and OD_{570} of the supernatants was determined.

Plasma membrane ATPase-mediated proton pumping

The proton pumping activity of *C. albicans* was estimated by monitoring the glucose-induced acidification of the external medium due to pH changes as previously reported with slight modifications (Tian et al. 2012). Overnight cultures of *C. albicans* were grown in YEPD broth for 18 h at 30 °C on a shaker at 160 rpm. The cells were collected by centrifugation at 3000 rpm for 5 min at 4 °C and washed with sterile distilled water and 50 mM KCl (pH 6.5). The washed cells were resuspended in 40 ml of 50 mM KCl (pH 6.5) and incubated at 4 °C overnight to deplete their carbon reserves. The carbon-starved cells were harvested by centrifugation, and approximately 1.0 g wet weight of the pellet was again resuspended in 40 ml of 50 mM KCl (pH 6.5). To a 40 ml aliquot of the cell suspension, Ger at MIC_{80} was added to obtain the required concentration and mixed well, and the volume was adjusted to 45 ml with 50 mM KCl. The cell suspension was incubated at room temperature with gentle stirring for 10 min, and then, 5 ml of 20 % glucose (final concentration, 55 mM) was added and the pH of the external medium was monitored at regular intervals for 60 min at indicated time points. The experiment was performed in the presence of a comparable concentration of the solvent DMSO (control) to measure the extent of acidification of the external medium in the absence of Ger.

Intracellular pH (pHi)

Intracellular pH was measured as described earlier with slight modifications (Manzoor et al. 2002). Mid-log phase cells grown in YEPD medium were harvested and washed twice with distilled water. Cells (0.1 g) were suspended in 5 ml solution containing 0.1 M KCl and 0.1 mM $CaCl_2$. Desired concentration of Ger (MIC_{80}) was added to the suspension and pH was adjusted to 7.0 in each case. Following incubation for 30 min at 37 °C with constant shaking at 200 rpm, pH was again adjusted to 7.0. Nystatin (20 mM) dissolved in 10 % DMSO was added to the unbuffered cell suspension and incubated at 37 °C for 1 h as control. The change in pH of suspension was followed on pH meter with constant stirring. The value of external pH at which nystatin permeabilization induced no further shift was taken as an estimate of pHi.

Yeast to hyphal transition

Studies of hyphal induction on *C. albicans* were carried out on hyphal induction media such as spider (1 % nutrient broth, 1 % mannitol, 0.2 % K_2HPO_4), 10 % (v/v) horse serum and SLAD (0.17 % yeast nitrogen base without amino acids and ammonium sulfate, 2 % glucose, 50 µM ammonium sulfate, 2 % Bacto Agar). The dimorphic switching was performed using the protocol described elsewhere (Munro et al. 2007). Briefly, the culture was grown overnight at 30 °C in YEPD broth before each study. The revived cells were harvested by centrifugation at 5000 *g* rpm for 3 min and washed twice and incubated at 37 °C for 6 h with PBS to induce starvation. After incubation, the cells were transferred to the required media for hyphal growth and hyphae were observed under microscope at magnification 40× and 4× for liquid and solid media, respectively.

Electron microscopy

Cells treated with Ger at its MIC_{80} value were observed by using SEM (Zeiss EVOMA10) and TEM (JEOL JEM-1011). The cells ($\sim 10^6$ cells) were administered to the media with and without Ger and were incubated for 24 h at 30 °C. Sample preparation and analysis were performed by using the method as described elsewhere (Chen et al. 2011; Kumar et al. 2014). Briefly, all cells were fixed with 2 % glutaraldehyde in 0.1 % phosphate buffer for 1 h at room temperature (20 °C), washed with 0.1 M phosphate buffer (pH 7.2), and post-fixed with 1 % OsO_4 in 0.1 M phosphate buffer for 1 h at 4 °C. Then the cells were dehydrated in acetone, dropped on round glass coverslip with hexamethyldisilazane (HMDS), dried at room temperature, and then sputter coated with gold and observed

under the SEM (Zeiss EVOMA10) at 10 K magnification and TEM (JEOL JEM-1011) at 10 K magnification.

Biofilm formation and cell adhesion

To see the effect of Ger on biofilm development and cell adhesion, *Candida* biofilms and cell adhesion were checked on polystyrene surface of 96-well plates (Chauhan et al. 2010; Chen and Lan 2015). An overnight culture was prepared and cell suspension of 1×10^7 cells/ml was made in PBS and 100 µl was inoculated in each well. The plates were incubated at 37 °C at 50 rpm for 90 min to adhere the cells on the surface. The wells were gently washed two to three times with PBS after 90 min to remove the non-adhered cells. The biofilm was formed by suspending 200 µl of YEPD medium along with subinhibitory concentrations of Ger (135 µg/ml) and one control without Ger to each well of adhered cells to polystyrene 96-well plates and the plates were incubated at 37 °C for 24 h. After incubation, wells were washed to remove any planktonic cells and biofilms were observed under light microscope. To monitor the biofilm on the polystyrene surface of 96-well plate 50 µl (stock solution containing 5 mg/ml, diluted 1:5 in prewarmed 0.15 M PBS prior to addition) of tetrazolium salt, 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) was added in each well. The plates were incubated for 5 h at 37 °C. Dimethyl sulfoxide (200 µl) was added to each well to solubilize MTT formazan product, and optical density was measured at 450 nm. The metabolic activity of biofilm formation was calculated in percentage by comparing the drug-free control with the treated cells. For cell adhesion assay, same protocol was followed except that primarily treated and non-treated cells were grown till OD_{600} 1.0, and after washing the non-adhered cells, they were directly quantified through MTT assay without forming biofilm.

Statistical analysis

All experiments were performed in triplicates ($n = 3$). The results were reported as mean ± standard deviation (SD) and analyzed by using Student's t test in which $P < 0.05$ was considered as statistically significant.

Results and discussion

Antifungal activity of Ger against *C. albicans*

In order to find out the antifungal effect of Ger against *C. albicans*, we have performed various methods of drug susceptibility assays namely minimum inhibitory concentration (MIC_{80}) using broth microdilution method, spot assay and filter disk assay. Broth microdilution assay confirmed that Ger was showing the antifungal effect against *C. albicans* at MIC of 225 µg/ml (Fig. 1a). These results were also confirmed by spot assay (Fig. 1b) and filter disk assays (Fig. 1c). The antifungal activity of Ger was further assessed against seven clinical isolates of *C. albicans*, and we found that all the tested strains displayed susceptibility to Ger albeit at 250 µg/ml instead of 225 µg/ml depicting their little resistance (Fig. 1a, b, c). Thus, all the drug susceptibility testing results clearly indicate that Ger is inhibitory against reference as well as clinical isolates of *C. albicans.*

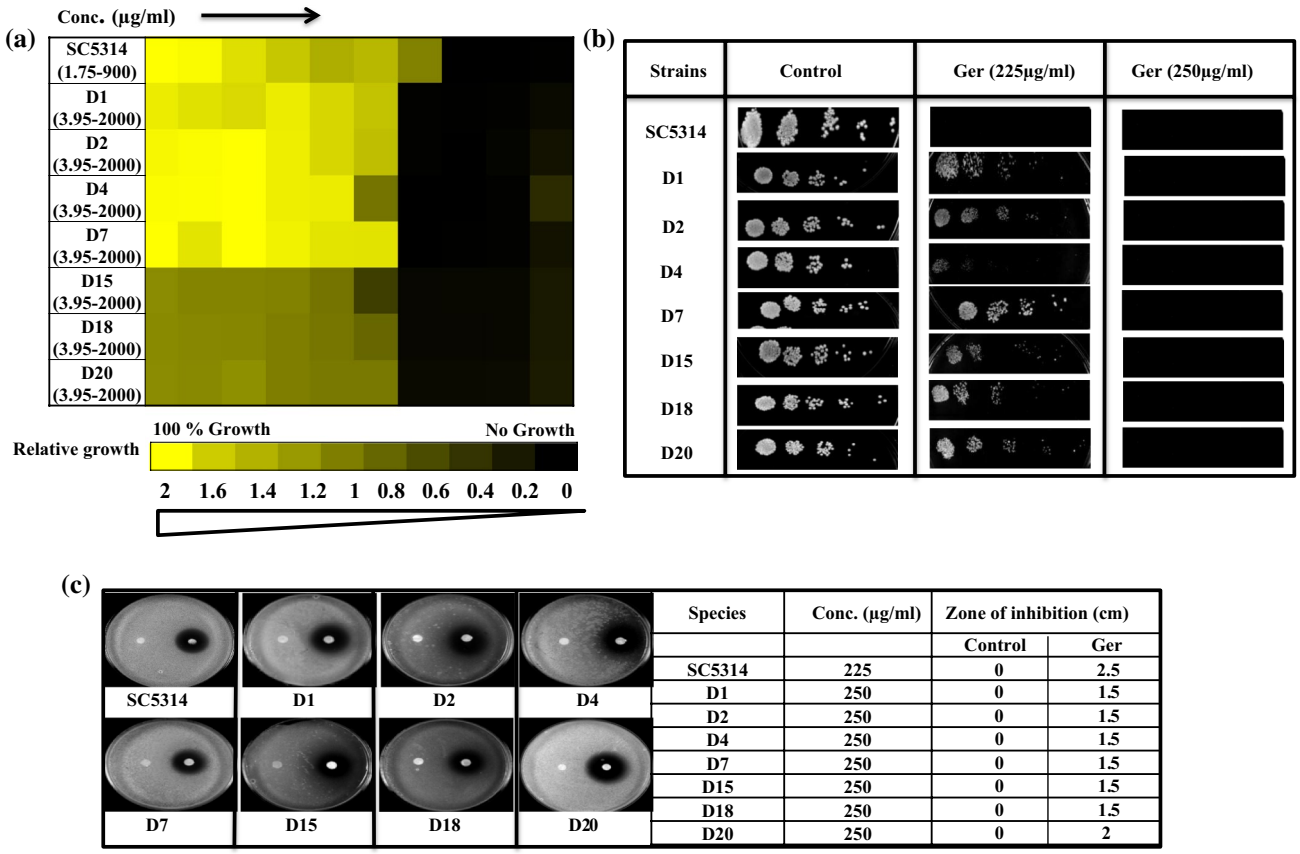
Antifungal activity of Ger against non-*albicans* species

To further elaborate our study and confirm whether Ger is also efficient against other *Candida* species, similar drug susceptibility assays were performed on non-*albicans* species namely *C. krusei, C. tropicalis, C. parapsilosis and C. glabrata.* The minimum inhibitory concentration determined by broth microdilution assay was observed at 300 µg/ml (Fig. 2a) which was further confirmed by both spot assay and filter disk assay expressed in the form of inhibitory zones (Fig. 2b, c). These results establish the broad repertoire of Ger not only against *C. albicans* but other *Candida* species as well.

Ger tampers the cell membrane

Cell membrane is among the primary target for most commonly used antifungal drugs particularly of the class azoles (Khodavandi et al. 2014). To ascertain whether the antifungal mechanism of Ger is associated with any cell membrane disruption, spot assays were performed in the presence of FLU, a well-known drug that targets cell membrane and SDS, a commonly used membrane disrupting cationic detergent. Interestingly, we observed that Ger leads to hypersensitivity in the presence of both FLU and SDS suggesting its possible role in membrane damage (Fig. 3a). Membrane disturbance was further assessed by micrographic images through TEM that also confirmed the spoilage of membrane integrity in Ger-treated *C. albicans* cells (Fig. 3b).

Disruption of membrane homeostasis in the presence of Ger prompted us to further examine the membrane composition which in turn may affect the ability of the drug to permeate the cell membrane. For this we estimated the ergosterol level in the presence of Ger which is one of the main components of the cell membrane in *C. albicans*. To our expectation, we observed that there is a marked decrease (P value < 0.05) in ergosterol levels by more than 50 % in the presence of Ger as shown in Fig. 3c. However, whether altered ergosterol levels also



Species	Conc. (µg/ml)	Zone of inhibition (cm)	
		Control	Ger
SC5314	225	0	2.5
D1	250	0	1.5
D2	250	0	1.5
D4	250	0	1.5
D7	250	0	1.5
D15	250	0	1.5
D18	250	0	1.5
D20	250	0	2

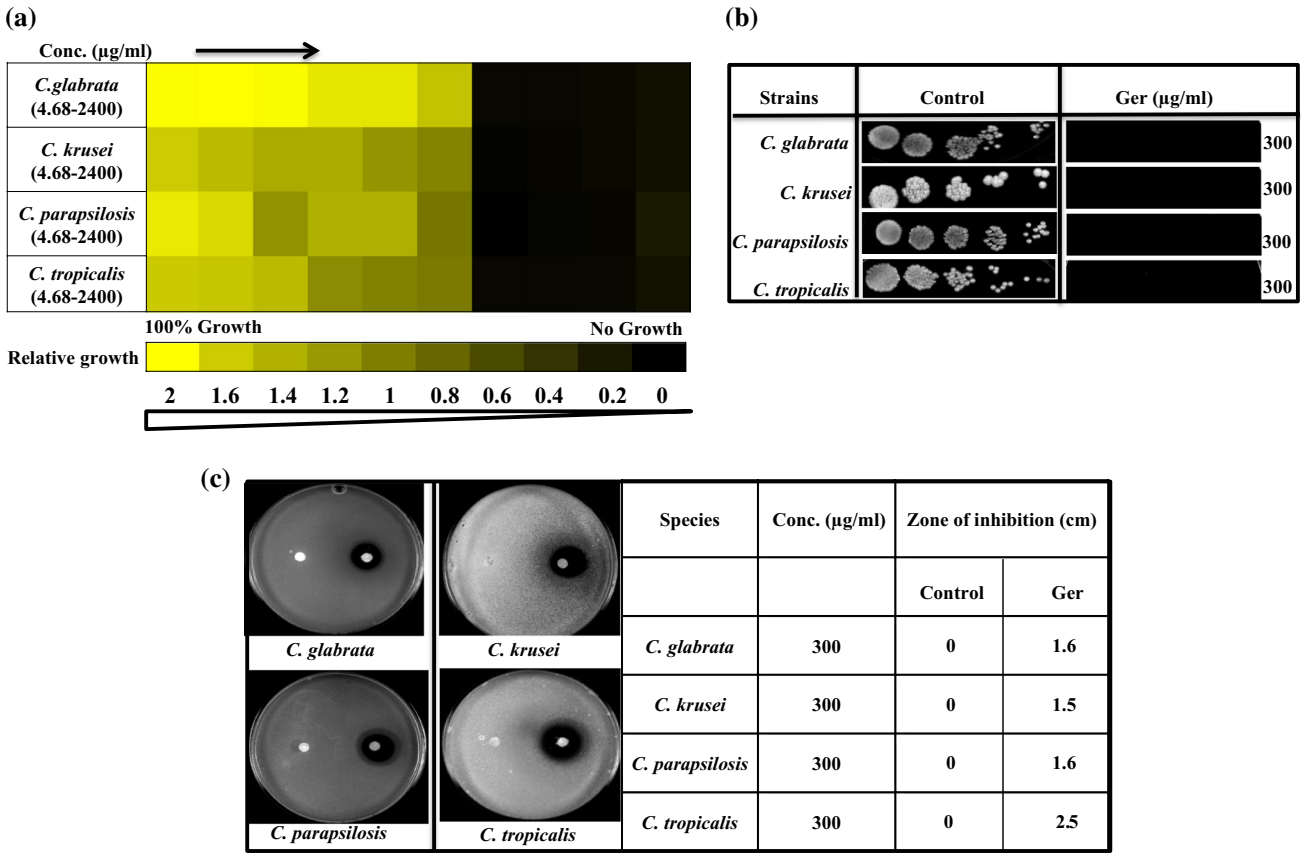
Fig. 1 Drug susceptibility assays against *C. albicans* in the presence of Ger. **a** Broth microdilution assay to determine the MIC_{80} of *C. albicans* reference strain (SC5314) and clinical isolates (D1, D2, D4, D7, D15, D18 and D20) in the presence of Ger. Data were quantitatively displayed with color (see *color bar*), where each *shade of color* represents relative optical densities of the cell. The minimum drug concentration that inhibits growth by 80 % relative to the drug-free growth control is indicated as MIC_{80} for each strain. **b** Spot assay of *C. albicans* reference strain (SC5314) and clinical isolates (D1, D2, D4, D7, D15, D18 and D20) in the absence (control) and presence of Ger. **c** Disk diffusion assay against *C. albicans* reference strain (SC5314) and clinical isolates (D1, D2, D4, D7, D15, D18 and D20) of *C. albicans* and their respective zone of inhibitions in the absence (control) and presence of Ger. For control, disks were spotted with the solvent of Ger (DMSO)

lead to any effect on faster entry of drugs still remains to be validated. These observations led us to believe that Ger tampers with the cell membrane veracity of *C. albicans* cells.

Ger leads to alteration in plasma membrane ATPase activity and intracellular pH

Proton pumps associated with plasma membrane require functional plasma membrane ATPase activity to pump the protons to maintain the pH (Manavathu et al. 1999). Fungal cells starved for carbon source (glucose) when exposed to glucose in the external medium are compelled to take up glucose through proton motive force generated by the proton gradient due to the pumping out of intracellular protons. This change results in medium acidification due to efflux of protons into surrounding leading to changed external pH that can be measured by pH electrode. Thus, we ascertain whether the disrupted membrane homeostasis in the presence of Ger also leads to any abrogated plasma membrane ATPase activity. We observed that Ger significantly ($P < 0.05$) delayed the glucose-induced reduction in external pH of *C. albicans* at its MIC_{80} concentration (Fig. 4a). This confirms that Ger-treated *Candida* cells have abrogated ability to pump intracellular protons to the external medium.

Next, we tested whether the abrogated pumping ability of intracellular protons in the presence of Ger will lead to further acidification of internal pH which is also maintained by PM ATPase activity in yeast (Shreaz et al. 2011). Intriguingly, we explored that only control cells with normal PM ATPase activity maintain the mean pHi near neutrality at 6.78, whereas Ger-treated cells showed mean pHi at 6.56 (Fig. 4b) possibly due to increased internal acidification. These observations positively led us to believe that disrupted membrane homeostasis in



Species	Conc. (µg/ml)	Zone of inhibition (cm)	
		Control	Ger
C. glabrata	300	0	1.6
C. krusei	300	0	1.5
C. parapsilosis	300	0	1.6
C. tropicalis	300	0	2.5

Fig. 2 Drug susceptibility assays against non-*C. albicans* species of *Candida* in the presence of Ger. **a** Broth microdilution assay to determine the MIC_{80} of *C. glabrata, C. krusei*, *C. parapsilosis* and *C. tropicalis* in the presence of Ger. Data were quantitatively displayed with color (see *color bar*), where each *shade of color* represents relative optical densities of the cell. The minimum drug concentration that inhibits growth by 80 % relative to the drug-free growth control is indicated as MIC_{80} for each strain. **b** Spot assay of *C. glabrata, C. krusei*, *C. parapsilosis* and *C. tropicalis* in the absence (control) and presence of Ger. **c** Disk diffusion assay against *C. glabrata, C. krusei*, *C. parapsilosis*, *C. tropicalis* and their respective zone of inhibitions in the absence (control) and presence of Ger. For control, disks were spotted with the solvent of Ger (DMSO)

the presence of Ger leads to disrupted pH homeostasis as well.

Ger disrupts the cell wall integrity

The membrane-perturbing effect of Ger promoted us to further assess cell wall integrity of *C. albicans* being another target of commonly used antifungals of class echinocandins (Song and Stevens 2015). To test whether Ger effects the cell wall integrity, we again performed the spot assay in the presence of CAS, a commonly used antifungal targeting cell wall and well-known cell wall disrupting agents, namely CFW and CR. Intriguingly, *Candida* cells were showing hypersensitivity with all the tested cell wall disrupting agents in the presence of Ger suggesting its possible role in disturbance of cell wall integrity (Fig. 5a). To further validate the cell wall mutilating effect of Ger, scanning electron microscopy (SEM) was performed which clearly depicts that cell wall is distorted in Ger-treated cells (Fig. 5b). Thus, our data demonstrate that Ger leads to disruption of not only membrane but cell wall integrity as well.

Ger phenocopies compromised calcineurin signaling pathway

Calcineurin is a multifunctional regulator which is involved in regulating the cell growth in various stress responses including membrane stress, alkaline pH, serum, ionic and ER (Juvvadi et al. 2014). Cell wall integrity pathway (CWI) is another signaling cascade known to regulate key cellular responses crucial for survival of exposure to antifungal drugs targeting the cell wall and known to be linked with calcineurin signaling (LaFayette et al. 2010). Thus, the functional indispensability of calcineurin to sustain membrane and cell wall stress from previous studies and

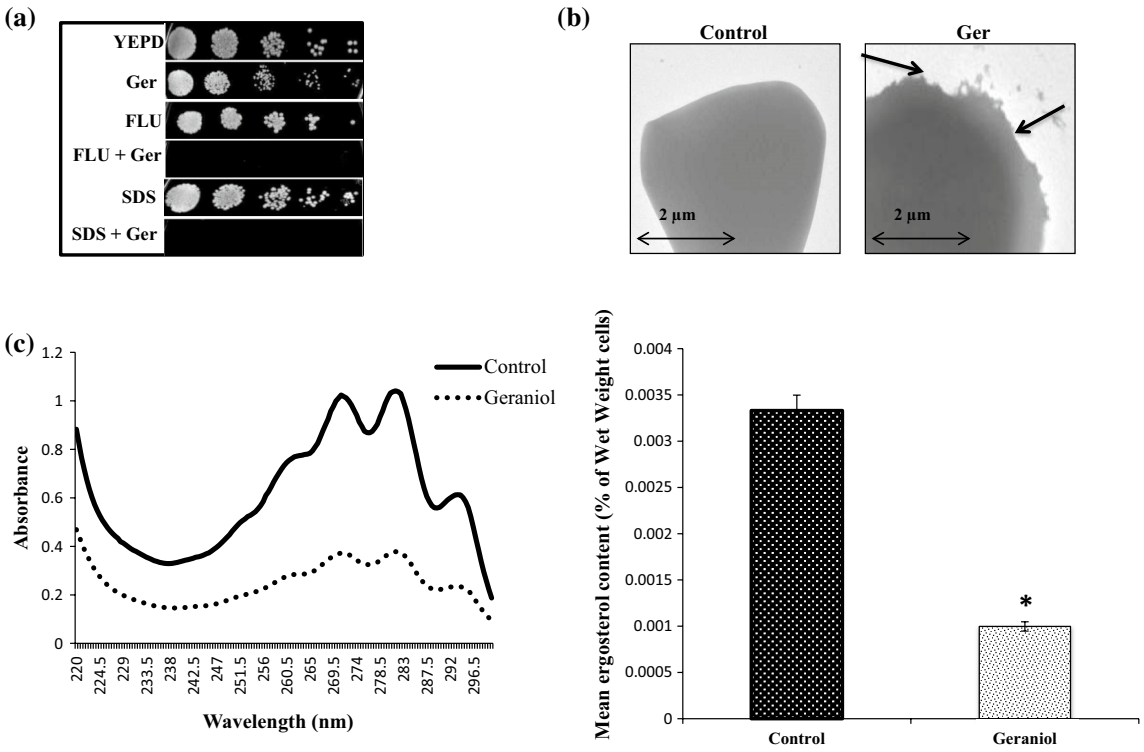


Fig. 3 Effect of Ger on membrane integrity. **a** Spot assays in the absence (control) and presence of Ger (135 µg/ml) with membrane-damaging agents FLU (0.5 µg/ml) and SDS (0.02 %). **b** Transmission electron micrographs, as described in "Materials and methods," showing the smooth surface of untreated cell (control) and the disrupted cell membrane (marked with an *arrow*) because of the excessive damage caused due to Ger. **c** UV spectrophotometric ergosterol profiles of *C. albicans* scanned between 220 and 300 nm from a culture grown for 16 h with and without Ger (135 µg/ml). Relative percentages of ergosterol content in the absence (control) and presence of Ger (135 µg/ml). Mean of % ergosterol levels is calculated as described in "Materials and methods" normalized by considering the untreated control as 100 ± SD of three independent sets of experiments are depicted on y-axis and * depicts P value < 0.05

membrane and cell wall integrity disruption effect of Ger from this study necessitated and in-depth analysis to ensure any possibility of linkage with calcineurin signaling. For this, we performed phenotypic susceptibility assays in the presence of Ger under the stress conditions which require functional calcineurin signaling pathway. We observed that cells without Ger were efficiently able to grow in all the tested conditions viz. 50 % serum, DTT (ER stress), pH 10 (alkaline stress), $CaCl_2$, LiCl and NaCl (ionic stress). However, *Candida* cells grown in the presence of Ger with the above stresses were hypersensitive clearly depicting that antifungal action of Ger is linked with hindered calcineurin pathway (Fig. 6a).

Involvement of calcineurin signaling pathway with antifungal action of Ger became further apparent when we used calcineurin mutants such as *Δcnb1* (regulatory B subunit), *Δcrz1* and calcineurin strain having hyperactive allele. We observed that in contrast to the WT cells, *Δcnb1* mutant was hyper susceptible to Ger while *Δcrz1* mutant was not. Furthermore, in contrast to calcineurin mutant *Δcnb1,* the calcineurin over expressing strain, i.e., strain having hyperactive allele remained resistant to Ger (Fig. 6b). Thus, our data reveal that functional calcineurin signaling pathway is indispensible for *C. albicans* cells to sustain Ger stress. However, whether abrogated calcineurin signaling leads to disruption of cell membrane and cell wall integrity still needs to be validated.

Ger affects mitochondrial functioning, iron homeostasis, genotoxicity but not ROS formation

Mitochondria play an important role in cellular energy metabolism and provide ATP for various cellular processes (Guo et al. 2014). Moreover, many natural compounds have been known to be associated with the mitochondrial dysfunction (Wu et al. 2009). To address this issue, we compared the growth of *Candida* cells in both fermentable (glucose) and non-fermentable (glycerol) source of carbon in the media in the presence of Ger. We observed that in contrast to fermentable carbon media where cells were efficiently growing, growth was severely hampered in Ger-treated cells in non-fermentable carbon media (Fig. 7a). This observation indicates that Ger causes mitochondrial dysfunction affecting cellular respiration. It has been reported in the

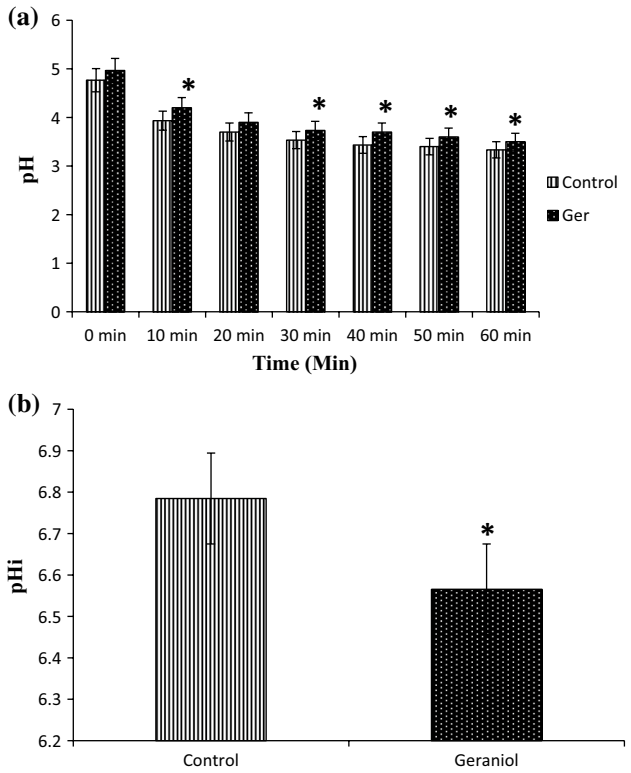


Fig. 4 Effect of Ger on the medium acidification and internal pH of *C. albicans*. **a** Effect of Ger on the glucose-dependent acidification of the medium by *C. albicans* cells. Mean of pH ± SD for untreated (control) and Ger-treated cells of three independent sets of experiments are depicted on y-axis with respect to time (minutes) on x-axis. (* depicts *P* value <0.05). **b** Intracellular pH (pHi) in the presence of Ger in *Candida* cells. Mean of pH ± SD of three independent sets of experiments are depicted on y-axis with respect to control & Ger on x-axis (* depicts *P* value <0.05)

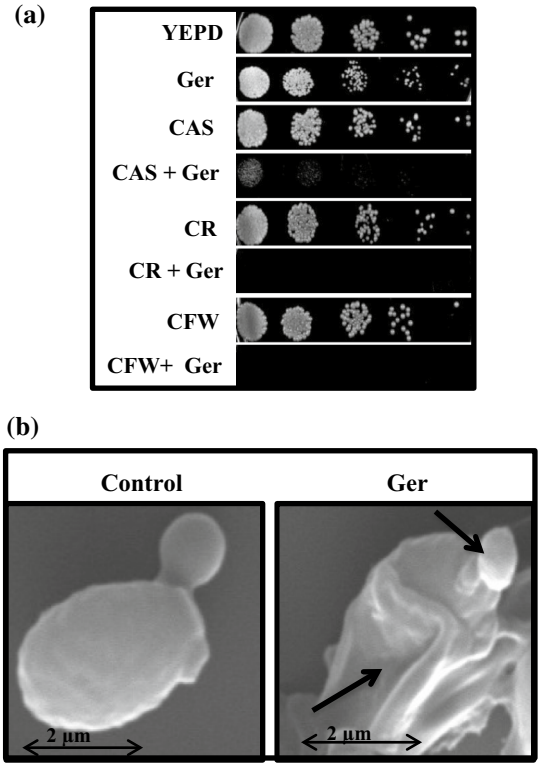


Fig. 5 Effect of Ger on the cell wall of *C. albicans*. **a** Spot assay showing hypersensitivity to Ger (135 µg/ml) in the presence of cell wall-perturbing agents; CAS (0.15 µg/ml), CR (10 µg/ml) and CFW (10 µg/ml). **b** Scanning electron micrographs, as described in "Materials and methods," showing the smooth surface of untreated cell (control) and the wrinkled cell wall with the leakage of its cell contents (marked with an *arrow*) because of the extensive damage caused due to Ger

literature that cell wall stress caused by antifungal agents also affects mitochondrial activity (Qilin et al. 2014) which can be estimated by using MTT assay, an indicator of metabolic activity. To our expectation, we found that Ger significantly inhibits mitochondrial activity in comparison to the control Candida cells (Fig. 7b). Since mitochondria being and a significant player in MDR development in *C. albicans* (Thomas et al. 2013; Hameed and Fatima 2013), effect of Ger on mitochondria could be an important strategy that may be efficiently exploited.

Next we assessed, whether mitochondrial dysfunction due to Ger has any effect on iron homeostasis as mitochondria influence iron homeostasis apart from drug susceptibility and cell wall integrity (Thomas et al. 2013). Moreover, previous studies have already established a link between iron and drug susceptibilities of *C. albicans* that has also been linked to calcineurin signaling (Prasad et al. 2006; Hameed et al. 2011). To confirm this fact, we performed spot assay in the presence of Ger under iron deprivation (BPS is a well-known iron chelator). Our result revealed that iron deprived cells were hypersensitive in the presence of Ger in contrast to control (iron sufficient) cells (Fig. 7c). This observation suggests that Ger also interferes with iron homeostasis.

Terpenoids are known to arrest cell cycle at different stages in *C. albicans* (Zore et al. 2011). In view of this, we speculated whether Ger has any effect on DNA repair machinery. We tested this by growing cells in the presence of EtBr, a known DNA-damaging agent at a concentration in which there was no appreciable inhibition depicting the intact DNA repair machinery. In comparison, when the same cells were treated with Ger, then we could clearly observe hypersensitivity implying some possible defect caused by Ger in DNA repair machinery (Fig. 7c). However, to conclusively say that Ger affects DNA repair machinery in *C. albicans*, we certainly need further validation.

Generation of ROS in fungal cells is an integral part of oxidative stress machinery (Alonso-Monge et al. 2003).

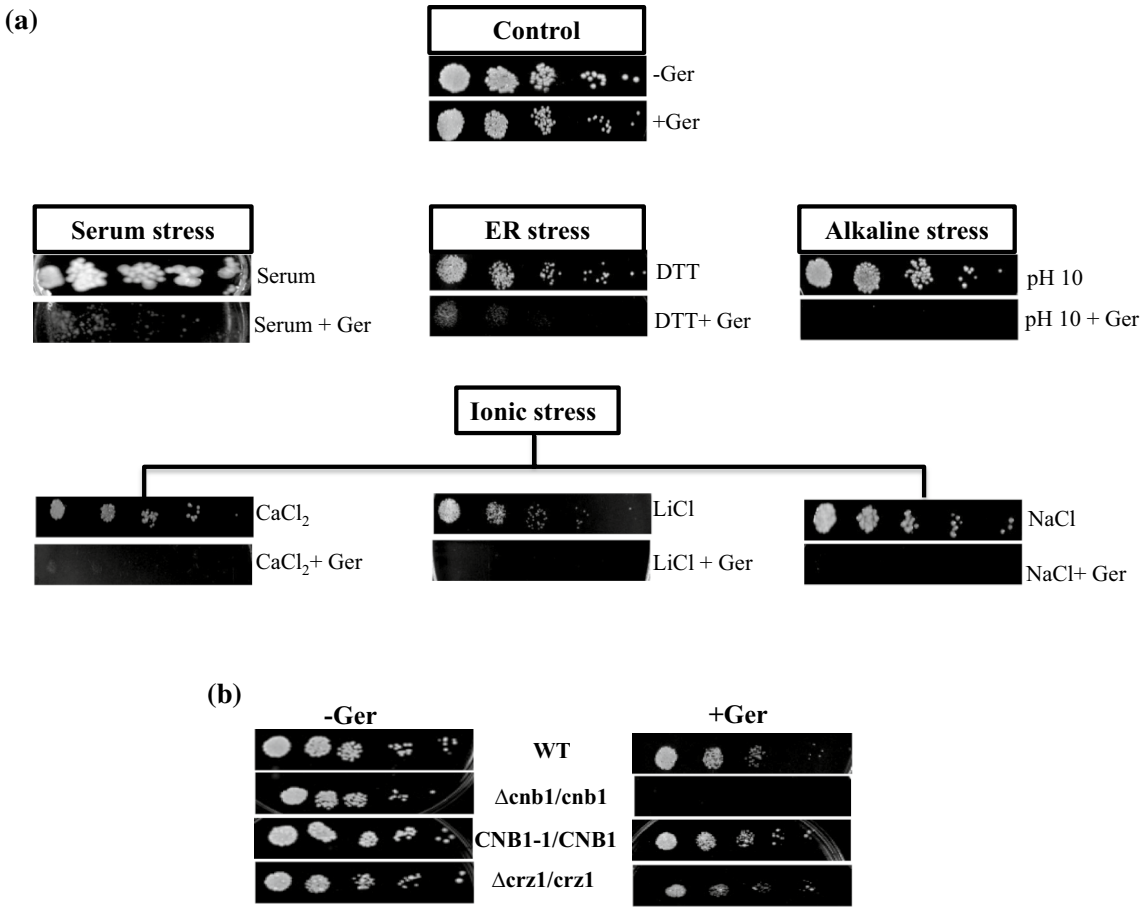


Fig. 6 Phenotypic susceptibility assays to reveal the effect of Ger on the calcineurin-dependent phenotypes in *C. albicans*. **a** Spot assays with and without Ger (135 μg/ml) as controls and under various stresses viz. serum (50 % w/v), DTT (20 mM), pH 10, ions [$CaCl_2$ (0.3 M), LiCl (0.2 M), NaCl (1.5 M)]. **b** Spot assay depicting loss of growth in *Δcnb1* mutant in the presence of Ger (135 μg/ml) while the *Δcrz1* mutant and CNB1-1/CNB1, calcineurin overexpressing strain were efficiently growing in the presence of Ger

Moreover, many antifungal drugs act via generation of ROS itself as their mechanism of action (Li et al. 2015a, b). Thus, we checked whether altered mitochondria function, iron homeostasis and genotoxicity due to Ger also lead to any oxidative stress and therefore involve formation of ROS. For this, we perform the spot assay in the presence of Ger at its MIC_{80} and ascorbic acid (AA), a known antioxidant. We observed that neither *C. albicans* cells were able to grow in the presence of Ger nor addition of AA could revert the hypersensitivity caused by Ger (Fig. 7c). This implies that antifungal action of Ger may not be associated with ROS formation.

Ger is potent yeast to hyphae inhibitor

One of the major virulence factors responsible for success of *C. albicans* to cause infection is yeast to hyphae transition (Gil-Bona et al. 2015). Moreover, this potential attribute has also been linked to calcineurin signaling in many pathogenic fungi (Chen et al. 2012). Many natural compounds have also been studied which are known to inhibit the yeast to hypha conversion (Li et al. 2015a, b; Vediyappan et al. 2013). We therefore tested the effect of Ger on the yeast to hyphae transition of *C. albicans* cells in the presence and absence of Ger (135 μg/ml). It was observed that Ger efficiently inhibited this transition in both liquid and solid various hyphae inducing media namely serum, spider and SLAD (Fig. 8). Thus, our results demonstrates that Ger is potent yeast to hyphae inhibitor; however, whether this morphological inhibition is due to any effect of Ger on calcineurin signaling requires further validation.

Ger inhibits biofilm formation and cell adhesion

Biofilms formed by microorganisms including *C. albicans* still remain a cause of concern being a major contributor of MDR development (Yu et al. 2012). The same drugs which are efficient otherwise are rendered ineffective by *C. albicans* due to biofilm formation (Mishra et al. 2007). Yeast to hypha transition is an important factor responsible for the formation of mature biofilms in *C. albicans*(Ramage

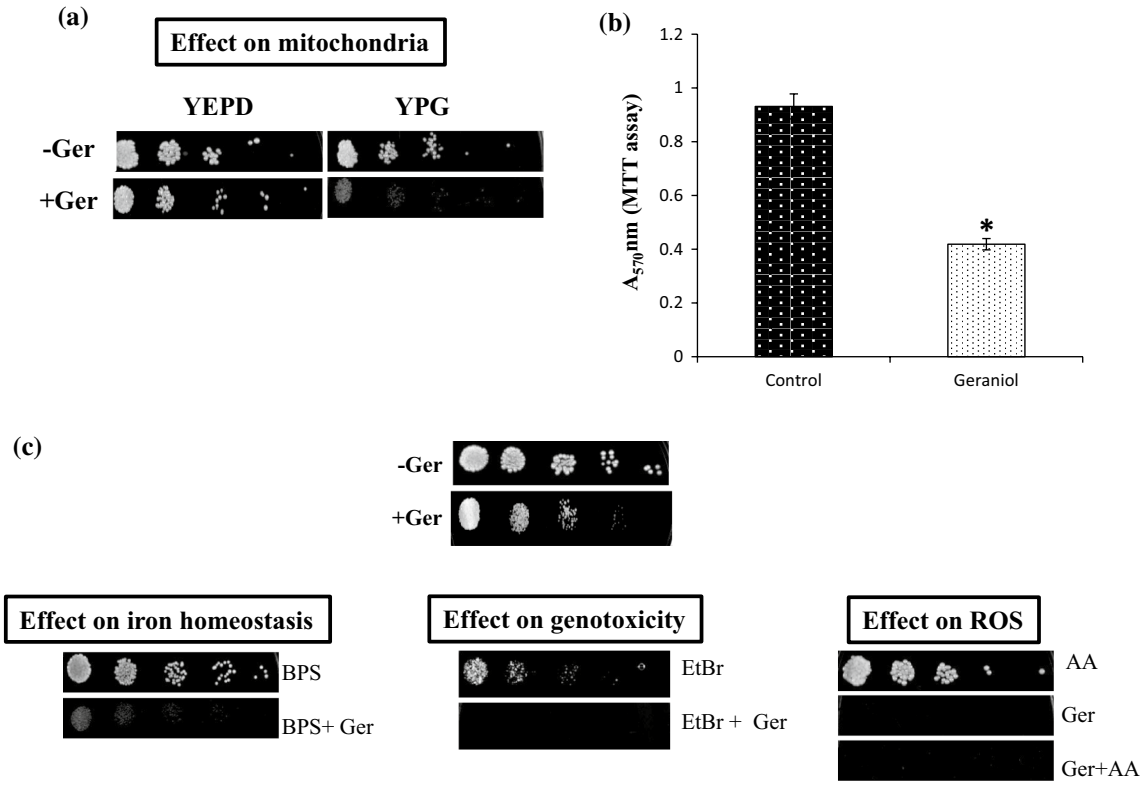


Fig. 7 Effect of Ger on mitochondria dysfunction, iron homeostasis, genotoxicity and ROS formation in *C. albicans*. **a** Spot assays with and without Ger (135 µg/ml) in YEPD (fermentable carbon source) as controls and YPG media (non-fermentable carbon source). **b** Effect of Ger on mitochondrial activity of *C. albicans* depicted as bar graph and quantified by using MTT assay as described in "Materials and methods." Mean of $O.D_{450}$ nm as described in "Materials and methods" ± SD of three independent sets of experiments are depicted on y-axis and * depicts *P* value <0.05. **c** Spot assay depicting loss of growth with BPS (150 µM), EtBr (50 µg/ml) in the presence of Ger (135 µg/ml) while AA (5 mM) treated alone cells were efficiently growing but could not rescue cells treated with MIC_{80} of Ger (225 µg/ml)

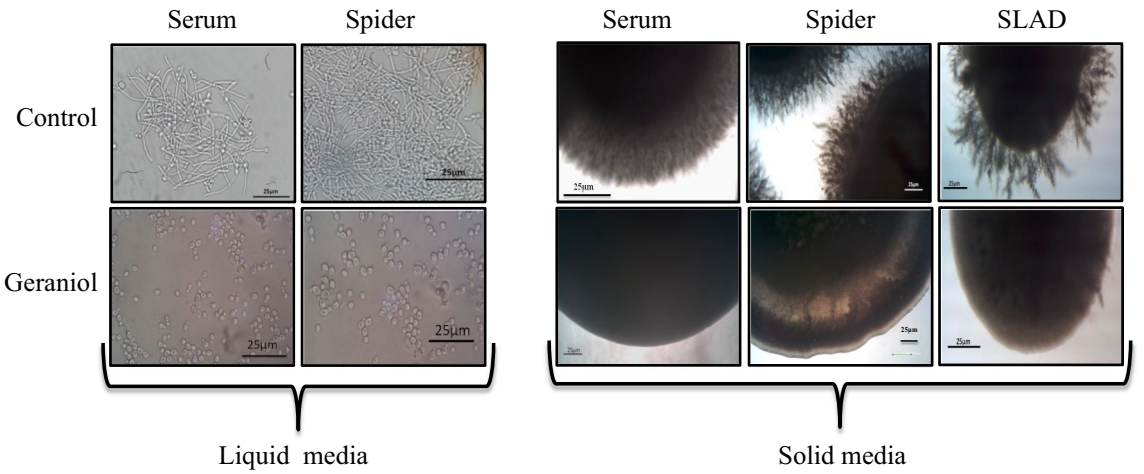


Fig. 8 Effect of Ger on dimorphic switching of *C. albicans*. *Left panel* shows hyphal morphogenesis in the liquid hyphal inducing media (YEPD with 10 % serum and spider media) in the absence (control) and presence of Ger (135 µg/ml) in *C. albicans* (SC5314) at 4 h (magnification 40 ×). *Right panel* shows hyphal morphogenesis in the solid hyphal inducing medium (10 % serum, spider and SLAD medium) in the absence (control) and presence of Ger (135 µg/ml) in *C. albicans* (SC5314) at 6 days (magnification 4 ×)

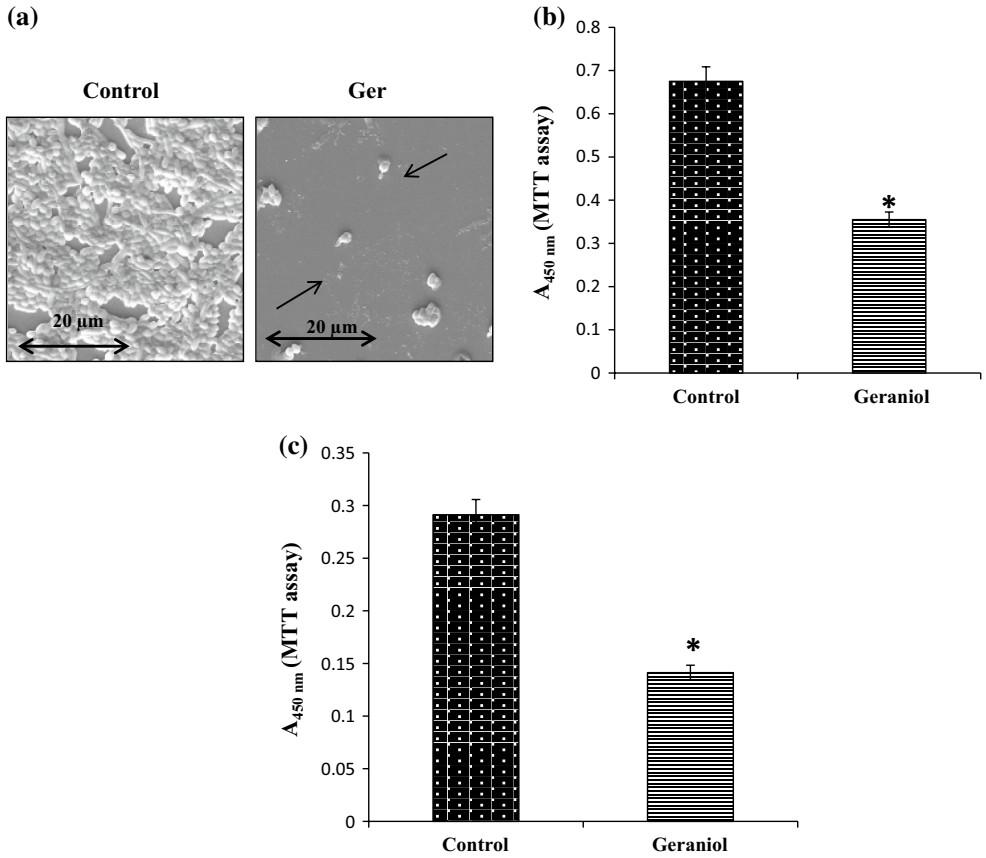


Fig. 9 Effect of Ger on Biofilm formation and Cell adhesion of *C. albicans*. **a** Scanning electron micrographs, as described in "Materials and methods", showing the biofilm formation in the absence (control) and presence of Ger. **b** Effect of Ger on biofilm formation of *C. albicans* depicted as bar graph and quantified by using MTT assay as described in "Materials and methods". Mean of $O.D_{450}$ nm ± SD of three independent sets of experiments are depicted on y-axis and * depicts *P* value < 0.05. **c** Effect of Ger on cell adherence of *C. albicans* quantified by using MTT assay as described in "Materials and methods". Mean of $O.D_{450}$ nm ± SD of three independent sets of experiments are depicted on y-axis and * depicts *P* value <0.05

et al. 2005; Sun et al. 2015). Inhibition of yeast to hyphae by Ger positively led us to hypothesize that biofilm formation may also be affected in the presence of Ger. To test this, biofilm formation was visualized by performing the scanning electron microscopy (SEM) of *C. albicans* biofilms in the absence (control) and presence of Ger (Fig. 9a). We observed that biofilm formation was hampered in *C. albicans* in the presence of Ger. This result was further validated quantitatively by performing MTT assay which showed that biofilm formation was considerably ($P < 0.5$) inhibited by more than 50 % in the presence of Ger in *C. albicans* (Fig. 9b).

Cell adherence is a primary step in formation of biofilm because a cell must adhere to the surface for biofilm formation (Chen and Lan 2015). Therefore, we hypothesized whether the inhibition of biofilm formation in the presence of Ger is due to the inhibition of cell adherence. Interestingly, we observed that cell adherence was significantly ($P < 0.5$) disrupted by (> 50 %) in the presence of Ger (Fig. 9c). Thus, our results show that Ger is an efficient inhibitor of virulence attribute as apart from inhibiting yeast to hyphal transition it also inhibits cell adherence leading to inhibited biofilm formation in *C. albicans*.

Conclusion

Natural compounds such as Ger that can be used as antifungal agents have become a renewed source of interest owing to their natural origin. Our data generated from this study clearly point out toward the promising antifungal activity of Ger. Moreover, potential of Ger to be used in combination therapy with known antifungal drugs may also be studied. Taken together, the multifaceted antifungal targets (Fig. 10) of Ger may provide options to improve

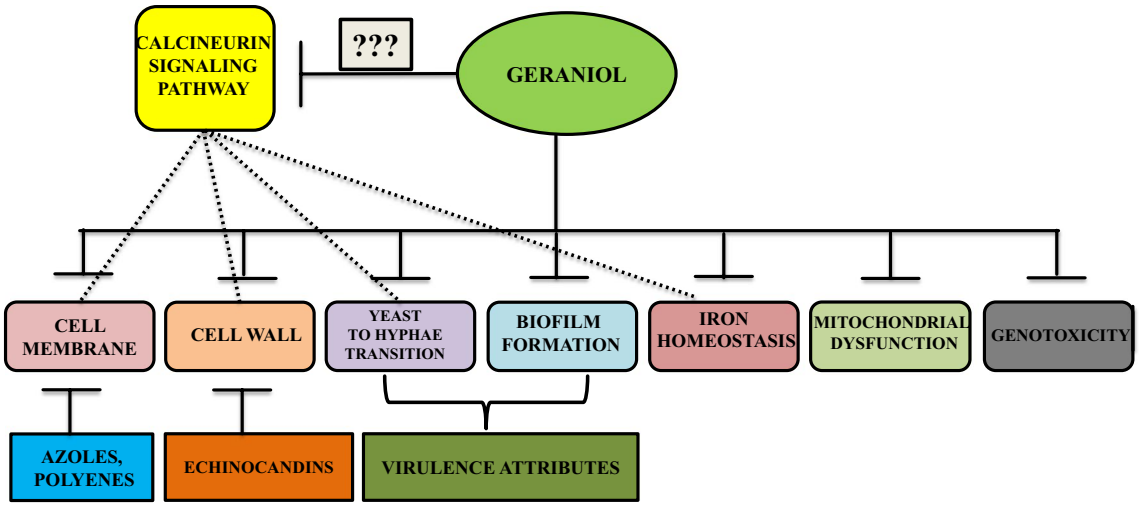


Fig. 10 Summary of mode of action of Ger in *C. albicans*. Ger causes blockage in calcineurin signaling and affects membrane (known target for azoles, polyenes), cell wall integrity (known target for echinocandins), yeast to hyphal transition and biofilm formation (known virulence attributes), iron homeostasis, mitochondrial dysfunction and genotoxicity (shown by dark lines). Compromised calcineurin signaling caused by Ger possibly (?) leads to abrogated membrane homeostasis, cell wall integrity, yeast to hyphal transition and iron homeostasis (denoted by *dashed lines*). All these disrupted mechanisms due to Ger are necessary to sustain drug tolerance and virulence in *C. albicans*

therapeutic strategies and further elucidate mechanisms and pathways responsible for MDR development.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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