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Oxidative stress induced by piperine leads to apoptosis in *Candida albicans*

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Abstract

Candida albicans is a member of pathogens with potential drug resistance threat that needs novel chemotherapeutic strategies. Considering the multifarious biological activities including bioenhancer activity, anti-*Candida* potential of piperine was evaluated against planktonic/biofilm and hyphal growth of *C. albicans* alone or in combination as a synergistic agent with fluconazole. Piperine inhibits planktonic growth at or less than 15 μg/ml, hyphae induction at 5 μg/ml concentration, and exhibits stage-dependent activity against biofilm growth of a fluconazole-resistant strain of *C. albicans* (ATCC10231). Though piperine couldn't kill inoculum completely at minimum inhibitory concentration (MIC), it is fungicidal at higher concentrations, as shown in apoptosis assay. FIC index values indicate that piperine exhibits excellent synergistic activity with fluconazole against planktonic (0.123) and biofilm (0.215) growth of an FLC resistant strain. Mode of anti-*Candida* activity was studied by identifying piperine responsive proteins wherein the abundance of 25 proteins involved in stress response, signal transduction and cell cycle were modulated (22 up and 3 down-regulated) significantly in response to piperine (MIC50). Modulation of the proteins involved suggests that piperine affects membrane integrity leading to oxidative stress followed by cell cycle arrest and apoptosis in *C. albicans*. Flow cytometry-based mitochondrial membrane potential (MMP), cell cycle and apoptosis assay, as well as real-time quantitative polymerase chain reaction analysis of selected genes, confirms piperine induced oxidative stress (TRR1), cell cycle arrest and apoptosis (CaMCA1). Based on our results, we conclude that piperine inhibits planktonic and difficult-to treat-biofilm growth of *C. albicans* by affecting membrane integrity thereby inducing oxidative stress and apoptosis.

Lay Abstract

Piperine inhibit *Candida albicans* growth (planktonic and biofilm) significantly in our study. Piperine exhibits excellent synergistic potential with fluconazole The proteome analysis suggests that piperine induced membrane damage leads to oxidative stress followed by cell cycle arrest and apoptosis.

Key words: *C. albicans*, synergy, piperine, drug resistance, proteomics, apoptosis.

Introduction

Among the 150 species of the genus *Candida*, *Candida albicans* is the most successful opportunistic pathogen of warm-blooded animals including humans.[1–3] Candidiasis is the common term for infections caused by *Candida* that ranges from superficial infections of skin, nails, epithelial (oropharyngeal and vulvovaginal candidiasis, etc.) cells to systemic infections.[4,5] Superficial infections are common and recurrent even in healthy individuals

while systemic infections are frequent and the major cause of mortality among immunocompromised individuals.[3–6] Systemic infections like bloodstream infections (BSI), deep seated tissue invasion and nosocomial infections in the form of biofilm exhibit very high mortality (40–50%) especially among immunocompromised individuals.[6] It is because, invasive infections including biofilm indwelling medical devices (pacemaker, heart valves, catheters, stents, etc.) are considered as difficult-to-treat ones.[6] Cells in different morphological forms and those in biofilm are reported to exhibit differential responses towards both, host immune responses and antifungal agents facilitating emergence of drug resistance.[7–9] Emergence of resistance against the currently available antifungal antibiotics among the clinical isolates further worsened the situation.[3] Considering its impact (clinical and economical), the Center for Disease Control and Prevention (CDC) has included *C. albicans* in the list of pathogens with potential drug resistance threat.[3] It was decided to develop novel and more potent strategies like identifying novel drugs and drug targets against these pathogens.[10–13]

There are already limited options of drugs for antifungal chemotherapy viz. members of azoles, polyenes, allylamines, echinocandins, and other groups of molecules.[3,6,13,14] Emergence of drug resistance against azoles and severe side effects associated with polyenes limited their use though these antifungals were considered as the drugs of gold standard.[6,13–15] Among the several alternative options being explored, combination therapy based on exploiting synergistic potential of different drugs/molecules seems to be the most promising one.[14,15] Combination therapy has shown promising results in case of infectious diseases like tuberculosis, human immunodeficiency virus, and so forth, and noninfectious disease like cancer.[16–19] In addition to this several recent studies have reported that plant molecules exhibit excellent synergy with antifungal agents including azoles and polyenes.[14–18]

The plant products and their derivatives are providing wide variety of lead compounds for the treatment of various diseases alone or in combination with other drugs.[20,21] Considering the excellent synergistic potential of plant molecules, combinatorial therapy is becoming an important option especially against difficult-to-treat diseases and antimicrobial resistance.[21,22] Several plant molecules have shown excellent synergistic activity with fluconazole especially against tolerant organisms.[22] In the present study, we have evaluated anti-*Candida* potential of piperine and its synergy with fluconazole against *C. albicans* strains and isolates. Piperine is used as preventive agent for deterioration and pathogenic diseases in traditional and folk medicines and is a well-known bioenhancer.[23] It is a naturally occurring alkaloid found in black pepper (*Piper nigrum*) and is the major active principle associated with antioxidant, antibacterial, anti-fungal, anti-parasitic, anticancer activity as well as bioenhancer activity of black pepper.[24–28] Piperine is reported to enhance drug uptake by affecting membrane integrity and modulating fluidity. As it is apolar in nature, it was reported to interact with membrane lipids as well as protein microvicinity.[29] Membrane active molecules and piperine-lipid and piperine-protein interactions modulate conformation of membrane lipids and/or proteins thereby affecting membrane integrity leading to increased permeability.[29–31] As piperine is reported to enhance drug uptake by destabilizing membranes, it could be exploited especially against drug resistant *C. albicans* infections and biofilms. In the drug resistant strains/isolates and in the biofilm form growth, achieving threshold concentration of fluconazole is a challenge. Thus in the present study, we have evaluated anti-*Candida* potential of piperine alone and in combination with fluconazole against planktonic and biofilm (adhesion, development and maturation) growth and morphogenesis. To understand its mechanism, we have identified piperine responsive proteins of *C. albicans* using Micro LC-MS/MS analysis and confirmed proteomic data using apoptosis and real-time quantitative polymerase chain reaction (qPCR) analysis.

Methods

Chemicals and growth media

Piperine (97%) was purchased from Sigma-Aldrich India Pvt. Ltd., Bangalore (India). Roswell Pak Memorial Institute (RPMI) 1640 medium, horse serum, YPD (yeast extract peptone dextrose) broth and MTT (3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide) were purchased from Hi-media Laboratories, Pvt. Ltd. Mumbai (India). Polystyrene made 96 well microtiter plates were obtained from Tarson India Ltd., Mumbai (India).

All the fine chemicals for proteomic analysis viz., NaOH, Ethylenediaminetetraacetic acid (EDTA), Sodium dodecyl sulfate (SDS), beta Mercaptoethanol, protease inhibitor cocktail, Dithiothreitol (DTT), Urea, Thiourea and 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate (CHAPS) were procured from Sigma-Aldrich India Pvt. Ltd., Bangalore (India). Analytical grade solvents used in this study, such as acetic acid, methanol, chloroform were purchased from S. D. Fine Chemicals Limited (SDFCL), Bangalore. Phenyl methane sulphonyl fluoride (PMSF) was purchased from Himedia Laboratories, Pvt. Ltd. Mumbai (India). The apoptosis detection kit was a generous gift from ThermoFisher Scientific India Pvt. Ltd. RNeasy Mini kit (50 reactions) (Cat. No. 74104) and KAPA SYBR FAST qPCR Kit Master Mix (2X) Universal were purchased from Qiagen Pvt. Ltd., Mumbai (India), High-Capacity cDNA Reverse Transcription Kit (Cat. No. 4368814) was purchased from Applied Biosystems, Mumbai (India).

Candida albicans isolates and strains

Thirty-one clinical isolates and two standard strains of *Candida albicans*, differentially susceptible to fluconazole (FLC)

Table 1. Effect of piperine on planktonic growth of *isolates* and strains of *Candida albicans* differentially susceptible to fluconazole.

		Candida albicans strains ($n = 2$) and isolates (N = 29)		
Compounds	MICs (mg/l)	Susceptible (N = 17)	S-DD (N = 2)	Resistant (N = 12)
Piperine	25	5	…	3
	5	…	…	1
	10	7	2	2
	15	5	…	6

MIC, minimum inhibitory concentration; S-DD, susceptible dose dependent.

(Resistant-N = 12, Susceptible dose dependent (S-DD)-N = 02 and susceptible-N = 17) used in this experiment were obtained from Swami Ramanand Teerth Culture Collection (SRTCC), School of Life Sciences, Swami Ramanand Teerth Marathwada University, Nanded (MS) India. A FLC resistant strain of *C. albicans* ATCC 10231 (Member of CaDR MP-8 Panel developed by ATCC for drug testing, Application Note 3. 2014) and a susceptible strain ATCC 90028 were obtained from the Microbial Type Culture Collection, Institute of Microbial Technology (IMTECH), Chandigarh (India), and included as a quality control in this study. Yeast extract peptone dextrose (YPD) agar slants were used to maintain all the cultures at 4°C.

Anti-*Candida* activity of piperine against planktonic growth

Anti-*Candida* activity of piperine against 31 clinical isolates and two standard strains of *C. albicans* was evaluated using broth microdilution assay (CLSI M27-A3) as mentioned previously with slight modification.[15,16] Briefly, 100 μl YPD broth containing 24 h grown *C. albicans* yeast phase cells (2×10^3 cfu/ml) and 0.001% (v/v) Tween 20 was transferred aseptically to each well of the 96 well microtiter plates. Double volume (200 μl) was added in the 12th well of each row; the highest concentration of piperine was added to this well and serially diluted up to eightfold. Well number 3 and 2 of each row served as respective solvent controls and well number 1 as control without solvent. Piperine concentration ranged from 1.5 to 15 μg/ml. Plates were observed visibly after 24 h of incubation at 35°C, and concentrations causing complete inhibition of visible growth were considered as minimum inhibitory concentration (MIC). Triplicates were used for each concentration and experiment was repeated three times.

Minimum fungicidal concentrations (MFCs) of piperine were determined by plating 5 μl cultures from these wells on YPD agar plates. Number of colonies appeared on the agar plates were counted after 48 h of incubation at 35°C. The lowest concentration killing 99.9% inoculums was considered as MFC.[15,16]

Effect of piperine on morphogenesis

Effect of piperine on serum inducedmorphogenesis of *C. albicans* (ATCC 10231) was evaluated by using microtiter plate based morphological assay as done previously by Zore et al.[15,16] In brief, 100 μl of YPD broth supplemented with 25% horse serum and 24 h grown *C. albicans* yeast phase cells (10^5 cfu/ml), were treated with different concentrations of piperine (15 to 1.25 μg/ml). Three wells were used for each concentration and wells without piperine, containing respective solvents were used as control. Plates were incubated at 37°C for 90 minutes and cells

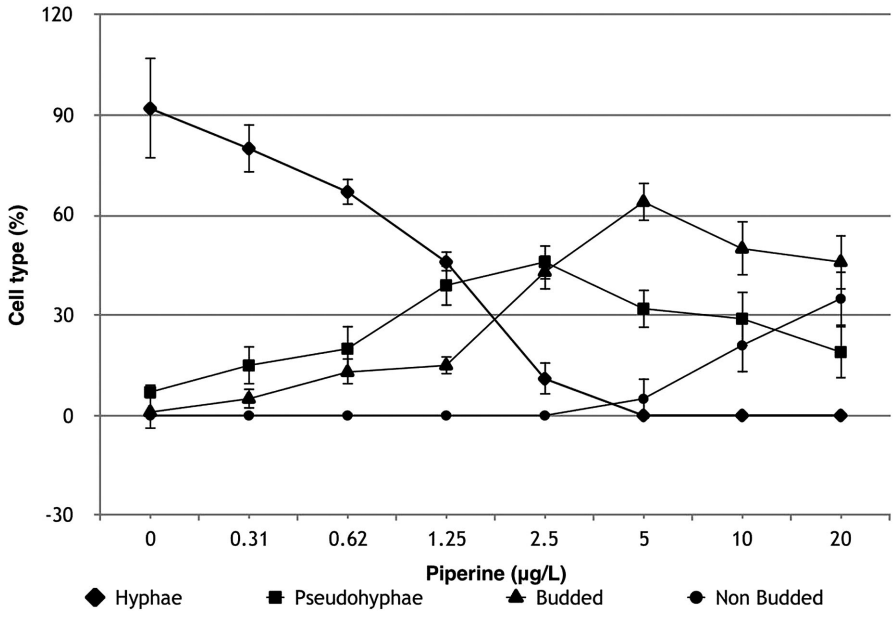

Figure 1. Inhibition of budded morphological forms of *Candida albicans* (ATCC 10231) (hyphae, pseudohyphe, budded, and nonbudded).

Table 2A. Effect of piperine on biofilm growth (adhesion, development, and maturation) of *Candida albicans* (ATCC 10231).

Developmental stage	Biofilm formation (%)					
	Concentrations (mg/l)					
Piperine	0	1.25	2.5	5	10	15
Adhesion	100 ± 0	61 ± 13	56	31	3	2
Developing biofilm	100 ± 0	72	66 ± 9.3	55 ± 7.6	54 ± 8.2	52 ± 6.3
Mature biofilm	100 ± 0	100 ± 0	100 ± 0	93 ± 7	60 ± 15	54 ± 15
Fluconazole	**0**	**16**	**32**	**64**	**128**	**256**
Developing biofilm	100 ± 0	52 ± 5	39 ± 3	38 ± 4	38 ± 3	38 ± 3
Mature biofilm	100 ± 0	62 ± 14	54 ± 10	46 ± 14	45 ± 10	45 ± 11

*Stage of development: Adhesion = after 90 min, Developing biofilm = 24 h, Mature biofilm = 48 h.

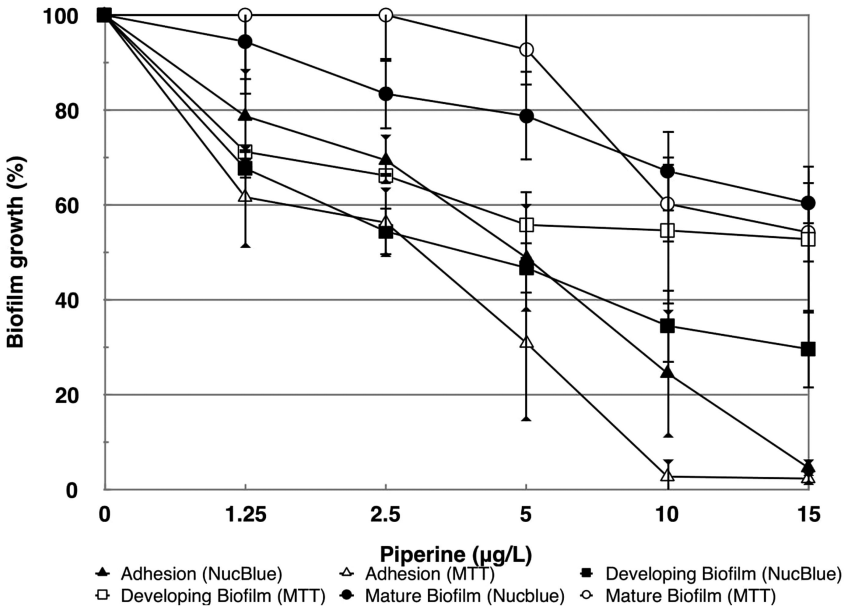

Figure 2. Inhibition of *Candida albicans* (ATCC 10231) biofilm by piperine at adhesion, development, and maturation stage of growth using MTT assay. Values represent mean of three reading and error bar indicates standard deviation.

of different morphological types (budded, un-budded, hyphae and pseudohyphae) were observed microscopically, and percentage of cell types were calculated using hemocytometer (Fig. 2).

Inhibition of *Candida albicans* biofilm formation (adhesion, development, and maturation) by piperine

Preparation of inoculum

Inoculum was prepared using *C. albicans* cells grown in YPD broth for 24 h at 30°C were harvested, washed with sterile distilled water and resuspended in phosphate-buffered saline (PBS).

Biofilm formation: adhesion, development, and maturation

Inhibition of biofilm formation by piperine (at 15 to 0.3 μg/ml) at different developmental stages viz. adhesion, development and maturation were evaluated using biofilm assay.[30] Inhibition of biofilm formation by piperine (15 to 0.3 μg/ml) was assessed by measuring adhesion (90 min), development (24 h) and maturation (48 h) by using MTT assay after removing nonadhered cells.[30]

In brief, adhesion was performed by dispensing 100 μl inoculum without and with Piperine (concentrations mentioned above) aseptically to the wells and incubated at 37°C for 90 min. Non-adhered cells were removed by washing the wells three times with PBS and adhesion was measured by comparing with the respective solvent controls. To evaluate efficacy against biofilm development, cells were allowed to adhere (adhesion) as mentioned above. In sum, 100 μl of fresh RPMI 1640 medium with and without piperine was added to these wells and incubated further. Growth was measured after 24 h incubation at 37°C by comparing with respective solvent controls. To evaluate efficacy of Piperine against mature biofilm, 24 h old biofilm were further incubated for 48 h at 37°C after addition of 100 μl

Table 2B. Synergistic activity of Piperine with fluconazole against planktonic and biofilm growth of *Candida albicans* (ATCC 10231).

	Concentrations		FICs		
Combinations	FLC (mg/l)	Piperine (mg/l)	FLC	Piperine	FIC index/Interaction
Planktonic growth (MIC 100)					
FLC alone	256	0	1	0	1.0/ADD
FLC + piperine	8	0.92	0.03	0.18	0.123/synergistic
Piperine	0	10	0	1	1.0/ADD
Developing biofilm (MIC 50)					
FLC alone	256	0	1	0	1.0/ADD
FLC + piperine	8	0.92	0.03	0.09	0.12/synergistic
Piperine alone	0	10	0	1	1.0/ADD
Mature biofilm (MIC 50)					
FLC alone	256	0	1	0	1.0/ADD
FLC + piperine	8	1.85	0.03	0.18	0.21/synergistic
Piperine	0	10	0	1	1.0/ADD

FIC, fractional inhibitory concentrations; FIC index, ΣFICs; FLC, fluconazole; FICI, FIC index; FICI value ≤ 0.5 = Synergy; FICI>0.5–≤4.0 = no interaction; FICI>4, antagonistic (Odds 2003).

of fresh RPMI 1640 medium with and without piperine. Growth was measured after 48 h incubation by comparing with respective solvent controls. Triplicates were used for each concentration and experiment was repeated three times.

Synergy with FLC against planktonic growth and developing biofilm of a FLC resistant strain of *Candida albicans* (ATCC 10231)

Synergistic activity of piperine with FLC was studied by microdilution checkerboard assay using different combinations of piperine (1.5 to 15 μg/ml) and FLC (2 to 128 μg/ml)[7] and growth inhibition was determined by MTT assay.[19]

MTT assay

MTT assay was performed as described in Ramage et al. (2007).[30] In brief, biofilm growth was measured by monitoring the reduction of MTT (0.1 mg/well) after 5 h of incubation under dark condition. Reduction product was solubilized in DMSO (100 μl /well), and OD was read at 570 nm. Percentage inhibition of biofilm formation (adhesion, development and maturation) by Piperine was calculated by considering growth in control wells as 100%.

Identification of piperine responsive proteins

Lysis and extraction of proteins

C. albicans cells exposed to piperine MIC 50 for 24 h was harvested using 0.22-micron membrane filters. The cells were washed and proteins were extracted using optimized protocol.[20] In brief, 5×10^6 cells were transferred to the vials containing 2000 μL lysis buffer (0.1 M NaOH, 0.05 M EDTA, 2% SDS, 2% Beta Mercaptoethanol) containing protease inhibitor cocktail (10 μL/ml), vortexed briefly and lysed by incubating at 90°C for 10 min. Cell lysates were neutralized by adding 50 μl of 4 M acetic acid, with gentle vortexing and incubating further for 10 min at 90°C. Phenyl methane sulphonyl fluoride (5 μl/ml) were added to the supernatants separated by centrifugation (5000 rpm for 5 min) and proteins were precipitated using methanol: chloroform: water (4:1:3) with simultaneous vortexing. Protein pellets were separated by centrifugation (5000 rpm for 5 min) under chilled condition, washed using 3 volumes of methanol, centrifuged (5000 rpm for 5 min), air dried, and resuspended in rehydration buffer (CHAPS 2%, DTT 1%, Urea 6 M, Thiourea 2 M). Protein concentration was estimated by Bradford method using bovine serum albumin as a relative standard.

Sample processing for LC-MS

Trypsin digestion Proteins extracted from yeasts were subjected to trypsin digestion. Briefly, the proteins were reduced, alkylated and digested into peptides, which were cleaned up by means of Zip tip C_{18} chromatography (Millipore; Billerica, MA, USA).[32–36]

Liquid chromatography and mass spectrometry All the samples were analyzed on Triple-TOF 5600 (AB Sciex; Concord, Canada) mass spectrometry coupled with Micro LC 200 (Eksigent; Dublin, CA, USA) in high-sensitivity mode.[33–35] To generate the SWATH spectral library equal amounts of peptide samples from each treatment were pooled together and analyzed via LC-MS/MS (Information dependent acquisition IDA).

SWATH MS analysis SWATH MS data sets were acquired (in triplicate) on Micro LC-Triple TOF 5600, briefly peptides were directly injected onto a Eskigent C18-RP HPLC column (100 × 0.3 μg/ml, 3 μm, 120 Å) and then separated using a

Table 3. Piperine responsive downregulated and upregulated proteins of *Candida albicans* (ATCC 10231).

Sr. no.	Uniport ID	Protein name	Description	Fold change
Downregulated proteins				
Cell cycle	CDC37	Q5ACE0	Necessary for passage through the START phase of the cell cycle and stabilizes protein kinase and its folding.	7.34
Translation	TIF11	Q5A5P8	Putative uncharacterized protein predicted as TIF11 (Translation initiation factor eIF1a)	5.14
Transport	BRE5	Q59QX3	Possible de-ubiquitination complex subunit as have role in protein deubiquitination and regulation of ER to Golgi and Golgi to ER vesicle-mediated transport.	3.00
Upregulated proteins				
Regulation of gene expression	C4YFG3	CAWG_01280	DNA-directed RNA polymerase II	3.62
Translation, PTM, elongation factor	C4YN67	CAWG_02307	Eukaryotic translation initiation factor 4E	2.11
	Q5AI93	FRS2	Putative uncharacterized protein FRS2: likely as an alpha subunit of active phenylalanyl-tRNA synthetase forms tetramer with Frs1p in translation	2.62
	B9W7Y5	Rpl7/CD36_05400	60S ribosomal protein L7:	2.87
	M3JF49	G210_0332	Uncharacterized protein: predicted to have function in inter-relay of apoptosis.	4.40
	Q5AL21	Rpn1	Likely 26S proteasome regulatory particle subunit	1.99
	C4YPV8	CAWG_02512	Alpha-1,2 farnesyltransferase	2.85
Energy generation and mitochondrial protein	Q59QN7	SDH2	Succinate dehydrogenase	2.00
	Q5AHK8	PYC2	Pyruvate carboxylase,	2.04
	Q5A2L3	GDH2	Putative glutamate dehydrogenase	2.08
Cytoskeleton protein	Q5A094	SLA2	Putative uncharacterized protein, ortholog has role as adapter protein which involved in linking actin-clathrin and regulating endocytosis.	2.95
Metabolism	G8BFQ0	CPAR2_203270	Putative uncharacterized protein, carboxy-lyase activity,	2.11
	Q5AMQ5	CTN2	Carnitine acetyl transferase	5.57
	…	SOL3	Potential 6-phosphogluconolactonase	2.22
Transport protein	Q5A330	PEX19	Potential peroxisomal biogenesis protein	2.03
	C4YPG0	CAWG_02359	Putative uncharacterized protein, ADP-ribosylation factor GTPase activating protein.	3.11
Apoptosis	Q59NB3	SNL1	Putative BAG-1 protein involved in anti-apoptotic mechanism through binding to BCL2.	2.49
Nucleotide binding		DEHA2B14322	Nucleotide binding protein have role in signal transduction.	3.74
Stress response	Q59Z58	CQR1	Potential reductase, flavodoxin.	4.12
	Q5A8Z9	CaO19.10846	Putative uncharacterized protein; It is a plasma membrane protein was reported to be repressed by nitric oxide stress	2.99
	Q59Z38	ZTA1	Zinc ion binding, involved in oxidoreductase activity.	3.00
	B9WBH7	WHS11	White colony protein, putativeIt is Ortholog protein have role in pathogenesis, phenotypic switching and cytoplasm localization. It also plays important role in stress response	1.99

90-min gradient of 3% to 35% mobile phase (Mobile phase A: 100% water with 0.1% (v/v) formic acid, Mobile Phase B: 100% acetonitrile with 0.1% (v/v) formic acid) at a flow rate of 8 μl/min. In SWATH-MS mode, the instrument was specifically tuned to optimize the quadrupole settings for the selection of precursor ion selection windows 25 *m/z* wide. Using an isolation width of 25 *m/z* (containing 1 *m/z* for the window overlap), a set of 34 overlapping windows was constructed covering the precursor mass range of 400–1250 *m/z*. SWATH MS/MS spectra were collected from 100 to 2000 *m/z*. The collision energy was optimized for each window according to the calculation for a charge 2+ ion centered upon the window with a spread of

Table 4. Primers used in this study for real-time expression analysis of selected genes.

Gene	Gene ID	Accession No.	Forward (5-3)	Reverse (5-3)	Amplicon size
KRE9	3635303	NW_139424	CGGATCAAGCTTCAGGATTT	CATTTGCATTGGTGCGTATC	107
RPL11	3643557	NW_139516	TCCCAAAATGTTATGCGTGA	CTAAAACTTTGGCGGCTCTG	100
MCA1	3635381	NW_139424	TGGTACTGCCACTGGTGCTA	TGGGAAGCAGATAATTGTGG	144
TRR1	3637778	NW_139442	TCTACGCCATTGGTCACATC	ATCACCAGCTGCAAACACAC	131
ERG11	3641571	NW_139483	CCATTTGGTGGTGGTAGACA	GGGTCAGGCACTTTATAACCA	125
GAPDH	…		CGGTCCATCCCACAAGGA	AGTGGAAGATGGGATAATGTTACCA	…

15 eV. An accumulation time (dwell time) of 100 ms was used for all fragment-ion scans in high-sensitivity mode, and for each SWATH-MS cycle a survey scan in high-resolution mode was also acquired for 100 ms resulting in a duty cycle of 3.4 s.[33–36] Statistical analysis was performed by Student *t*-test, probability *P* value less than .05 were considered to be significant, Number of matching peptides ≥2 and 2-fold change difference of protein expression (biological samples were acquired in triplicate).[33–36]

Mitochondrial membrane potential (MMP) assay

C. albicans were grown for 24 hours at 30°C were used for evaluating effect of piperine on membrane potential. In brief, *C. albicans* cells (5×10^6 cells/ml) were exposed to piperine (MIC) for 6 hours. Afterward, 30 minutes of incubation at 37°C in an atmosphere of 5% CO_2, 1 μl of the 10 mM stock solution of MitoTracker Red dye was added to each well. Thereafter, cells washed with PBS were resuspended into 100 μl of 1X annexin-binding buffer followed by addition of 5 μl of Alexa Fluor 488 annexin V (Component A) in each well. Afterward, 15 min incubation at room temperature, 400 μl 1X annexin-binding buffer was added in each well and analyzed the stained cells by flow cytometer, measuring the fluorescence emission at 530 nm and 585 nm.[37]

Cell cycle assay

C. albicans cells were exposed to Piperine for cell cycle assessment. After 24 hours of treatment with piperine (1 mg/ml and measured by flow cytometry analysis).[14,15]

Apoptosis assay

Detection of piperine induced apoptosis was carried out in *C. albicans*. Cells that were exposed to piperine (MIC50) were evaluated after 24 hours by Annexin V Alexa FluorR 568 conjugate/PI binding and measured by flow cytometry analysis.[14] Experiment was repeated three times in which samples were used in triplicates.

Analysis of piperine responsive genes using RT-qPCR

C. albicans cells (1×10^7) exposed to piperine were lysed by using lyticase, and the total RNA were extracted using RNeasy Mini kit (50 reaction) (Cat. no. 74104, Qiagen Pvt. Ltd) as per the manufacturer's instructions and as described previously by Thakre et al. (2017).[14] cDNA were synthesized using 2 μg of total RNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems Cat. no. 4368814) as per the manufacturers' instructions. Expression analysis of five genes was carried out using KAPA SYBR FAST qPCR Kit Master Mix (2X) Universal and using CFX96 TouchTM Real-Time PCR Detection System (Bio-rad Pvt. Ltd) as per manufacturers' instructions and parameters. The optimized primers used in this study were shown in Table 4. The targeted genes were analyzed in triplicates using biological replicates, and data are reported as mean ± SD. Statistically significance was calculated using ANOVA, and *P*-values less than .05 were considered statistically significant. Initially, gene expression was normalized with GAPDH levels and then to untreated control cells.

Results

Inhibition of planktonic growth

Our results showed that piperine is equally effective against all the isolates and strains differentially susceptible to fluconazole (Table 1). Among the tested ($n = 31$), all the isolates were inhibited completely at or less than 15 μg/ml concentration of piperine (Table 1). Among the 17 susceptible isolates, five are inhibited at 2.5 μg/ml, seven at 10 μg/ml, and 5 at 15 μg/ml of piperine (Table 1). Among the 12 resistant isolates, three are inhibited at 2.5 μg/ml, one at 5 μg/ml, two at 10 μg/ml, and six at 15 μg/ml of of piperine (Table 1). Two of the S-DD isolates are inhibited at 10 μg/ml of piperine (Table 1). MFC analysis suggests that piperine was fungistatic at MIC; however, higher concentration could kill *C. albicans* cells significantly.

Effect of piperine on *C. albicans* morphogenesis

Piperine exhibited concentration dependent activity against *C. albicans* morphogenesis, that is,. percentage of hyphae induction decreased with increasing concentrations (Fig. 1). Piperine

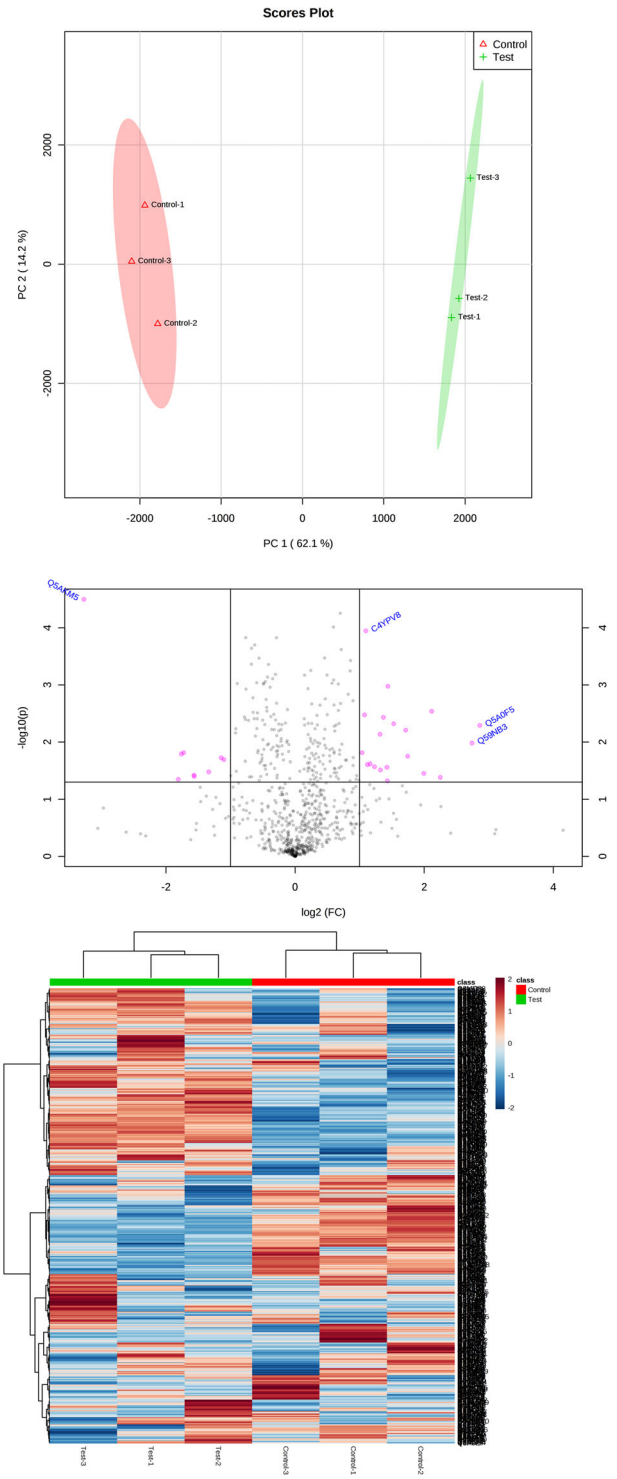

Figure 3. (A) PCA plot. All replicate acquisitions of control and test are clustered together in PCA suggesting reproducibility of replicate acquisitions (upper image). (B) Volcano plot. Pink or red color dots indicate differentially expressed proteins with ≥2-fold change and ≤.05 *P* value (middle image). (C) Heat map of fold change of proteins in individual replicates. Red color is for upregulated and blue is for downregulated proteins (lower image)

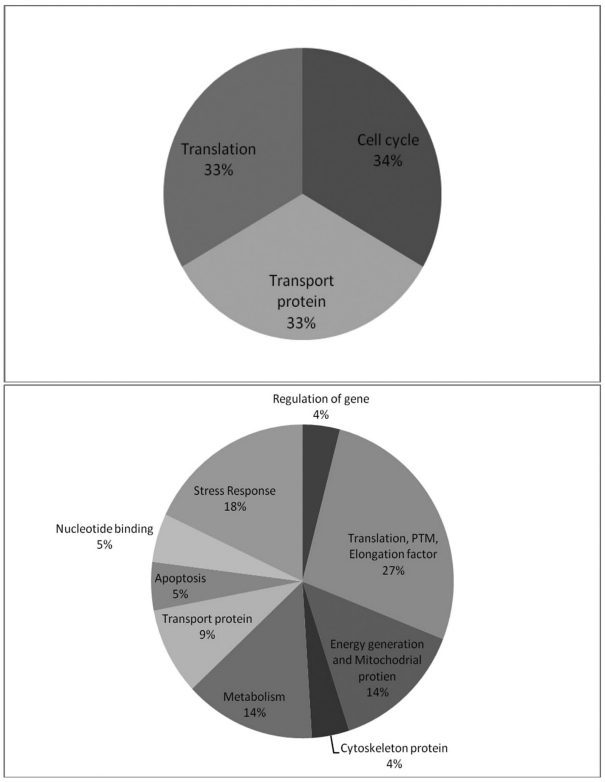

Figure 4. Distribution of piperine responsive downregulated (a), and upregulated (b) proteins of *Candida albicans* (ATCC 10231) identified by LC-MS analysis.

inhibited hyphae induction by 50% only at 1.25 μg/ml concentration while complete inhibition was achieved at 5 μg/ml (Fig. 1). Interestingly, percentage of pseudohyphae increased up to 46% at 2.5 μg/ml of piperine compared 7% at control (Fig. 1). However, further increase in concentration decreased pseudohyphae formation (Fig. 1). Similarly, percentage of budded cells increased to maximum of 30% at 5.5 μg/ml concentration while further increase in concentration increased percentage of unbudded cells (Fig. 1)

Effect of piperine on *C. albicans* biofilm formation (adhesion, development, and maturation)

Anti-biofilm activity of piperine was tested against three developmental stages (viz. adhesion, development and maturation) of biofilm form growth of *C. albicans*. Adhesion of *C. albicans* (ATCC 10231) cells to 96 well microtiter plates (basal surface) in presence of various concentrations of piperine (15 to 1.25 μg/ml) was tested. The minimum concentration of piperine used in this study (1.25 μg/ml) inhibited adhesion by 40% (Table 2A, Fig. 2). Though piperine couldn't inhibit adhesion completely, 10 μg/mL concentration caused 98% inhibition (Fig. 2, Table 2A). Piperine inhibited biofilm development by 30% at 1.25 μg/ml piperine while 5 μg/ml concentration caused 45% inhibition (Fig. 2, Table 2A). Further increase in

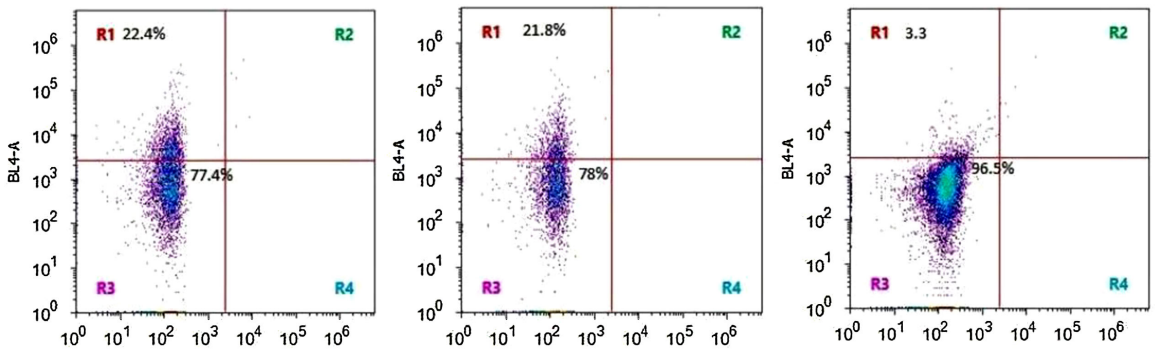

Figure 5. Effect of piperine on membrane integrity of *C. albicans* cells treated with (A) piperine (MIC50), (B) fluconazole (MIC50), (C) untreated (from right to left). Quadrant analysis of fluorescence intensity of gated cells in RL2-H (Alexa Fluor 488 annexin V (Component A) and BL4-A (PI) channels was from 10,000 events. Values shown were percentages of each quadrant. $^{*}P < .05$, in comparison to control.

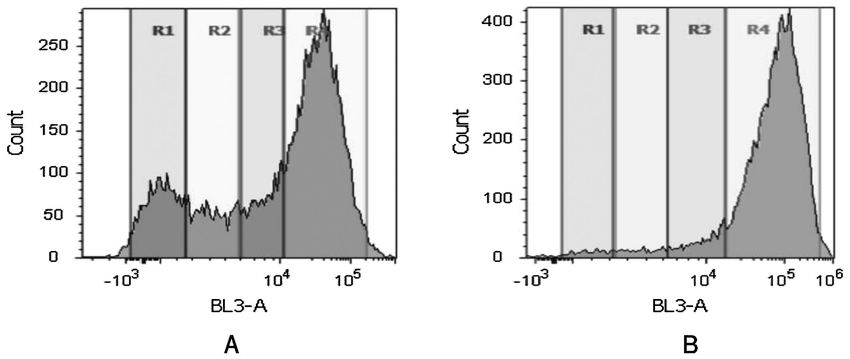

Figure 6. Cell cycle progression in *Candida albicans* (ATCC 10231) in (A) absence and (B) presence of piperine (1 μg/ml). Phases of cell cycle viz. R1 = G1, R2 = G0-G1, R3 = S, R4 = G2-M.

concentration by threefold (15 μg/ml) could achieve maximum of 48% inhibition of biofilm development (Fig. 2). Mature biofilm seem to be more tolerant, as 10 μg/ml of piperine could cause 40% inhibition of mature biofilm while 15 μg/ml could increase it up to 47% (Fig. 2, Table 2A).

Synergy with FLC against planktonic and biofilm development of *C. albicans* (ATCC 10231)

Piperine (0.92 μg/ml) in combination with FLC (8 μg/ml) sensitized and inhibited planktonic growth completely, indicating excellent synergistic activity (Table 2B). Similarly, piperine at 0.92 and 1.85 μg/ml concentration could inhibit 50% growth of developing and mature biofilm respectively, in presence of 8 μg/ml FLC (Table 2B). FIC index value of 0.123 and 0.215 indicates very good synergistic activity of piperine with FLC against developing and mature biofilm, respectively (Table 2B).

Identification of piperine responsive proteins using LC-MS/MS analysis

LC-MS/MS analysis could identify 1000 proteins, out of which abundance of 25 proteins was found to be significantly modulated in response to piperine. The functional annotation was carried out using a software David (functional annotation bioinformatics microarray) and databases like NCBI, CGD, SGD, and so forth (Table 3, Fig. 3A,B,C; Fig. 4a,b). The detailed description of these proteins with significant changes in expression level in response to piperine is provided in Figure 3A,B,C; and Figure 4a,b; Table 3. Among the 25 identified proteins, 22 were upregulated and 3 were downregulated in response to piperine (Table 3).

Three proteins viz. Cdc37 (cell division cycle protein 37) (33.33%), Tif11 (translation initiation factor eIF1A) (33.33%) and Bre5p (33.33%) involved in cell cycle, translation, and transport were downregulated by 7.34, 5.14, and 3.00-fold, respectively (Table 3, Fig. 4a,b).

Twenty-two piperine responsive up regulated proteins were involved in Regulation of gene expression (4%), translation Elongation Factor and PTM (27%), Energy generation and mitochondria (14%), cytoskeletal (4%), energy generation and metabolism (14%), stress response (18%), transport protien (9%), apoptosis (5%), and nucleotide binding (5%) are provided in Table 3, and Figure 4a,b.

Piperine modulate gene expression significantly as one protein involved in transcription, that is, CAWG_01280

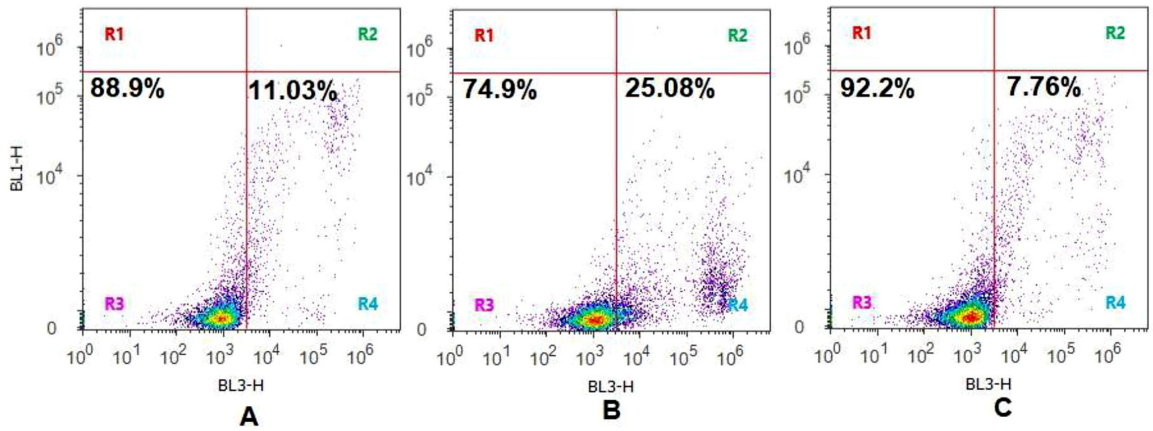

Figure 7. Induction of apoptosis in *Candida albicans* (ATCC 10231) cells treated with piperine (A) 0.5 μg/ml, (B) 1 μg/ml, (C) untreated (from right to left). Piperine treated apoptotic *Candida albicans* cells were stained with Annexin V/PI and subjected to flow cytometry analysis. The four quadrants represent living cells R3 (Annexin V-PI-), early apoptotic R4 (Annexin V + PI-), late apoptosis R2 (Annexin + PI+) or necrotic or dead R1 (Annexin V-PI+) stages. Quadrant analysis of fluorescence intensity of gated cells in BL3-H (Annexin V-FITC) and BL1H (PI) channels was from 10,000 events. Values shown were percentages of each quadrant $P < .05$, in comparison to control.

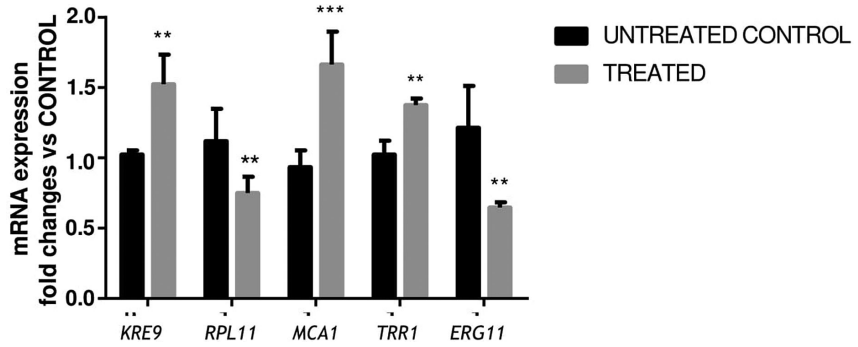

Figure 8. Expression analysis of selected genes of *Candida albicans* (ATCC 10231) by real-time qPCR in response to piperine. *Data are expressed as the mRNA copies in cells, where significance refers to the differences between Untreated control and treated cells* ($n = 3$; $^{***}P < .001$, $^{**}P < .01$. Error bars indicate SD.

(subunit of RNA Pol II) and six proteins involved in translation and PTM viz. G210_0332, CD36_05400, CAWG_02512, Frs2, CAWG_02307/ Eif4E and Rpn1 were upregulated in response to piperine in our study by 3.62-, 4.40-, 2.87-, 2.85-, 2.62-, 2.11-, and 1.99-fold, respectively (Table 3). Piperine modulated energy generation as three proteins involved in energy generation viz. Sdh2, Pyc2 and Gdh2 were upregulated by 2.00-, 2.04-, and 2.08-fold, respectively (Table 3). Over expression of Sla2 in response to piperine indicates that remodeling of cytoskeleton assembly was induced by 2.95-fold (Table 3). Piperine enhanced three proteins in metabolic processes as CPAR2_203270 with carboxy-lyase activity, Sol3 that hydrolyze 6-phosphogluconolactone to 6-Phosphogluconate in pentose phosphate pathway and Ctn2 that transfer acetyl CoA intracellularly were upregulated significantly by 2.11-, 2.22-, and 5.57-fold, respectively (Table 3). Piperine seems to affect transport in *C. albicans*, as two proteins viz. Glo3 and Pex 19 involved were upregulated (Table 3). Pex19 (Potential peroxisomal biogenesis protein) a peroxins involved in biogenesis and replication of peroxisome was upregulated by 2.03-fold and Glo3 (uncharacterized protein) involved in coating COP-I of Golgi vesicle and mediate ER to Golgi and Golgi to ER transport was upregulated by 3.11-fold in response to piperine (Table 3).

Upregulation of Snl1 (ribosome associated protein) plays major role in translation and likely to be BAG-1 protein in mammal involved in anti-apoptotic mechanism was upregulated by 2.49 (Table 3). Four proteins viz., Whs11 (white colony protein, putative), Zta1 (threefold), Cqr1 (potential reductase, flavodoxin), and CaO19.10846 (putative uncharacterized protein) involved in stress response were upregulated by 1.99-, 3.00-, 4.12-, and 2.99-fold, respectively (Table 3). DEHA2B14322g binds with GTP (nucleotide binding) and plays important role in signal transduction was upregulated by 3.74-fold (Table 3).

Validation of LC-MS/MS data

MMP assay

Piperine induced membrane integrity damage evaluated by Alexa Fluor 488 annexin V (Component A) using flow cytometry method indicates that piperine (MIC50) and FLC (MIC50) affect 22.4% and 21.8% cells, respectively, compared to 3.3% in control (Fig. 5A,B,C).

Cell cycle assay

Piperine (1 μg/ml) arrested 87% cells inG_2-M Phase of cell cycle in *C. albicans* compared to 57% in control (Fig. 6A,B).

Apoptosis assay

The apoptotic *Candida albicans* cells were evaluated by Annexin V FITC using flow cytometry assay. Piperine (0.5 μg/ml and 1 μg/ml) induces apoptosis in 11.03% and 25.08% of cells, respectively, as compared to 7.76% in control (Fig. 7A,B,C).

Real time qPCR analysis of piperine responsive genes

The expression analysis of five genes viz., *KRE9, RPL11, MCA1, TRR1,* and *ERG11* was carried out using real-time qPCR to support our proteomic and flow cytometry data. Out of the five, three viz. *KRE9* (cell wall biosynthesis), *TRR1* (oxidative stress) and *MCA1* (apoptosis) were upregulated while two viz. *RPL11*(nucleolar stress) and *ERG11 (ergosterol biosynthesis*) were downregulated as compared to control. The expression pattern is shown in Figure 8.

Discussion

Piperine (1-peperoylpiperidine) an alkaloid molecule and a major active principle associated with a variety of biological activities of black pepper (*Piper nigrum)*.[23–25] Black pepper is one of the major components of spices being used worldwide, especially in south Asian countries.[23–26,38] Black pepper exhibits a variety of biological activities viz. antioxidant, gastroprotective, antidiarrhoeal, antimicrobial (antibacterial, antifungal, antiparasitic, etc.), antidiabetic, anti-inflammatory, anti-ulcer, hepatoprotective, antidepressant, hypocholesteremia, analgesic, immunomodulatory, anticancer, antihypertensive, antiasthmatic, and bioavailability enhancer.[23–26,38–43]

In the present study, piperine inhibited *C. albicans* growth (planktonic and biofilm) significantly; although MIC could not kill inoculums completely, apoptosis assay indicates that piperine is fungicidal (Table 1). Piperine as reported to exhibit broad spectrum antimicrobial activity, inhibited isolates of *C. albicans,* differentially susceptible to FLC, equally in our study.[44–47] However, mode of antimicrobial activity of piperine is not yet understood. It is being used in several traditional drugs as a bioenhancer. It was reported to interact with biological membranes, that is, both lipids and proteins as it is apolar in nature.[29] Piperine-lipid and piperine-protein interaction affects membrane integrity thereby enhancing fluidity and absorption.[29] In case of intestinal cell membranes, drug absorption was reported to be increased within 5–15 min after oral administration of piperine.[29] In brief, bioenhancer activity could be ascribed to the Piperine induced modification of membrane properties viz. fluidity, micro-viscosity, elasticity and permeability due to the nonpolar nature.[29] Bioenhancer activity was validated in recent studies also where it was reported to enhance antibiotic susceptibility in pathogens like *Escherichia coli* and methicillin-resistant *Staphyloccus aureus* (MRSA).[46,47] The potential of antibiotic activity in drug resistant *S. aureus* is ascribed to inhibition of bacterial efflux pumps by piperine.[46] Similarly, piperine enhanced fluconazole susceptibility in a FLC resistant strain of *C. albicans* (ATCC10231) in our study, indicating modulation of membrane permeability.

In addition to permeability, membrane integrity damage also modulates other membrane functions like transport and signal transduction and induces oxidative stress.[28,29] Our proteomic data suggest enhanced oxidative stress in response to piperine as four proteins involved in oxidative stress were upregulated. Real-time expression analysis of *KRE9* and *TRR1*, involved in cell wall biosynthesis and oxidative stress and MMP assay further confirms piperine induced oxidative stress (Fig. 8). Upregulation of four proteins involved in energy generation and four in PTM could be a compensatory response to maintain homeostasis under piperine induced oxidative stress.[48]

Our proteomic analysis shows that Piperine arrest cell cycle as Cdc37, essential for cell cycle progression is downregulated by 7.34-fold.[49–52] Cdc37, a chaperone involved in folding and activation of cyclin-dependent kinases (CDKs) that facilitate entry into START phase of the cell cycle.[49–52] Cell cycle analysis supports proteomic results as piperine arrest *C. albicans* cell cycle in G2-M phase, that is, failed to enter into start phase (G1). Piperine induced cell cycle arrest is further supported by upregulation of a pro-apoptosis (Snl1) and an anti-apoptotic (G210_0332) protein by 4.4- and 2.49-fold, respectively.[52,53] Snl1 (ribosome associated protein) plays a major role in translation and likely to be a BAG-1 protein in mammals involved in anti-apoptotic mechanism through *BCL2*.[54] Piperine induced apoptosis is further confirmed by evaluating real-time expression of apoptosis regulator *MCA1* at transcriptional level and apoptosis assay (Figs. 7A,B,C, and 8).[53] Piperine induced oxidative stress is reported to induce apoptosis in a rat model.[55]

In general, our study showed that piperine inhibits planktonic and biofilm (adhesion, development, and maturation) growth, as well as hyphae induction in *C. albicans*, significantly. Our LC-MS/MS data suggest that piperine induced membrane integrity damage affect membrane functions like permeability and signal transduction and generate ROS. ROS affect several processes and thus homeostasis (viz. enhance energy generation, biosynthesis of molecules required for oxidative stress tolerance) was upregulation in *C. albicans*. Modulations in signal transduction pathway affect cell cycle progression, activate pro-apoptotic pathways and induce apoptosis in response to piperine. Mitochondrial membrane potential (MMP) assay, Annexin V FITC based flow cytometry assay confirms piperine induced enhanced ROS and apoptosis in *C. albicans*, respectively. Real-time qPCR analysis further confirms both proteomic and flow cytometry results as *KRE9 and TRR1* involved in oxidative stress response

and *CaMCA1* involved inapoptosis were up regulated in response to piperine. Considering its potential, piperine may find use in anti-*Candida* chemotherapy as a sole molecule or in combination with other drugs. However, toxicity studies are needed, although MIC in our study was considerably less compared to LD_{50} value of rat model (33.5 mg/kg body weight) reported earlier.[56]

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Author contribution

A.T., G.B.Z., S.M.K. conceived idea, planned, and designed experiments. A.T. carried out microbial work (MIC, Biofilm, synergy assay). R.K., R.P., G.B.Z. performed LC-MS/MS analysis; R.K. performed bioinformatics analysis. A.T., G.B.Z., R.P., A.S. performed real-time qPCR analysis. G.B.Z., V.J., K.K. performed MMP, cell cycle, and apoptosis assay; G.B.Z. wrote the manuscript.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

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