In Vitro Effect of Curcuma Longa Rhizome Extract on Growth and Ergosterol Biosynthesis in Clinical Candida Isolates

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Abstract: The rhizome (root) of turmeric (Curcuma longa) has long been used in traditional Asian medicine to treat various human body disorders. Laboratory and animal research have demonstrated enormous biological properties of Curcuma longa. Here we have carried out the phytochemical analysis and anticandidal examination of the ethanolic rhizome extract of this plant. Phytochemical analysis of the test extract revealed the presence of alkaloids, tannins, amino acids, cardiac glycosides and diterpenes. In GC-MS analysis, the major compound found was aromatic (ar-) tumerone (32.73%). Antifungal activity of the test extract was investigated against 10 fluconazole-resistant and 5 fluconazole-sensitive Candida strains. Minimum inhibitory concentrations (MIC90) ranged from 1000 to 2200 µg/ml for both sensitive and resistant strains. Ergosterol content was drastically reduced by the test extract (MIC90), 65% in clinical-sensitive and 71% in clinical-resistant strains. The drop in sterol content correlated well with MIC90 values. It appears that the test extract acts in a dose-dependent fashion to decrease the ergosterol content in test extract treated samples. Cellular toxicity of this extract against H9c2 rat cardiac myoblasts was less than 12% at the highest MIC value. These findings encourage further development of ethanolic rhizome extract of *Curcuma longa*.

Keywords: Candida, Curcuma longa, Ergosterol, Ethanolic rhizome extract.

I. Introduction

Fungal infections have increased at an alarming rate, resulting in the cause of morbidity and mortality [1]. The advancement of antimicrobial resistance and populations of patients at some risk, in relation with the extremely limited number of commercially available antifungal drugs that still create many side effects, are the main cause for this problem [2, 3]. Of the two major classes of antifungals; polyenes guide to intense host toxicity [4, 5]. While as azoles are fungistatic, and their utilization leads to the expansion of drug resistance [6, 7]. The need of an hour is to develop effective strategies for the treatment of candidiasis and other fungal infections.

Curcuma longa is a tropical plant belonging to Zingiberaceae family. The rhizome part of this plant is considered to have beneficial medicinal properties because it contains a number of sesquiterpenoids, monoterpenoids, and curcuminoids [8, 9]. Dried *C. longa* rhizome is the source of the spice turmeric, and it is because of this ingredient that gives curry powder its characteristic yellow color [10]. *C. longa* rhizome has been traditionally used as antimicrobial agent as well as an insect repellent [9]. In Indian folk medicine, this herb has been used to fight a wide variety of ailments [11]. Extracts from *C. longa* have shown broad-spectrum antifungal, antibacterial, antiviral and antimalarial activities [12-17]. Recent research has all ears on turmeric's anti-inflammatory, antioxidant, anticarcinogenic, hepatoprotective, and antimicrobial properties.

However, to examine the effect of *C. longa* rhizome, little work has been done to examine its effect on growth and total ergosterol content of yeast cells. In our earlier studies we have confirmed the the antifungal role of methanolic rhizome extract of *C. Longa* by studying its effect on membrane integrity by confocal microscopy [18]. In this effort, we carried out GC-MS analysis for phytochemical characterization of ethanolic rhizome extract and then examined its antifungal effect against different *Candida* isolates. Primary screening for anticandidal activity was carried out by evaluating MIC, growth curve study and time-kill assays. To gain insight into the mechanism of action, we made an effort to assess the antifungal role of this test extract by studying its effect on ergosterol biosynthesis in different *Candida* isolates. The test extract displayed potent anticandidal activity and showed limited toxicity against H9c2 rat cardiac myoblast cells.

II. Materials and Methods

A. Collection and Extraction

The dried rhizome (4 kg) from *C. longa* was purchased from a medicinal herb shop, Old Delhi Market (Delhi, India). A voucher of specimen (R 18) was stored in laboratory for further reference. After collection the rhizomes were washed with distilled water and then kept in -20°C deep freezer until extraction. At the time of extraction the rhizomes were finely powdered, extracted twice with ethanol (3 L) in Soxhlet apparatus at room temperature (24-26°C) for 2 days. The extract was then filtered with whattman filter paper and filtrate was evaporated to dryness. The dried extract was then used for phytochemical and anticandidal investigation after clearance of biosafety and ethical committee of the Institute.

B. Phytochemical Examination

To find out the major constituents, the ethanolic rhizome extract was subjected to various phytochemical tests. The tests were performed for Alkaloids (Wagner's / Dragendroff's / Hagers test), amino acids (Ninhydrin test), phenols (Ferric chloride test), diterpenes (Copper acetate test), cardiac glycosides (Legals / Kellar-Killiani test) and for tannins (Ferric chloride test) [19-21].

C. Gas Chromatography, Mass Spectrometry Analysis

Mass Spectrometry analysis of the extract was carried out using a Shimadzu 2010 gas chromatograph fitted with an AB-Wax column. Helium was used as carrier gas. Sample (0.1 ml) was injected in the splitless mode. The chemical component from the extract was identified by comparing the retention time of the chromatographic peaks with those of authentic compound with the use of NIST05s.LIB and WILEY8.LIB [18].

D. Growth Conditions and Determination of MIC_{90}

Clinical isolates of fluconazole sensitive and resistant *Candida* species were obtained from Sexually Transmitted Disease Centre, Safdarjung Hospital, New Delhi, India. Species identification was done through examining microscopic morphology on Corn Meal Agar and Hi Chrome Agar. Germ tube test, nitrogen assimilation test and ascospore production on malt-extract agar was also done.The yeast strains were cultured in Yeast Extract, Peptone and Dextrose (YEPD) broth as required, maintained on YEPD agar plates at 4ºC and restreaked every 4-6 weeks. The culture was initiated with a loop full of cells maintained on YEPD slants into a 50 ml of appropriate medium (YEPD) and grown at 37ºC in a rotary shaker at 150-170 rpm to the stationary phase (24 hours) of growth and for experimental purpose 5×10^6 cells (Optical density A_{600}=0.1) were inoculated into the fresh media. Growth was followed for further 24-48 hours and measured turbidometrically using LaboMed Spectrophotometer at 600 nm. For long term storage cultures were stored at -20ºC with 1:1 glycerol as glycerol stocks. Minimum inhibitory concentration (MIC), defined as the lowest concentration of test entity that causes 90% decrease in absorbance (MIC_{80}) compared with control. This sensitivity testing procedure was carried out in microtitre plates as done earlier [22].

E. Growth Curve Studies

For growth studies, inoculation was done from *Candida* (10^6 cells/ml) (optical density A_{595} = 0.1) into yeast extract, peptone and dextrose (YEPD) broth in 250 ml flasks containing 150 ml medium and grown aerobically at 35ºC and 120 rpm, in orbital shaker (Scigenics, India). Required concentrations of the test extract were added in the medium. At pre-determined time periods (0, 2, 4, 8, 12 and 24 hours) growth was recorded turbidometrically at 595 nm using LaboMed Inc. Spectrophotometer (USA). Optical density was recorded for each concentration against time (hours) [23].

F. Time Kill Kinetics

Yeast isolates were sub cultured at least twice and grown for 24 h at 35ºC on YEPD plates, and initial inoculum was adjusted to 4.5×10^5c.f.u/ml as described earlier [24]. Final concentrations of test extractwere MIC, MIC/2 and MIC/4 for each test sample. 5 ml final volume cultures were incubated at 35ºC with agitation at 200 rpm. At pre-determined time points (0, 2, 4, 8, 12 and 24 h), 100 µl aliquots were removed and transferred to eppendorf tubes, centrifuged (3,900g at 4ºC for 1 min) and rinsed twice with 0.9 ml of sterile distilled water to obtain compound free cells. Pellets were suspended in 100 µl of sterile distilled water and were serially diluted as required. Diluted culture (50 µl) was spread onto YEPD plates and incubated at 35ºC for 48 h to determine the numbers of c.f.u/ml.

G. Ergosterol Extraction and Estimation Assay

Total intracellular sterols were extracted following the method prescribed by Brevik and Owades [25]. A single *Candida* colony from an overnight YEPD agar plate culture was used to inoculate 50 ml of YEPD broth for control and for various concentrations of the test extract. The cultures were incubated for 16 h and harvested by centrifugation at 2,700 rpm for 5 min. The wet weight of the cell pellet was determined. 25% alcoholic potassium hydroxide solution was added to each pellet and vortex mixed for 1 min. Sterol extraction was carried out by the 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. After this 20-µl aliquot of the sterol extract was diluted fivefold in 100% ethanol and scanned spectrophotometrically between 240 and 300 nm. The detailed procedure can be obtained from the method reported by Brevik and Owades (Breivik and Owades 1957). Ergosterol content is calculated as a percentage of the wet weight of the cell by the following equations:

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

% 24(28) DHE = [(A_{230}/518) x F]/pellet weight,

% ergosterol = [% ergosterol + % 24(28) DHE] - % 24(28) DHE,

Where F is the factor for dilution in ethanol and 290 and 518 are the E values (in percentages per centimetre) determined for crystalline ergosterol and 24(28) DHE, respectively.

H. MTT Cell Viability Assay

H9c2 rat cardiac myoblasts were cultured and maintained as monolayer in Dulbecco's modified Eagle's medium (DMEM), high glucose, supplemented with 10% fetal bovine serum (heat inactivated), 100 units/ml penicillin, 100 µg/ml streptomycin, and 2.5 µg/ml amphotericin B, at 37°C in humidified incubator with 5% CO_2 [26]. For treatments, test extract stock solutions were prepared in dimethyl sulfoxide (DMSO) and added to wells to give the indicated final concentrations. Final DMSO concentration was 0.2% in all wells including the untreated cells (control) and fluconazole controls. Cells were incubated for 48 hrs at 37ºC in 5% CO_2 humidified incubator together with untreated control sample. After incubation cells were washed in PBS and incubated with MTT solution for 45 minutes at 37ºC. After discarding the supernatant, MTT crystals were dissolved in acid iso-propanol and the absorbance was measured at 570 nm. Percent viability was defined as the relative absorbance of treated versus untreated control cells.

III. Results and Discussion

After preliminary phytochemical analysis of the ethanolic rhizome extract of *C. longa*, the results obtained showed the presence of alkaloids, diterpenes, cardiac glycosides, amino acids and tannins, whereas phenols were absent in the test extract (Table 1). Presence of 61 different compounds were confirmed by the GC-MS analysis of this extract, out of which 5 were found in majority (range 4.64 – 32.73%) and rest 56 compounds were found in almost minor quantities. Phytochemical identification is based on the molecular weight, molecular formulae, retention-time and % of presence. In table 2, the compounds showing less than 0.32 % presence were expelled and so we have shown only 34 compounds. The major compounds of the extract were Ar-tumerone (32.73%), Curlone (13.36%), Beta-cedrene (5.98%), Bergamotene (5.39%) and 1-Isobutyl-2,5-dimethyl benzene (4.64%), respectively (Table 2).

Table I: Phytochemical Analysis of Ethanolic Rhizome Extract of *C. Longa*. The Test is Based on the Colour Intensity

Phytochemical	*Name of Test*	*Colour observed*	*Colour intensity*
Alkaloids	Wagner's	Brown reddish ppt.	++
	Dragendroff's	Creamy ppt.	+
	Hager's	Yellow colored ppt.	+
Tanins	Ferric chloride	Green	++
Phenols	Ferric chloride	Bluish black	–
Diterpenes	Copper acetate	Emerald green	+
Amino acids	Ninhydrin	Violet	++
Cardiac Glycosides	Legal's	Pink to red	+
	Kellar-Killiani	Brown colour ring	+

– = (absent), + = (slightly present), ++ = (moderately present)

Table II: Chemical Compositions of Ethanolic Rhizome Extract of *C. Longa*

Name of Compound	*Molecular Formula*	*Molecular weight*	*Retention Time*	*% of Presence*
Durenol	$C_{10}H_{14}O$	150	11.41	1.13
Caryophyllene	$C_{15}H_{24}$	204	13.69	1.34
Curcumene	$C_{15}H_{22}$	202	15.27	2.17
Bergamotene	$C_{15}H_{24}$	204	15.61	5.39
Beta-Bisabolen	$C_{15}H_{24}$	204	15.88	1.39
.Beta.-Cedrene	$C_{15}H_{24}$	204	16.31	5.98
Cis-Nerolidol	$C_{15}H_{26}O$	222	17.23	1.22
Epiglobulol	$C_{15}H_{26}O$	222	17.88	1.56
1-Isobutyl-2,5-dimethylbenzene	$C_{12}H_{18}$	162	18.23	4.64

Cedr-8-ene	$C_{15}H_{24}$	204	18.83	1.11
Curlone	$C_{15}H_{22}O$	218	20.61	13.36
Ar-tumerone	$C_{15}H_{20}O$	216	19.97	32.73
Artemisia ketone	$C_{10}H_{16}O$	152	21.76	1.06
Tumerone	$C_{15}H_{22}O$	218	21.60	1.12
Cyclopentancarbonsaeure	$C_{19}H_{30}O_2$	290	21.40	2.13
Germacron	$C_{15}H_{22}O$	218	21.99	2.40
3-Phenylpropyl cyclohexanecarboxylate	$C_{16}H_{22}O_2$	246	24.30	1.19
Cyclohexane ketone	$C_{13}H_{22}O$	194	26.46	1.63
Veridiflorol	$C_{15}H_{26}O$	222	19.35	0.99
2-Norpinanone, 3,6,6-trimethyl	$C_{10}H_{16}O$	152	24.68	0.89
12-O-Acetyl-ingol-8-Tigliat	$C_{27}H_{38}O_8$	490	26.91	0.86
2-Propenoic acid, 1,7,7-trimethylbicyclo	$C_{13}H_{20}O_2$	208	22.55	0.85
Phenol, Dodecyl	$C_{18}H_{30}O$	262	25.53	0.73
Zingiberenol	$C_{15}H_{26}O$	222	18.41	0.72
Butanetetrol	C4H10O4	122	4.22	0.71
Dehydrozingerone	$C_{11}H_{12}O_3$	192	23.31	0.49
Chrysanthenyl Acetate	$C_{12}H_{16}O_2$	192	22.97	0.43
Cyclohexanecarboxylic acid, 1-adamantylmethyl ester	$C_{18}H_{28}O_2$	276	20.93	0.51
Alpha.-Benzylphenethylamine	$C_{15}H_{17}N$	211	18.97	1.34
ChrysanthenylAetcate	$C_{12}H_{16}O_2$	192	22.97	0.43
Cloven N	$C_{15}H_{24}$	204	16.43	0.40
Pentadecanoic Acid	$C_{15}H_{30}O_2$	242	25.96	0.61
Methyl 10,12-pentacosadiynoate	$C_{26}H_{44}O_2$	388	20.16	0.39
Dehydrozingerone	$C_{11}H_{12}O_3$	192	25.14	0.32

Available data shows that ethanolic rhizome extract of *C. longa* has antifungal properties against large number of dermatophytes [27]. Precise mechanism by which crude rhizome extract exerts its antifungal action is not clear. Till now there are no reports that show the effect of ethanolic rhizome extract of *C. longa* on ergosterol biosynthesis in different yeast species. Only the initial screening experiments have been performed till date. In this study we have measured one membrane parameter: ergosterol content of the treated samples. Ergosterol is unique to fungi, and the potential fungicidal effect of the test extract used in this study hints affinity for this specific target, and hence its biosynthesis pathway. In an attempt to discover plant derived principles with significant activities against human opportunistic fungi, we examined the antifungal effect of ethanolic rhizome extract of *C. longa* by performing some initial screening experiments and then studying the effect on the sterol biosynthesis. The test extract was found to be effective against all tested *Candida* strains. Initial screening for antifungal activity of test extract was carried out by evaluating MIC against 5 clinical sensitive and 10 clinical resistant isolates of *Candida* species. MIC results obtained in this study showed that the test extract exhibited varying degrees of antifungal activity. Generally, all the yeast isolates investigated were found sensitive to the test extract. The use of total mean MICs obtained gave a good evidence of the overall antimicrobial effectiveness. This may indicate that the yeast physiology may not be better equipped to counteract the antifungal properties of the extract. Ethanolic rhizome extract of *C. longa* showed the MIC ranging from 1000 µg/ml to 1700 µg/ml and 1400 µg/ml to 2200 µg/ml against clinical sensitive and clinical resistant isolates used in this study (Table 3). The antifungal activity of ethanolic rhizome extract of *C. longa* may be attributed to the presence of Ar-tumerone in higher quantity (32.73%).

Growth curve has been plotted by recording in time vs absorbance at 595 nm. Fig. 1 shows growth curves of *C. albicans* STD 31, *C. tropicalis* STD 189 and *C. krusei* STD 1342 in presence of MIC and sub-MIC concentrations of ethanolic rhizome extract of *C. longa*. In these growth curves, the top curve depicted the normal or control cells which showed the lag phase of 2-4 hours and after which the cells entered log phase which lasted till 10-12th hour. Log phase of control cell could be divided in to initial (4-6 hours), active (6-9 hours) and late log phase (9-12 hours). Second curve from top shows growth pattern at MIC/4 of test extract, third curve from top depicted growth pattern at MIC/2 and finally last curve depicted growth at MIC of the test extract. All the *Candida* isolates used in the present study were sensitive to the extract tested. Increase in concentration of test extract leads to significant decrease in growth with suppressed

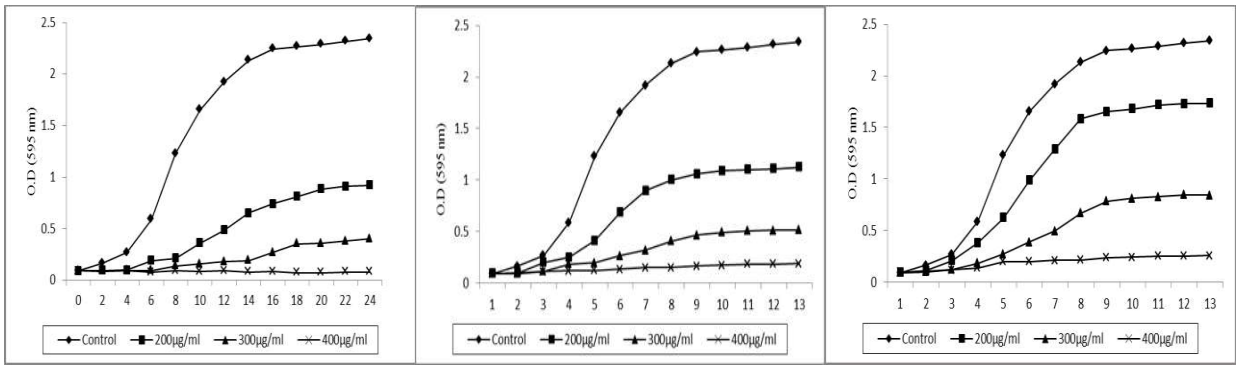
and delayed exponential phases with respect to control. At MIC values almost complete cessation of growth was observed for all the yeast species. Besides the growth inhibition, an increase in the lag phase was also observed with increasing concentrations of the test extract. It was observed that at MIC/4 and MIC/2 concentrations of the test extracts lag phase was extended by couple of hours respectively with respect to control. It was encouraging to see that the test extracts used in this study decreased cell growth and the decrease was concentration dependent. At higher concentrations of test extracts, the normal sigmoid growth curve could not be seen and almost a flat line was observed indicating negligible growth.

Table III: Minimum Inhibitory Concentration (MIC_{90}) Of Ethanolic Rhizome Extract of *C. Longa* Against Fluconazole Sensitive and Resistant Isolates used in the Study

Strains	*S. No*	*Species*	*MIC (µg/ml)*
Clinical Sensitive (n=5)	1.	*C. albicans* STD 32/33	1500
	2.	*C. albicans* STD 31	1000
	3.	*C. tropicals* STD 961	1700
	4.	*C. kruesi* STD 116	1600
	5.	*C. tropicals* STD 189	1300
Clinical Resistant (n=10)	1.	*C. glabrata* STD 1121	1500
	2.	*C. guilliermondii* STD 1689	1900
	3.	*C. glabrata* STD 64	1400
	4.	*C. kruesi* STD 1342	1500
	5.	*C. tropicals* STD 985	2200
	6.	*C. tropicals* STD 1004	1400
	7.	*C. albicans* STD 1141	1700
	8.	*C. albicans* STD 977	1600
	9.	*C. albicans* STD 41	1800
	10.	*C. glabrata* STD 2728	2000

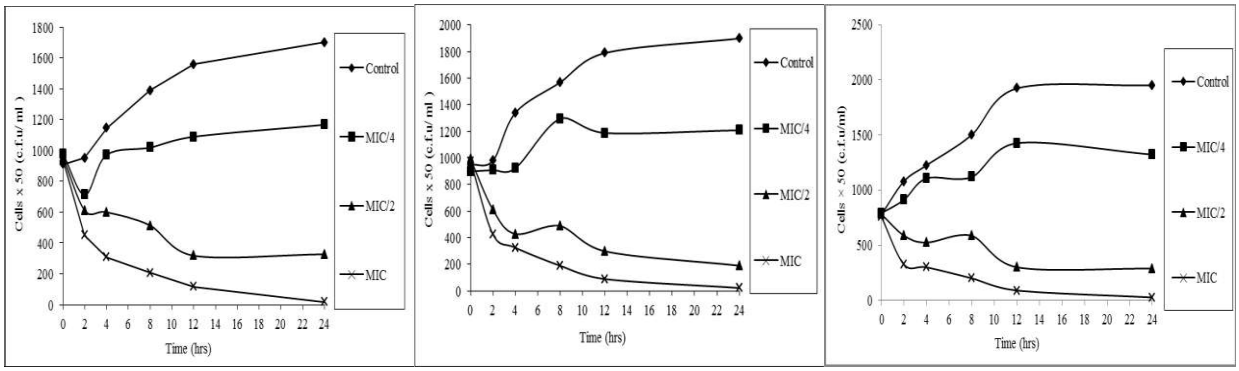
In time kill assay, Fig. 2 shows the killing activity of ethanolic rhizome extract of *C. longa* against *C. albicans* STD 31, *C. tropicalis* STD 189 and *C. krusei* STD 1342, respectively. At its respective MIC and MIC/2 values the test extract showed potential killing activity against all the tested *Candida* isolates. A significant decrease in the number of CFU per ml was observed in both sensitive and resistant *Candida* strains. Complete fungicidal endpoint was reached in an average time of 14-16 h following incubation with MIC of the test extract. No systematic difference was observed between fluconazole sensitive and resistant isolates. Results obtained demonstrated that the ability to kill *Candida* species is concentration as well as time dependent. Increase in test extract concentrations leads to a significant decrease in CFU/ml. This decrease in CFU/ml was obtained for all the *Candida* species used in this study (data not shown).

Ergosterol is important component of cell membrane of yeasts which differentiates it from animal and plant cells. In this assay as described in materials and methods, a characteristic four peaked curve is obtained due to presence of ergosterol and the late sterol intermediate 24 (28) Dihydroergosterol (DHE) in the extracted sample. Ergosterol biosynthesis is thus an important target pathway of existing antifungals like azoles, as also for development of new antifungals. Ergosterol biosynthesis is also affected by changes in physical and chemical environment of yeasts. Conditions that affect membrane also play a role in regulating ergosterol biosynthesis. Decreased biosynthesis of ergosterol leads to decreased viability of yeasts. Effect of promising antifungals can therefore be explained by estimating ergosterol in their presence. Fig. 3, shows the effect of ethanolic rhizome extract of *C. longa*on the UV spectrophotometric sterol profiles of three *Candida* strains (2 clinical sensitive and 1 clinical resistant): *C. albicans* STD 31, *C. tropicalis* STD 189 and *C. krusei* STD 1342 when grown for 16 hours. In graphs, control cell with normal ergosterol content show characteristic four peaked curve (top curve). As the concentration of test extract increases from MIC/4, MIC/2 to MIC suppression of four peaks is observed which is clearly shown in fig 3. We can clearly distinguish the inhibitory effect of various concentrations of test extract on the sterol profile of *Candida* strains. Average percent decrease in total ergosterol content for 5 clinical-sensitive and 10 clinical-resistant *Candida* isolates after exposure to MIC and MIC/4, MIC/2 concentrations of ethanolic rhizome extract of *C. longa*has been summarized in Table 4. The mean decrease in ergosterol content for clinical sensitive strains ranged from 19%, 48% and 65% for cells grown in MIC/4, MIC/2 and MIC of the test extract. Similarly, for clinical resistant strain decrease in total ergosterol content ranged from 24%, 57% and 71% for cells grown in MIC/4, MIC/2 and MIC of ethanolic rhizome extract of *C. longa*, respectively. From the results, it is clear that as the concentration of test extract increases, ergosterol content decreases and finally at MIC value, almost flat line indicates near absence of ergosterol in the sample.



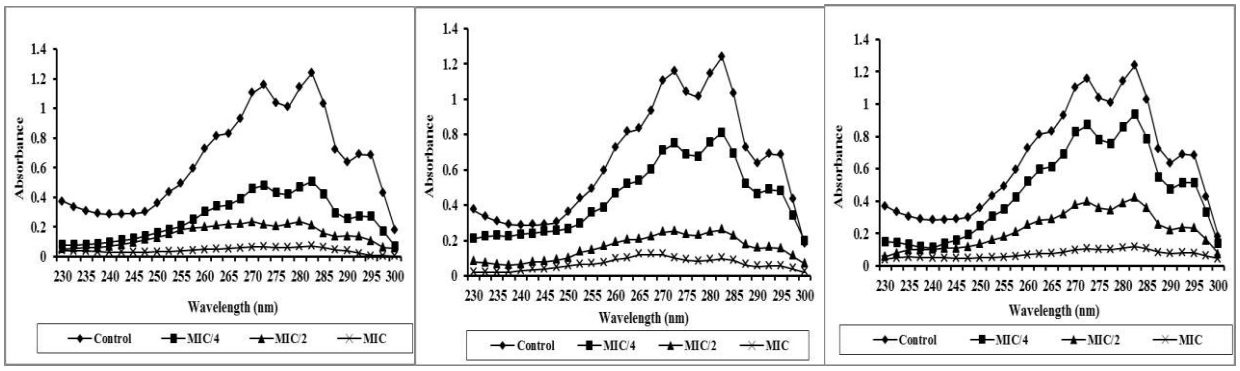
(a) (b) (c)

Fig. 1. Growth curves of *C. albicans* STD 31(a), *C. tropicalis* STD 189 (b), and *C. krusei* STD 1342 (c) in presence of ethanolic rhizome extract of *C. longa*



(a) (b) (c)

Fig. 2. Representative time-kill plots for (a) *C. albicans* STD 31 (b) *C. tropicalis* STD 189 (c) *C. krusei* STD 1342 following exposure to ethanolic rhizome extract of *C. longa*. At MIC_{90} values almost complete cessation of growth was observed



(a) (b) (c)

Fig. 3. UV spectrophotometric sterol profile of (a) *C. albicans* STD 31 (b) *C. tropicalis* STD 189 (c) *C. krusei* STD 1342grown for 16 hours in YEPD broth containing MIC/4, MIC/2 and MIC of ethanolic rhizome extract of *C. longa*. UV- Spectral profiles of extracted sterols were read between 230-300 nm

To evaluate the toxicity of the test extract, ethanolic rhizome extract of *C. longa* was tested against H9c2 rat cardiac myoblasts. Subconfluent populations of H9c2 cells were treated with increasing concentrations of test extract, and the number of viable cells was measured after 48 h by MTT cell viability assay. Fig. 4 shows the percentage toxicity after 48 h pre-treatment of H9c2 myoblasts with fluconazole (FCZ) and ethanolic rhizome extract of *C. longa*. It was observed that 50 µg/ml of test extract offered a remarkable viability of 97%, while as at the same concentration fluconazole offered only 74% viability. It was observed that 100, 250 and 500µg/ml of test extract showed only 5%, 8% and 11% toxicity, respectively. Toxicity of reference drug fluconazole at the same concentrations was 40%, 54% and 71%.

Table IV: % Ergosterol Decrease in Clinical-Sensitive and Clinical-Resistant *Candida* Isolates Grown in MIC/4, MIC/2 and MIC Of Ethanolic Rhizome Extract Of *C. Longa*

Ergosterol decrease in cells grown in presence of test extract			
Test extract	*Concentration*	*Clinical sensitive (n=5)*	*Clinical resistant (n=10)*
	Control	0	0
Ethanolic rhizome extract of *C. longa*	MIC/4	19	24
	MIC/2	48	57
	MIC	65	71

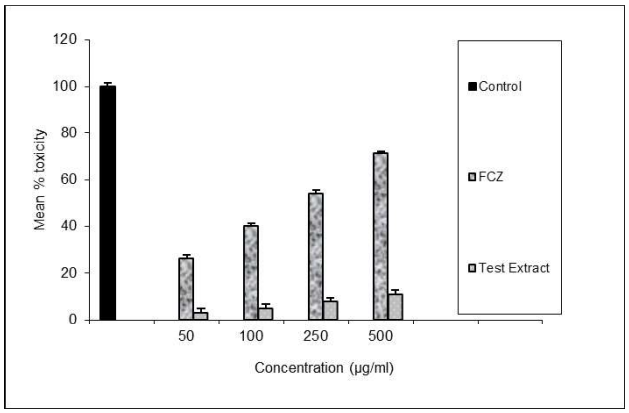


Fig. 4. Percentage toxicity after 48 h pre-treatment of H9c2 myoblasts with fluconazole (FCZ) and ethanolic rhizome extract of *C. longa* (test extract) evaluated by MTT assay

IV. Conclusion

Finally, plant derived ethanolic rhizome extract of *C. longa* is found to be very active anti-candidal agent. This antifungal effectiveness would be co-related with the presence of highly potent compound Ar-tumerone (32.73%). In our study decrease in ergosterol content co-related well with MIC_{90} suggesting that inhibition of ergosterol biosynthesis could be a leading / primary cause of antifungal action. With these studies, it was not possible to figure out whether this test extract causes direct inhibition of ergosterol biosynthesis or decrease in ergosterol is a consequence of disruption of membrane integrity. If mechanism of action of this test extract is taken as same, it would indicate that killing originates from loss of membrane integrity, and decrease in ergosterol content is only one consequence of this. These results taken together with the limited toxicity of ethanolic rhizome extract of *C. Longa* towards H9c2 rat cardiac myoblasts (3-11%) make it eligible for further development as antifungal.

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