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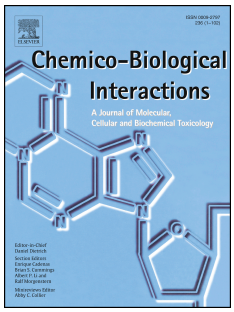

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Graphical Abstract

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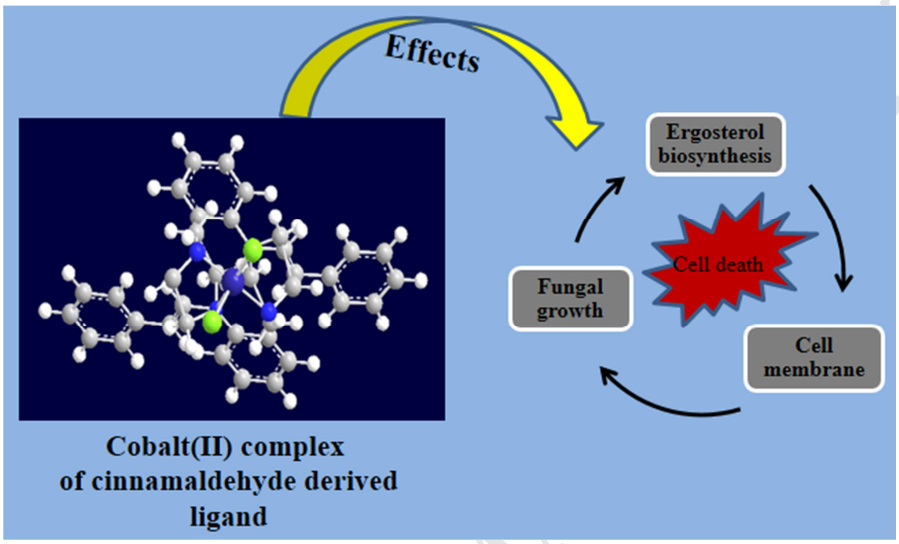

Impaired ergosterol biosynthesis mediated fungicidal activity of Co(II) complex with ligand derived from cinnamaldehyde

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A B S T R A C T

In this study, we have used aldehyde function of cinnamaldehyde to synthesize N, N'-Bis (cinnamaldehyde) ethylenediimine [$C_{20}H_{20}N_2$] and Co(II) complex of the type [$Co(C_{40}H_{40}N_4)Cl_2$]. The structures of the synthesized compounds were determined on the basis of physiochemical analysis and spectroscopic data (^{1}H NMR, FTIR, UV-visible and mass spectra) along with molar conductivity measurements. Anticandidal activity of cinnamaldehyde its ligand [L] and Co(II) complex was investigated by determining MIC_{80}, time-kill kinetics, disc diffusion assay and ergosterol extraction and estimation assay. Ligand [L] and Co(II) complex are found to be 4.55 and 21.0 folds more efficient than cinnamaldehyde in a liquid medium. MIC_{80} of Co(II) complex correlated well with ergosterol inhibition suggesting ergosterol biosynthesis to be the primary site of action. In comparison to fluconazole, the test compounds showed limited toxicity against H9c2 rat cardiac myoblasts. In confocal microscopy propidium iodide (PI) penetrates the yeast cells when treated with MIC of metal complex, indicating a disruption of cell membrane that results in imbibition of dye. TEM analysis of metal complex treated cells exhibited notable alterations or damage to the cell membrane and the cell wall. The structural disorganization within the cell cytoplasm was noted. It was concluded that fungicidal activity of Co(II) complex originated from loss of membrane integrity and a decrease in ergosterol content is only one consequence of this.

Key words: Cinnamaldehyde, *Candida*, Co(II) complex, Ergosterol biosynthesis, Anticandidal activity.

1. Introduction

Fungi are neglected pathogens as indicated by the fact that amphotericin B discovered in 1956 is still the gold standard to treat fungal infections. The incidence of infections caused by *Candida* species has increased dramatically worldwide due to increase in immunocompromised patients [1-5]. The majority of commonly used antifungals have serious drawbacks regarding toxicity to the host tissues, and their continuous deployment in treating infections has led to the emergence of drug-resistant fungal strains [6,7].

Cinnamaldehyde either in pure form or a crude extract of cinnamon has displayed exciting antifungal properties [8-10]. Na^+/K^+ ATPase and Cu^{2+}-ATPase as representatives of P- and CPx-type of ATPases have been established as possible intracellular targets for the action of cinnamaldehyde [11]. Importantly, cinnamaldehyde causes damage to cell walls, cell membranes, cytoplasmic contents and other membranous structures in a fungal cell [12,9]. Its antifungal action has been target specific and has demonstrated activities over a diversity of sensitive and resistant fungal species [8,11,13,14]. Cinnamaldehyde occurs naturally in the bark of cinnamon trees and in approved quantities it exhibits lower toxicities to host cells [8,15].

Macrocycles represent a class of cyclic macromolecules with diverse chemistry, a wide range of molecular topologies and sets of donor atoms [16]. Macrocyclic ligands and their coordination complexes are of significant interest as antitumor, antibacterial and antifungal agents [17,18]. The efficacies of such complexes can be easily optimized by complexing with various metals ions [19]. Organocobalt complexes of octanedioic acid and nonanedioic acid have shown high anticandidal activities [20]. Aside from the exciting biology of macrocycles other interesting classes of chemical compounds are Schiff base ligands and their complexes. Schiff bases and their complexes have shown significant antifungal activity towards *Candida albicans* and other human pathogenic fungi [21]. Cobalt (II)

complexes of ligands derived from reduced N, N'-o-Phenylenebis (Salicylideneimine) have been reported as effective against all tested *Candida* species [22].

Earlier we have reported the synthesis and anticandidal activity of a cinnamaldehyde derived ligand and its Ni(II) complex [23]. In the present study, Cobalt(II) has been employed to complex synthesized ligand with an aim to have increased antifungal activity. The initial antifungal screening was performed by determining minimum inhibitory concentration (MIC_{80}), disc diffusion assay and time-kill kinetics. Ergosterol biosynthesis is also an important target pathway of existing antifungals like azoles, as also for the development of novel antifungals. We have investigated the effect of cinnamaldehyde, its ligand, and metal complex [$CoLCl_2$] on total ergosterol content of yeast cells. To gain insight into the mechanism of antifungal action of Co(II) complex, we have carried out confocal scanning laser microscopy (CSLM) and transmission electron microscopy (TEM) analysis. Co(II) complex displayed markedly improved anticandidal activity with limited toxicity against H9c2 rat cardiac myoblast cells.

2. Materials and Methods

Cinnamaldehyde in oil form with 99% purity was obtained from Sigma-Aldrich, USA (Catalog No. C80687). The solvents were purchased from Merck, Ltd., India and used as received. The initial stock solution of cinnamaldehyde was 1g/ml, which was then dissolved in 1% dimethyl sulfoxide (DMSO) (control solvent) to dilute it further for obtaining lower concentrations as required to carry out different antifungal assays. Glass apparatus with standard joints were used throughout experimental work and stringent precautions were taken to exclude moisture. All the syntheses and handling were carried out under an atmosphere of dry oxygen-free di-nitrogen, using standard Schlenk techniques. To carry out microanalysis samples were vacuum dried to achieve a constant weight. Perkin Elmer 2400 CHNSO Elemental Analyser was used for elemental analysis. IR and far-IR spectra were

recorded as KBr pellets and CsI pellets (region 650-100 cm^{-1}), using Perkin Elmer 1620 FT IR and JASCO FT IR spectrophotometers, respectively. Bruker AVANCE DPX-600 spectrometer was used to record ^{1}H NMR spectra with TMS as internal standard and DMSO-d_6/$CDCl_3$ was used as a solvent. The splitting patterns were shown as: s, singlet; d, doublet; dd, doublet of doublets; t, triplet and m, multiplet. Values for chemical shifts were reported in ppm. Bruker (esquire3000_00037) instrument was used to measure positive and negative ESI mass spectra. Gouy method was used to carry out magnetic susceptibility measurements. UV-Vis Dual Beam 8 auto cell UVS–2700 LABOMED, INC, US spectrophotometer was used to record electronic spectra with DMSO as a solvent. Metrex melting point apparatus was used to record melting points.

2.1. Synthesis of ligand (L)

For the synthesis of ligand, the same procedure was followed as reported by Khalaji and Weil [24] and the yield obtained was 78%. The synthesis of ligand that involved the condensation reaction between cinnamaldehyde and ethylene diamine is shown in **Scheme 1.**

2.2. Synthesis of cobalt(II) complex

The synthesis of the ligand, and its Co(II) complex was carried out according to the steps shown in **Scheme 1**. A [2+1] condensation reaction of cinnamaldehyde and ethylenediamine formed the ligand (L) in excellent yield, which was further cyclised with cobalt metal chloride by mixing 2:1 ratio of the ligand and corresponding metal chloride. The obtained complexes are microcrystalline solids that are stable in air, soluble in DMF, DMSO, sparingly soluble in MeOH, EtOH and DCM but insoluble in water. The analytical data of the complexes corresponded well with the general formula $[M(L)X_2]$ where L = ligand; M = Co(II) and X = Cl^-. The molar conductance values in DMSO (10^{-3} M) are too

low to account for any dissociation of the complexes. Therefore, the complexes were considered to be non-electrolytes.

Yield: 65%; m.p.: > 300^{o}C; UV-Vis (DMSO) nm: 520, 570, 682, 739, 824; IR (KBr, cm^{-1}): 2977(C-H), 1420(C-N), 1043, 881, 748; Far IR (CsI, cm^{-1}): 445(Co-N), 330(Co-Cl); ^{1}H NMR (300 MHz, δ ppm from TMS in DMSO-d_6, 300 k): δ 7.40-7.58(20H, aromatic C-H), δ 3.41-3.87(8H, C=N-C-H_2), δ 6.92 (4H, C=C-CH), δ 7.79 (4H, C-N=CH); ESI-MS (m/z): 707 $[M]^{+}$, 709 $[M+2]^{+}$; Molar conductance, Λ_m (Ω^{-1}cm^{-1} mol^{-1}, 10^{-3} DMSO, r.t.): 34; μeff (r.t., BM): 4.82; Elem. anal. calcd.: C 68.02, H 5.66, N 7.93%; found: C 68.04, H 5.69, N 7.94%.

2.3. Growth conditions and determination of MIC_{80}

C. glabrata ATCC 90030, *C. tropicalis* ATCC 750 and *C. krusei* ATCC 6258 strains involved in the present study were obtained from Regional Research Laboratory, Jammu India. Clinical isolates of *C. albicans* STD number 1128 and *C. guilliermondii* STD number 1685 were obtained from Institute of Pathology, New Delhi, India, respectively. Species identification was done by 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 after 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 (150-170 rpm). For long term storage cultures were stored at -20ºC with 1:1 glycerol as glycerol stocks.

Minimum inhibitory concentration (MIC) was defined as the lowest concentration of test entity that causes 80% decrease in absorbance (MIC_{80}) compared with control. This sensitivity testing procedure was carried out as per NCCLS document M27-A2, 2002 [25],

in microtitre plates. Cultures were grown with/without test compounds in the media. Stock solutions of the test compounds and standard drug were prepared in 1% DMSO. The cells were grown in YEPD medium. The diluted cell suspensions were added to the wells of round-bottomed 96 well microtitre plates (100µl/well) containing equal volumes of media (100µl/well) and different concentrations of test compounds. A drug-free control was also included. The plates were incubated at 35ºC for 24 h. The MIC test end point was also evaluated both visually and by observing OD_{595} in a microplate reader (BIO-RAD, i Mark, US).

2.4. Disc diffusion assay

Strains were inoculated into liquid YEPD medium and grown at 37ºC. Overnight grown cells were then centrifuged and washed 2-3 times with distilled water. 10^5 cells/ml were added to molten agar media at 40ºC and then cells together with media was poured into 100-mm diameter petri plates. 4-mm-diameter sterile filter discs were impregnated with cinnamaldehyde, its ligand, and Co(II) complex, and then 2 mg/ml concentrations of the test compounds (dissolved in 1% DMSO) were spotted on the disc in 10 µl volume. The diameter of the zone of inhibition was recorded in millimeters after 48 h and was compared with that of control [26]. Sensitivity Index (SI) was calculated for all the five *Candida* isolates and was defined as:

Diameter of ZOI (mm)/ concentration of drug (mg/ml) = Clearing (mm/mg).

2.5. 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 4.5×10^5 c.f.u/ml as described earlier [8]. Final concentrations of cinnamaldehyde, its ligand, and Co(II) complex were 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 (20 µl) was spread onto YEPD plates and incubated at 35ºC for 48 h to determine the numbers of c.f.u/ml.

2.6. Ergosterol extraction and estimation assay

Total intracellular sterols were extracted following the method of Brevik and Owades [27]. A single *Candida* colony from an overnight YEPD agar plate culture was used to inoculate 50 ml of YEPD broth for control and various concentrations of cinnamaldehyde, its ligand, and Co(II) complex. 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 vortexed 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 aliquots 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 by Brevik and Owades [27]. Ergosterol content is calculated as a percentage of the wet weight of the cell by the following equations:

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

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

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

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

2.7. Confocal scanning laser microscopy (CSLM)

This microscopic experiment was used to evaluate the effect of Co(II) complex on the architecture of yeast cells as done earlier [28]. Mid-log phase *Candida* cells (10^6 cells/ml) were incubated without and with MIC of Co(II) complex. The samples were then incubated at 37 ºC for 30 min in the dark. After this, the suspensions were centrifuged, washed and resuspended in PBS. To determine general cell morphology, one µg/ml of propidium iodide (PI) was added to the cell suspensions. In case the test agent causes the damage to the cell, PI present at the surface finds its way inside the cell, showing increased red fluorescence [29]. The cells were examined with Olympus Laser Confocal Scanning Microscope equipped with green helium neon laser (543 nm) for PI.

2.8. Transmission electron microscopy (TEM)

Co(II) complex (MIC) treated cells (~1×10^6) were incubated for 12 h at 30 ºC and then fixed with 2% glutaraldehyde in 0.1 M phosphate buffer for 1 h at 20 ºC [30,31]. Cells were washed with 0.1 M phosphate buffer (pH 7.2) and post-fixed by 1% O_SO_4 in 0.1 M phosphate buffer for 1 h at 4 ºC. For ultrastructure study, the samples were dehydrated with graded acetone, cleared with toluene and infiltrated with toluene and araldite mixture at room temperature, and then finally in pure araldite at 50 ºC and embedded in eppendoff tubes (1.5 ml) with pure araldite mixture at 60 ºC. Sectioning was done with Ultramicrotome (Lecia EM UC6) and then observed under TEM. Other details can be obtained from the method by Mares and Borgers *et al*., [30,32].

2.9. 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% foetal 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 [33]. For treatments, test

compound 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 h at 37 ºC in 5% CO_2 humidified incubator together with the untreated control sample. After incubation, cells were washed with PBS and incubated with MTT solution for 45 min at 37 ºC. 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.

2.10. Statistical analysis

The descriptive statistics have been presented as Mean ± SD. The mean values were compared using t test between control and treated samples (MIC & sub-MIC values), and one-way analysis of variance (ANOVA) was used to test the significance among the different test agents. The two tailed probability value ($p< 0.001$) was considered statistically significant. The data management and statistical analysis was conducted using Statistical Package of Social Sciences, IBM SPSS, Version 22.0 (IBM Corp. in Armonk, NY, USA).

3. Results

The physical properties and analytical data of the Co(II) complex supported its proposed structure. A facile [2+1] condensation reaction of cinnamaldehyde and ethylenediamine produced ligand in excellent yield (78%). The complex was a microcrystalline solid and stable in air, soluble in DMF, DMSO, sparingly soluble in MeOH, EtOH and DCM; but insoluble in water The analytical data of complex corresponded well with the composition as shown in **Scheme 1**. The molar conductivity of Co(II) complex showed low value, which indicated that the complex was a non-electrolyte [34]. The FTIR, ^{1}H NMR, UV-Visible and ESI-MS results further confirmed the structure of ligand and its complex. The geometry of the complex was well ascertained by the

presence of characteristic UV-Visible absorption bands in the region of 520-824 nm and a magnetic moment of 4.82 BM.

3.1. IR spectra of ligand and complex

IR spectrum of ligand did not exhibit any band corresponding to the free primary diamine and carbonyl group [35,36]. A broad absorption band in the region of 3300-2800 cm^{-1} characteristic for hydrogen atom of (H-C=O), and the absence of absorption band in the region of 1675-1775 cm^{-1} characteristic for carbonyl group (HC=O) in cinnamaldehyde, indicated that the oxygen atom of cinnamaldehyde was detached from the HC=O group to form a bond between aldehydic carbon atom and amino nitrogen of ethylenediamine. It also suggested complete condensation of reactants and elimination of water molecule. This was confirmed by the appearance of a strong signal at 1460 cm^{-1} that might be attributed to the C=N bond [37].

This coordination behaviour shows how a ligand with multiple donor sites can coordinate to obtain a simple but stable geometry. The amide groups that are more close to the metal ion centre maintaining less metal-ligand bond distance, participate in the complexation. On complexation, the shifting in the band of υ(C=N) (1420) towards the lower wave number indicated that the coordination took place through the nitrogen of the υ(C=N) group. This further implied that the ligand is bidentate. The appearance of new vibrating signals at 445 and 330 cm^{-1} in the spectrum of metal complex gave clear proof for the presence of metal-nitrogen (M-N) and metal-chlorine (M-Cl) bonds in Co(II) complex, respectively [35,38].

3.2. ^{1}HNMR spectra

A multiplet in the range 7.36-7.54 ppm corresponded to the phenyl ring protons (10H). Another multiplet in the range 3.37-3.81 ppm has been ascribed to methylene protons (C=N-CH_2, 4H). The two sharp signals; one at the pseudo-aromatic region 6.85 ppm (2H,

C=C-CH) and another at 7.71 ppm (2H, C-N=C-H) have been ascribed to methine protons. The signal of C-N=C-H has been shifted downfield due to the deshielding effect of the nitrogen group attached to it. These aromatic and aliphatic proton signals undergo downfield shifting in Co(II) complex of the ligand due to the paramagnetic effect of Co(II) ion and hence support the coordination of ligand towards Co(II) ion [39,40].

3.3. Electro spray ionization mass spectra (ESI MS)

The ESI-MS of the ligand and complex were studied in DMSO solution. A negative ion ESI mass spectrum of ligand confirmed the proposed formula by showing a peak at m/z 289 corresponding to the moiety [($C_{20}H_{20}N_2$) of atomic mass m/z 288.21. The series of peaks at m/z 102.9, 192.9, 244.7, etc may be assigned to various fragments. All these suggested the 2+1 condensation of cinnamaldehyde and ethylenediamine, respectively. Similarly negative ion ESI-MS of Co(II) complex showed a peak at m/z 707 corresponding to the moiety [($CoC_{40}H_{40}N_4Cl_2$) of atomic mass m/z 706.25] (**Fig. 1**), which is consistent with the molecular ion fragment, and it supports the proposed structure of the complex. $[M+2]^+$ fragments were also observed in the metal complex. This may be due to the low quantities of isotopic chlorine. Molecular ion peak was also associated with water molecules, solvent and some adduct ions from the mobile phase solution in some cases [41-43].

3.4. Electronic spectra and magnetic measurements of Co (II) complex

The electronic spectrum of Co(II) complex recorded in DMSO supported the geometry around the respective metal atom. The presence of electronic transition bands at 520 and 565 nm; assigned to the transition $^4T_{1g} \rightarrow {}^4T_{1g}(P)$, 682 nm; assigned to the transition $^4T_{1g} \rightarrow {}^2T_{1g}$, and 739 and 824 nm; assigned to the transition $^4T_{1g} \rightarrow {}^4T_{2g}$ (**Fig. 2**) suggested an octahedral geometry around the Co(II) ion in the complex [44].

The Co(II) complex has an effective magnetic moment value (μ_{eff}) of 4.82 BM. This value is indicative of an octahedral geometry around the Co(II) ion. The magnetic properties of

octahedral Co(II) complexes are governed by the orbitally degenerate ground term $^4T_{1g}$. This provides an orbital contribution to the magnetic moment so that the room temperature moments are experimentally found to be in the range 4.7-5.2 B.M [41,45,46].

3.5. Minimum inhibitory concentration (MIC_{80}) and disc diffusion assay

Table 1 summarizes the minimum inhibitory concentration (MIC_{80}) values in microgram/ml (µg/ml) and micromolar (µM) of cinnamaldehyde, ligand and Co(II) complex against five different *Candida* species. On the concentration basis, average MIC of cinnamaldehyde was 3022 µM. The average MIC of ligand and Co(II) complex is 664 and 144 µM, respectively. Compared to cinnamaldehyde, ligand [L] is 4.55 times more efficient whereas Co(II) complex is 21.0 times more efficient. Anticandidal MIC of fluconazole varies between 6.5 µm (2 µg/ml) and 104.5 µM (32 µg/ml). Thus the synthesized complex is less effective than fluconazole by a factor ranging from 4.5 to 29.

Disc diffusion is one of the standard methods of measuring the antifungal activity of molecules against different *Candida* strains grown in agar culture plate. The test compounds used in this study showed varying zones of clearance at a particular concentration. Discs impregnated with the vehicle, 1% DMSO (negative control) showed no zone of inhibition. Our results showed that growth was inhibited by cinnamaldehyde its ligand and Co(II) complex. The halo was completely clear, which indicated potential fungicidal activity. Yeasts were found to be more sensitive to Co(II) complex as compared to ligand and cinnamaldehyde. Diameter of the inhibition zone (mm) by cinnamaldehyde, against *C. glabrata, C. tropicalis, C. krusei, C. albicans* and *C. guilliermondii* at 2 mg/ml concentration was 5 (± 0.3), 4 (± 0.5), 3 (± 0.2), 5 (± 0.5) and 3 (± 0.3), respectively. The average of five species being 4.0 mm. For ligand, these values increased to 9 (± 0.4), 7 (± 0.4), 6 (± 0.2), 11 (± 0.3) and 6 (± 0.2), respectively; with an average of 7.8 mm. For Co(II) complex the values were 12 (± 0.4), 11 (± 0.5), 9 (± 0.5), 14 (± 0.5) and 11 (± 0.4),),

respectively; with average being 11.4 mm. Sensitivity index (S.I) was calculated as the ratio of total clearing by test compounds (in mm) and the total amount of test compound (in mg). Results were calculated for cinnamaldehyde, its ligand and Co(II) complex for five sensitive isolates used in this study. Sensitivity index for all five strains was found to be the highest in the case of Co(II) complex with 5.7 mm/mg followed by ligand [L] with 3.9 mm/mg and then cinnamaldehyde with 2.0 mm/mg. On the concentration basis, ligand and Co(II) complex were more effective compared to cinnamaldehyde, but the results obtained implied that diffusion in solid medium restricts effectiveness of Co(II) complex to an extent.

3.6. Time kill kinetics

Fig. 3 shows the killing activity of Co(II) complex against *C. glabrata* ATCC 90030, *C. tropicalis* ATCC 750, *C. krusei* ATCC 6258, *C. albicans* STD 1128 and *C. guilliermondii* STD 1685, respectively. At its respective MIC and MIC/2 values the Co(II) complex showed potential killing activity against all the tested *Candida* isolates. A significant decrease in the number of cfu per ml was observed in both standard and clinical (susceptible) *Candida* strains. The complete fungicidal endpoint was reached in an average time of 12-14 h following incubation with MIC of the test agent. No systematic difference was observed between standard and clinical isolates. Results obtained demonstrated that the ability to kill *Candida* species is concentration as well as time dependent. The increase in compound concentrations leads to a significant decrease in cfu/ml. This decrease in cfu/ml was also obtained for cinnamaldehyde and ligand used in this study (data not shown). After 24 h incubation and at half MIC of the test compounds the average reduction in c.f.u/ml with respect to control for all the five *Candida* isolates was 43% in the presence of cinnamaldehyde, 59% in the presence of ligand, and 84% in the presence of Co(II) complex respectively. The aim was to examine the killing activity in presence of Co(II) complex therefore we have also given the decrease in cfu/ml after 24 h incubation when different

Candida isolates were grown in MIC and sub-MIC concentrations of Co(II) complex. The p values were calculated between control and treated samples (MIC & sub-MIC values) for each of the five strains (Table 2).

3.7. Ergosterol extraction and estimation assay

Ergosterol is an important component of cell membranes of yeasts, which differentiates them from animal and plant cells. 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. As the concentration of test molecules increases, suppression of four peaks is observed. Thus, we can clearly distinguish the inhibitory effect of various concentrations of test molecules on the sterol profile of *Candida* strains. **Table 3** summarizes the effect of cinnamaldehyde, its ligand, and Co(II) complex on ergosterol biosynthesis in five *Candida* isolates. The total ergosterol content was determined for each isolate grown in varying concentrations of test compounds. A dose-dependent decrease in ergosterol production was observed when isolates were grown in the presence of cinnamaldehyde, its ligand, and Co(II) complex. As shown in **Table 3**, the average decrease in total cellular ergosterol content for different *Candida* isolates after exposure to their respective MIC_{80} of cinnamaldehyde was 62%. The average decrease in total cellular ergosterol after exposure to MIC_{80} of ligand and Co(II) complex was 76 and 88%, respectively. Respective decrease at MIC_{80} of fluconazole was 92.73%. From the results, it is clear that the increase in the concentration of test molecules decreases ergosterol content. Finally, at MIC value, near absence of ergosterol occurs in the sample. MIC_{80} values of Co(II) complex co-relates very well with percent inhibition of sterol biosynthesis. It would thus appear that major mode of action of the complex is through inhibition of ergosterol biosynthesis.

3.8. Confocal scanning laser microscopy (CSLM)

It is a technique used for obtaining high-resolution optical images with depth selectivity. Studies were performed to validate the discrimination of live and dead *Candida albicans* STD 1128 cells due to MIC of Co(II) complex in the presence of PI. PI is used as a DNA stain for both flow cytometry and microscopy to evaluate cell viability, and to visualize the nucleus and other DNA containing organelles. It is membrane impermeant and generally excluded from viable cells. Therefore, it is used for identifying dead cells in a population. It can be used to differentiate necrotic, apoptotic and normal cells. Cells with severe membrane lesions leading to the inherent loss of viability will internalize PI, resulting in an increase in red fluorescence [29]. The laser confocal images of stained *C. albicans* STD 1128 cells exposed to MIC value of Co(II) complex are shown in **Fig 4**. The left panel shows the laser confocal images of stained *Candida* cells without any treatment, and both the images in the right panel show stained cells exposed to MIC of Co(II) complex, respectively. The right panel shows disruption in cell membranes of cells treated with Co(II) complex, which causes the imbibition of dye. More the damage to cell population more is the number of cells which turn red. Our results confirmed that PI penetrates in Co(II) complex treated cells implying that the structure of cell membrane was disrupted by the test agent. It was worth noticing that in the case of treated cells entire yeast cell appeared red (maximum entry of the dye inside the cell), which confirmed both cell wall and plasma membrane damage. These results formed firm basis to carry out further microscopic analysis of the treated cells by employing transmission electron microscopy.

3.9. Transmission electron microscopy (TEM)

Transmission electron microscopy was used to study the ultrastructural changes caused by Co(II) complex in *C. albicans* STD 1128 cells after 12 h exposure. **Fig 5. A**, at a direct magnification of 25000 X shows (untreated) cells of *C. albicans* STD 1128. Control cells showed the typical normal morphology of *Candida* with a uniform central density and

endomembrane system enveloped by a regular, intact cell wall and membrane. **Fig. 5 B** shows the Co(II) complex (MIC) treated cells at 20000 X magnification. The treated cells exhibited notable alterations or damage in the cell membrane and cell wall. The structural disorganization within the cell cytoplasm and leakage of intracellular content has also been noted (**Fig. 5 B**).

3.10. Toxicity profile

To evaluate the toxicity of synthesized compounds they were tested against H9c2 rat cardiac myoblasts. Sub-confluent populations of H9c2 cells were treated with increasing concentrations of these compounds, and the number of viable cells was measured after 48 h by MTT cell viability assay. Table 4 shows that cinnamaldehyde and its ligand showed almost 100% viability at the concentration range of 6.25 µg/ml, while as at the same concentration, Co(II) complex and reference drug fluconazole showed 98% and 90% viability, respectively. It was observed that 100 µg/ml of cinnamaldehyde, its ligand, and Co(II) complex offered a remarkable viability of 94, 90 and 84%, respectively. At 200 µg/ml concentration, cinnamaldehyde, ligand, and Co(II) complex showed 8, 11 and 20% toxicity, respectively. Toxicity of reference drug fluconazole at 200 µg/ml was 55%.

4. Discussion

Schiff base ligands and their transition metal complexes with macrocyclic architectures are being preferred for the development of drugs and drug candidates with exciting biological properties [47]. The ligand reported in the present study was prepared by a facile condensation involving the two primary amine groups of one ethylenediamine molecule and two aldehydic functional groups of two cinnamaldehyde molecules. The potentially bidentate ligand coordinated with Co(II) ions and formed a 1:2 metal to ligand complex with octahedral geometry. The structure of the complex was fully ascertained from the microanalysis, UV-Vis, FTIR, ^{1}H NMR, and ESI-MS spectral date. The low molar

conductance value of the complex in DMSO indicated its non-electrolytic nature, and thus it might be inferred that the chlorido ligands do not dissociate in the solution phase. The two chlorido ligands, therefore, satisfy both primary and secondary valencies of the Co(II) ion in this complex [48]. The absence of bands due to free primary amine and carbonyl groups in the FTIR spectrum of ligand, and the presence of bands due to C=N bond confirmed the condensation of cinnamaldehyde and ethylenediamine to form ligand. The coordination of ligand with Co(II) ion was ascertained by the shifting of υ(C=N) band towards the lower wave number. Besides, the appearance of vibrating frequencies of Co-N and Co-Cl further confirmed the complex formation. ^{1}H NMR spectral data was highly useful for the structure determination. The characteristic signals for phenyl ring, methylene, pseudo-aromatic and methine protons established the formation of ligand. The metal coordination of the ligand was ensured due to the downfield shifting of the signals of aromatic and aliphatic protons in the ^{1}H NMR spectrum of Co(II) complex. ESI-MS data further revealed the composition of the ligand and complex. The ESI-MS spectrum of the Co(II) complex (**Fig. 1**) confirmed the molecular composition of the complex as, [($CoC_{40}H_{40}N_4Cl_2$) with molecular mass m/z 706.25. Finally, the geometry of the complex was ascertained from the results of electronic spectral and the magnetic moment data of the Co(II) complex. The presence of electronic transitions such as $^{4}T_{1g} \rightarrow {}^{4}T_{1g}(P)$, $^{4}T_{1g} \rightarrow {}^{2}T_{1g}$ and $^{4}T_{1g} \rightarrow {}^{4}T_{2g}$ (**Fig. 2**) in the electronic spectrum of complex suggested an octahedral geometry around the cobalt(II) ion in the complex, which was further confirmed from the magnetic moment value of the complex (4.82 BM).

Drug resistance has become an important problem in a variety of infectious diseases, including human immunodeficiency virus (HIV) infection, tuberculosis, and the majority of fungal infections [49]. The rise in the incidence of fungal infections has exacerbated the need for the next generation of antifungal agents since many of the

currently available antifungal drugs like polyenes and azoles have undesirable side effects, are ineffective against new or re-emerging fungi, or lead to the rapid development of resistance [49-52]. Alternative forms of treatment may also address the growing problems of drug resistance in microbes. In this study, cobalt(II) has been employed to complex cinnamaldehyde based ligand with an aim to have increased antifungal activity. On the basis of spectroscopic analysis, it appears that ligand is formed through condensation of two molecules of cinnamaldehyde and one molecule of ethylene diamine. Cobalt(II) coordinated with four nitrogen atoms of two ligands as shown in **Scheme 1**.

Cinnamic aldehyde has antifungal properties against fungi involved in respiratory tract mycoses [53]. The precise mechanism by which cinnamaldehyde exerts its antimicrobial action is not clear. Being lipophilic, it permeates the membrane and hence, becomes accessible to various intracellular sites [54]. Antifungal effect of cinnamaldehyde may also originate from inhibition of fungal cell wall synthesizing enzymes, beta-(1,3)-glucan synthase and chitin synthase [55]. Structural component of cinnamaldehyde, which may contribute to its antimicrobial effects are aromatic ring, hydrocarbon chain outside the ring and aldehyde function. Most of the irritant effects of cinnamaldehyde especially at higher concentrations are attributed to aldehyde function [56]. Skin sensitization of cinnamaldehyde is probably initiated by the reaction of cinnamaldehyde with ε-amino groups on protein side chains. We used aldehyde function of cinnamaldehyde to form a ligand employing ethylenediamine (**Scheme 1**). The ligand formation led to increase in lipophilicity and extension of the hydrocarbon chain outside the ring. The ligand with structure twice that of cinnamaldehyde was further complexed employing cobalt as a central metal ion with no aldehyde function, and thus complex structure four times that of cinnamaldehyde was obtained. Chelation reduces the polarity of the metal ion because of partial sharing of its positive charge with the donor group within the whole chelate ring

system [57]. This process of chelation thus, increases the lipophilic nature of central metal ion, which in turn, favours its permeation through the lipid layer of membrane thus, causing the metal complex to cross the membrane more effectively.

Initial screening for antifungal activity of cinnamaldehyde, its ligand, and Co(II) complex was carried out by evaluating MIC_{80} against five *Candida* species of the fluconazole-sensitive category. MIC_{80} results obtained in this study showed that the test compounds exhibited varying degrees of antifungal activity. The use of total mean MICs obtained gave a good indication of the overall antimicrobial effectiveness of each test compound. MIC values as determined by NCCLS method decreased almost stoichiometrically with the multiplication of structure for all five species of *Candida*. Average MIC of five species for cinnamaldehyde is 3022 µM. Anticandidal MIC decreased tremendously for ligand as 664 µM, and complex as 144 µM, representing an increase in effectivity by 4.55 times and 21 times compared to cinnamaldehyde. Anticandidal MIC of fluconazole varies between 6.5 µm (2 µg/ml) and 1045 µM (32 µg/ml), thus the synthesized complex could be less effective as compared to fluconazole by a factor ranging from 4.5 to 29. Results obtained in time-kill assays demonstrated that the increase in the concentration of test compounds leads to significant killing activity. Full fungicidal endpoint was reached in an average time of 12-14 h following incubation with MIC of cinnamaldehyde, its ligand and Co(II) complex (**Fig. 3**). No systematic difference was observed between standard and clinical isolates. The average reduction in c.f.u/ml with respect to control was maximum in the presence of Co(II) complex (84%) followed by ligand (59%) and cinnamaldehyde (43%). Effectiveness of test molecules was evaluated on solid media to check whether the different state of media alters their antifungal approach. This was evaluated by disc diffusion assay in which filter paper disc impregnated with various concentrations of test molecules were put on agar plates. In the case of disc

diffusion, sensitivity index was calculated on the basis of zone of inhibition. Like MIC studies in liquid media, no systematic or significant difference was seen between various strains for all the three tested molecules. Accordingly, sensitivity index was calculated for all five tested isolates. Cinnamaldehyde showed the mean sensitivity index of 2.0 mm/mg; ligand showed 3.9 mm/mg and Co(II) complex showed 5.7 mm/mg of clearance. Therefore, Co(II) complex showed 2.85 folds and ligand showed 1.95 folds sensitivity index as compared to cinnamaldehyde. In comparison to the liquid medium, the anticandidal effect of synthesized compounds on solid medium showed that diffusion could be a constraint for the complex.

Ergosterol is important constituent of yeast cell membrane that distinguishes them from host cell membranes. Ergosterol biosynthesis is thus an important target pathway of existing antifungals like azoles, as also for the development of new antifungals. 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 content in their presence. Present study, therefore, explored the possible target sites for the antifungal actions of cinnamaldehyde, its ligand, and Co(II) complex. Earlier studies have shown that a major site of action of cinnamaldehyde is cell membrane and ergosterol biosynthesis [9]. One membrane parameter *viz.* ergosterol content was measured in this study. Inhibition of ergosterol biosynthesis correlates well with MIC_{80} values. Ergosterol reduction of 62, 76 and 88% was estimated in the presence of MIC_{80} of cinnamaldehyde, ligand, and Co(II) complex. This co-relation suggests that ergosterol is the primary site of action of cinnamaldehyde, its ligand, and Co(II) complex. Although cinnamaldehyde acts by disrupting the microbial cell membranes, however, specific mechanisms involved in its mode of antifungal action remain poorly characterized. Ergosterol is unique to fungi, and

the potential fungicidal effect of the compounds used in this study hints affinity for this specific target, and hence its biosynthesis pathway. In this study, an attempt has been made to compare the antifungal potential of cinnamaldehyde, its ligand, and Co(II) complex by performing some initial screening experiments and then studying the effect on the morphological aspects of membrane integrity and sterol biosynthesis. In case of Co (II) complex, ergosterol content co-related well with MIC_{80} 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 Co(II) complex causes direct inhibition of ergosterol biosynthesis or decrease in ergosterol is a consequence of disruption of membrane integrity. Co-relation of MIC_{80} with ergosterol decrease was found in case of Co(II) complex but was far off in case of cinnamaldehyde. If the mechanism of action of cinnamaldehyde, its ligand, and Co(II) complex 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. Other factors appear to have an equal contribution in cinnamaldehyde promoted killing.

To demonstrate the effect of Co(II) complex on yeast cells, microscopy studies were carried out against cells exposed to MIC concentration of Co(II) complex. It was observed that treated cells showed less viability as indicated by increased absorption of propidium iodide visualized by confocal scanning electron microscopy. The effect on membrane integrity, thus, appears to originate from inhibition of ergosterol biosynthesis as visible from confocal microscopy results **(Fig. 4**). TEM studies with Co(II) complex showed disruption of outer morphology and internal cellular structure to a greater extent. Cell wall breakage and leakage of intracellular content could be seen from TEM micrographs (**Fig. 5**). Hence, it can be concluded that fungicidal activity of Co(II) originates

from loss of membrane integrity, and a decrease in ergosterol content is only one consequence of this.

5. Conclusion

In summary, Schiff base ligand was synthesized from cinnamaldehyde in a good yield *via* facile condensation reaction. The synthesized ligand coordinated with Co(II) ions in a bidentate fashion. The basic approach behind the synthesis of ligand and its complex was to increase their size and so the lipophilicity. Ligand and Co(II) complex attained structures two times and four times, respectively the size of cinnamaldehyde. Both the compounds were markedly active against different *Candida* strains in liquid as well as solid media. Interestingly, the compounds also showed insignificant toxicity at MIC_{80}. The mechanistic studies revealed ergosterol biosynthesis as the primary site of action of Co(II) complex. Overall, the excellence of the anticandidal properties in addition to the insignificant toxicity of Co(II) complex; demands more insightful studies into all its possible mechanisms of action.

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Conflict of Interest

The authors declare no conflict of interest.

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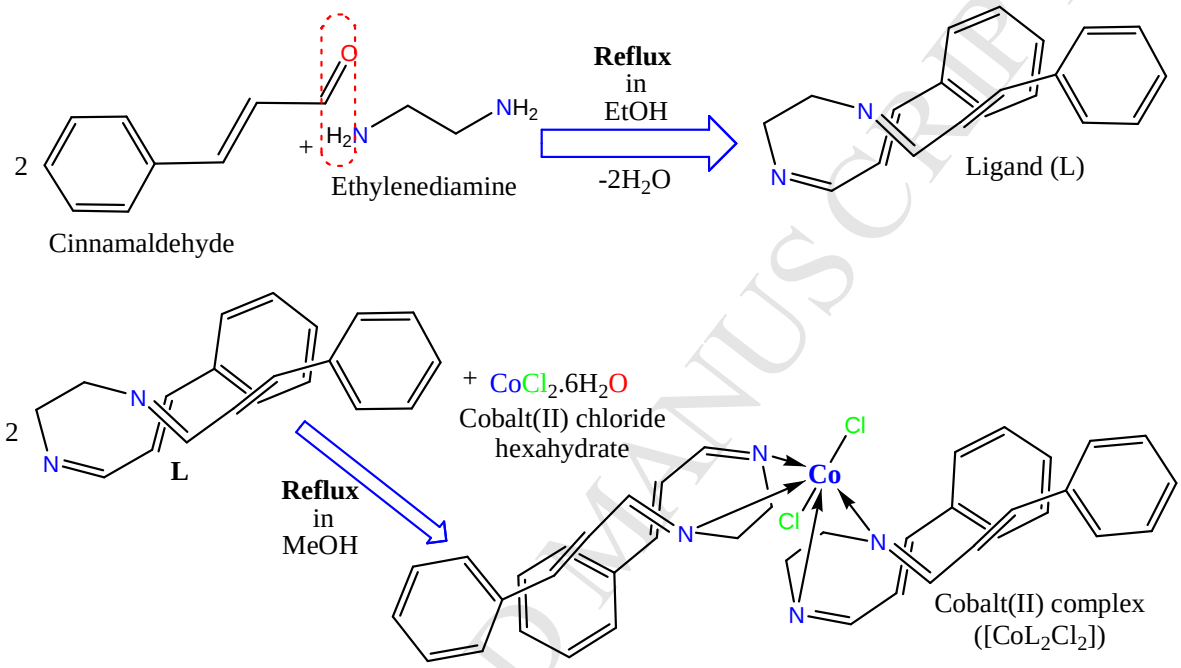
Schemes



Scheme. 1: Schematic representation of the synthesis of cinnamaldehyde derived ligand (L) and its Co(II) complex.

Figures

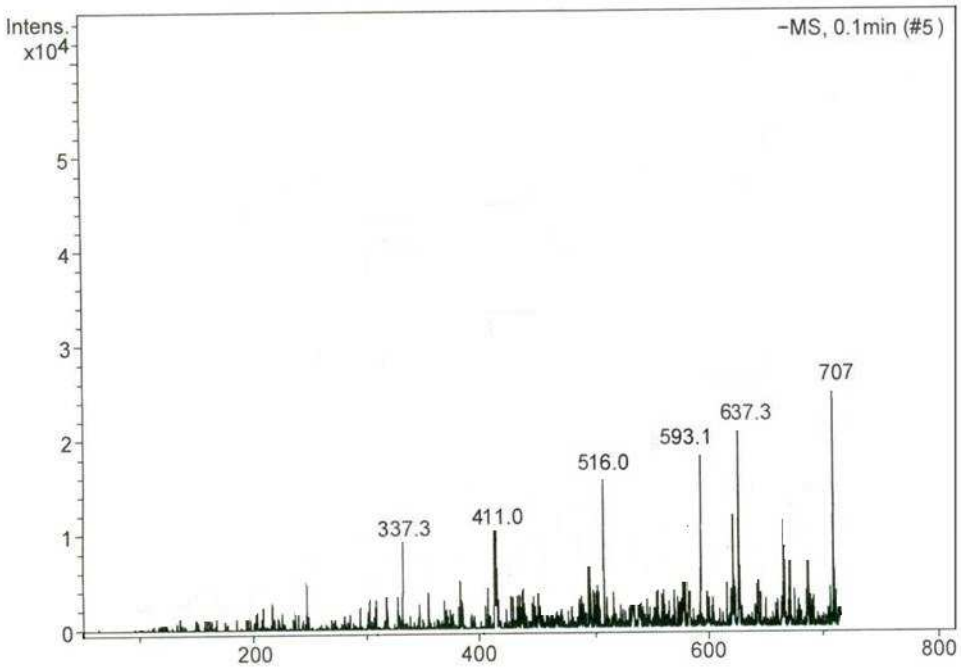


Fig 1. Electro spray ionization mass spectrum Co(II) complex.

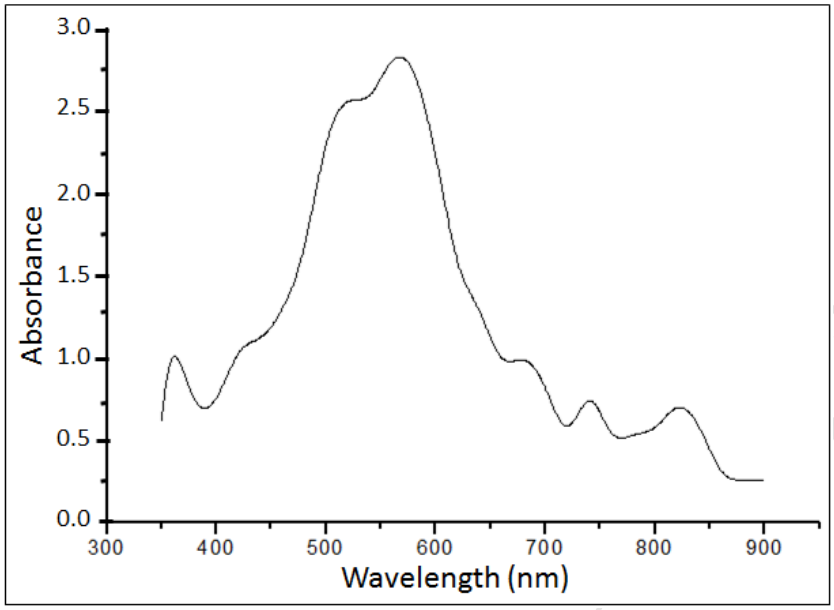


Fig 2. UV Visible spectrum of Co(II) complex

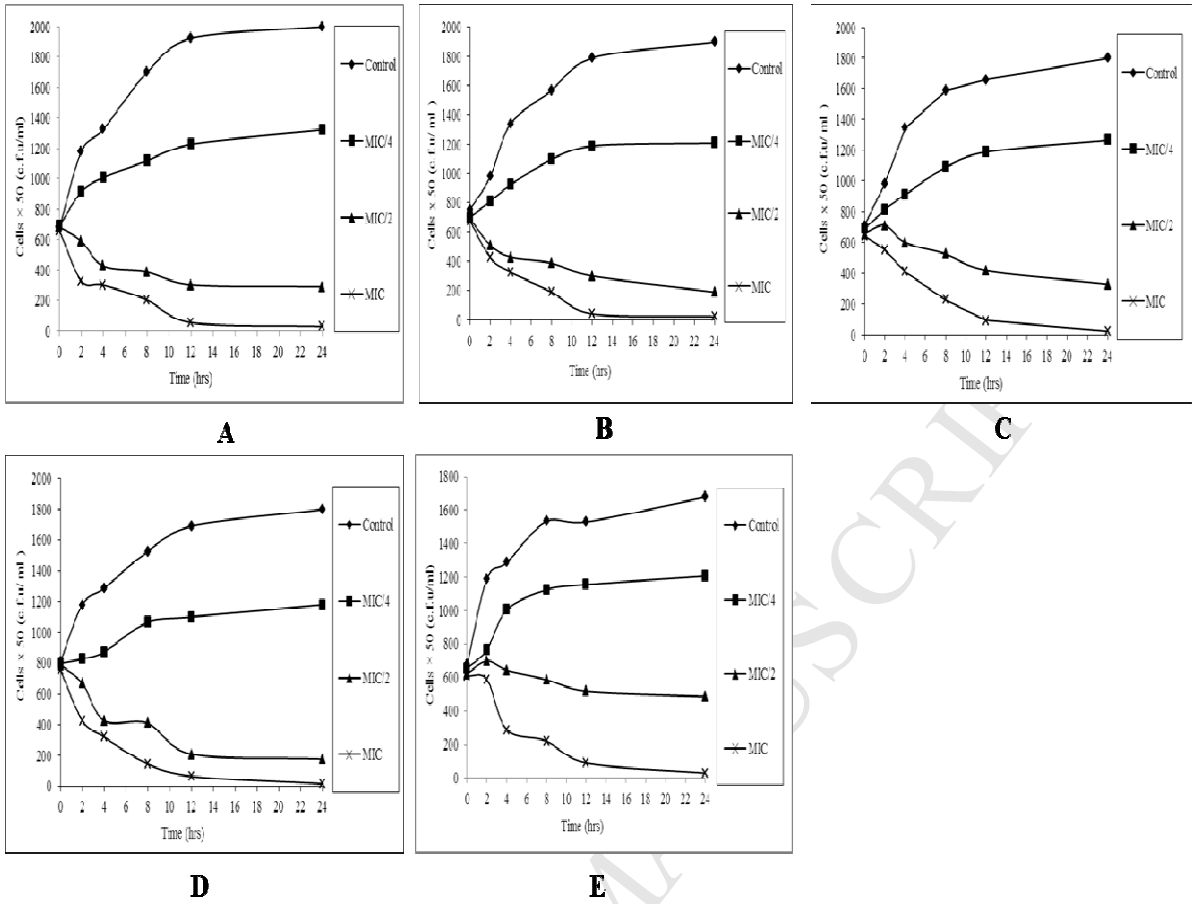
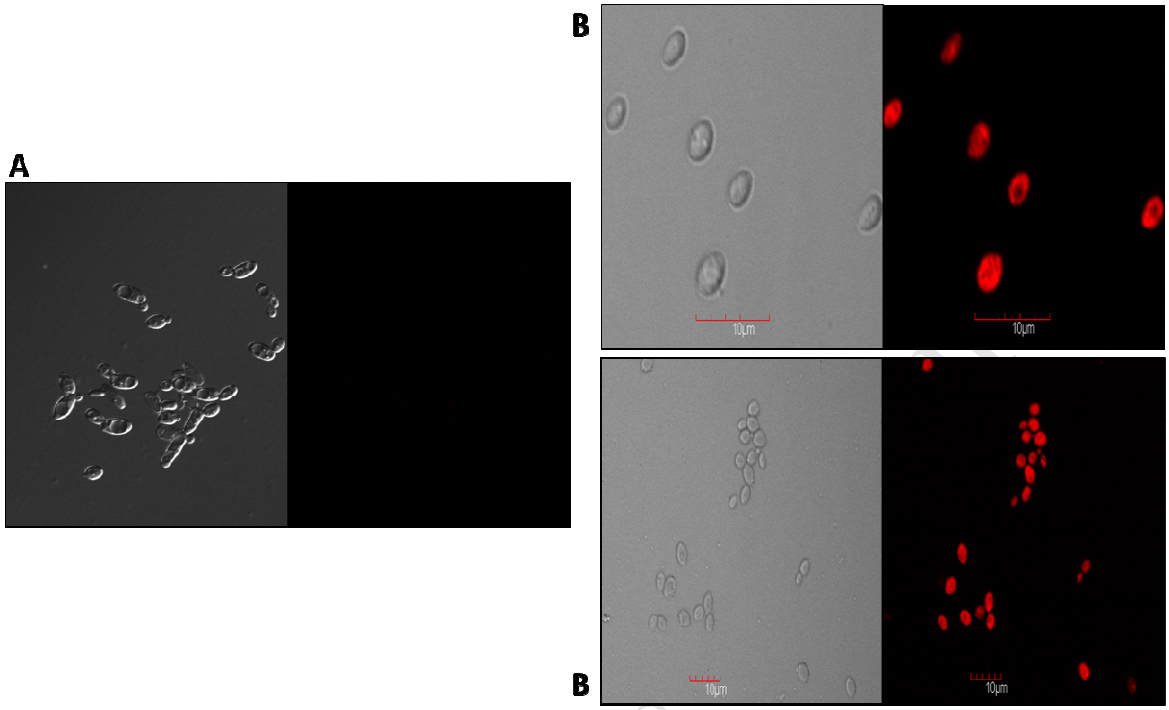
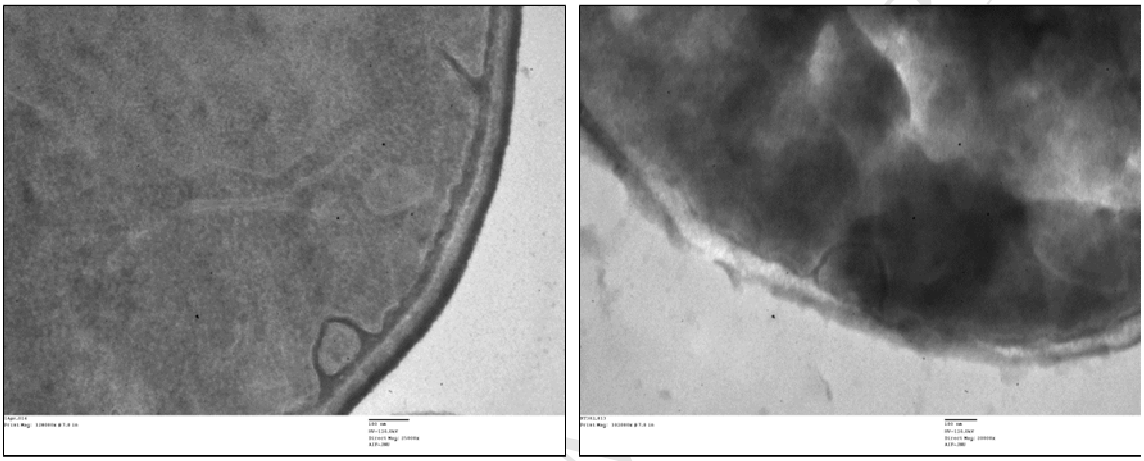


Fig. 3. Time kill curve for a fluconazole-sensitive strain of (A) *C. glabrata* ATCC 90030 (B) *C. tropicalis* ATCC 750 (C) *C. krusei* ATCC 6258 (D) *C. albicans* STD 1128 and (E) *C. guilliermondii* STD 1685 treated with MIC and sub-MIC values of Co (II) complex.



(A) Control cells **(B) Cells + PI + Co(II) complex (MIC)**

Fig. 4. Typical CSLM image of Propidium Iodide stained *C. albicans* STD 1128 cells to show differential staining patterns of live and dead yeasts. (A) Control cells. (B) Cells treated with MIC_{80} value of Co (II) complex. PI uptake was observed in cells treated with Co(II) complex (MIC_{80}).



(A) Control cells **(B) Cells + Co(II) complex (MIC)**

Fig. 5. Typical TEM micrographs of *C. albicans* STD 1128 (A) Control (B) Cells treated with Co(II) complex at MIC of 90 µg/ml. Treated cell indicating damage of the cell wall and cell membrane. The structural disorganization within the cell cytoplasm has been noted.

Tables

Table 1. Minimum Inhibitory concentration (MIC_{80}) values, in both (µg/ml) and (µM) of cinnamaldehyde, ligand [L] and Co (II) complex against different *Candida* species.

	Mean MIC_{80}					
	Cinnamaldehyde		ligand [L]		Co(II) complex	
Fungi	µg/ml	µM	µg/ml	µM	µg/ml	µM
C. *glabrata* ATCC 90030	300	2325	140	484.4	90	126.9
C. tropicalis ATCC 750	500	3875	200	692	100	141
C. krusei ATCC 6258	500	3875	250	865	120	169.2
C. albicans STD number 1128	250	1937.5	170	588.2	90	126.9
C. guilliermondii STD number 1685	400	3100	200	692	110	155.1

Anticandidal MIC of fluconazole varies between 6.5 µm (2 µg/ml) and 104.5 µM (32 µg/ml) for the five *Candida* species used in this study. MIC values were examined by performing serial microdilution in broth. In the first well, we fixed 3-4 times the highest concentration to be tested, whichever is greater. Different concentration of same test agent were kept in the first well in case of different strains to calculate the exact values. After this serial dilution was made and the wells were then inoculated with a standardized number of cells. The MIC end points were read visually after 48 h; repeated experiments showed growth inhibition in the same wells.

Table 2. Decrease in cfu/ml after 24 h incubation when different *Candida* isolates were grown in MIC and sub MIC concentrations of Co(II) complex .

	Cells × 50 (cfu/ml)				
	C. glabrata ATCC 90030	*C. tropicalis* ATCC 750	*C. krusei* ATCC 6258	*C. albicans* STD 1128	*C. guilliermondii* STD 1685
Control	2000 ± 164.31	1902 ± 166.11	1808 ± 95.03	1800 ± 61.69	1679 ± 96.00
MIC/4	1321 ± 55.53	1208 ± 168.01	1263 ± 145.41	1179 ± 177.83	1209 ± 94.55
p value	.002	.007	.006	.005	.004
MIC/2	288 ± 55.13	190 ± 36.49	327 ± 56.75	177 ± 37.75	487 ± 97.50
p value	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
MIC	29 ± 6.42	21 ± 4.50	19 ± 3.05	15 ± 2.64	28 ± 8.38
p value	.002	.003	.001	< 0.001	< 0.001

The data represents (Mean ± S.D) of three experiments. The p values were calculated between control and treated samples (MIC & sub-MIC values) for each of the five strains.

Table 3. Percent ergosterol content in three standard-sensitive and two clinical-sensitive *Candida* isolates grown in different concentrations of cinnamaldehyde, its ligand [L] and Co (II) complex.

Cinnamaldehyde									
C. glabrata ATCC 90030		*C. tropicalis* ATCC 750		*C. krusei* ATCC 6258		*C. albicans* STD 1128		*C. guilliermondii* STD 1685	
µg/ml	%Ergosterol content	µg/ml	%Ergosterol content	µg/ml	%Ergosterol content	µg/ml	%Ergosterol content	µg/ml	%Ergosterol content
100	88.29 ± 2.02	100	94.26 ± 1.63	100	93.15 ± 2.35	100	89.10 ± 1.75	100	90.11 ± 1.73
200	51.22 ± 1.62	250	65.25 ± 0.66	250	60.24 ± 0.79	200	58.49 ± 4.51	200	51.23 ± 2.02
300	43.46 ± 2.38	500	41.17 ± 1.93	500	36.42 ± 2.47	250	32.25 ± 2.13	400	33.47 ± 1.42
Ligand [L]									
50	70.21 ± 2.63	50	73.25 ± 3.00	50	78.11 ± 2.62	50	65.25 ± 1.90	50	69.30 ± 1.97
100	41.10 ± 1.53	100	49.50 ± 1.45	150	47.23 ± 1.68	100	36.77 ± 1.67	100	31.10 ± 2.04
140	21.25 ± 3.87	200	26.11 ± 0.76	250	28.25 ± 1.46	170	20.16 ± 1.43	200	23.29 ± 2.56
Co (II) complex									
30	60.08 ± 1.78	30	65.93 ± 1.51	40	63.70 ± 1.64	30	58.19 ± 1.71	30	59.02 ± 2.41
60	21.88 ± 1.36	60	30.19 ± 2.62	80	29.46 ± 3.00	60	25.32 ± 1.76	60	28.05 ± 1.61
90	12.42 ± 1.06	100	13.03 ± 1.50	120	14.00 ± 1.15	90	10.11 ± 1.32	110	9.23 ± 1.16
*	$p < 0.001$		$p < 0.001$		$p < 0.001$		$p < 0.001$		$p < 0.001$

L- [$C_{20}H_{20}N_2$]. The data represents (Mean ± S.D) of three experiments. *represents p values. ANOVA – was used to compare among three test agents (MIC values) for each of five strains and a significant difference was found ($p < 0.001$).

% Decrease in ergosterol content at respective MIC_{80} values of fluconazole: *C. glabrata* ATCC 90030; 96.23 ± 0.719, *C. tropicalis* ATCC 750; 93.12 ± 1.423, *C. kruesi* ATCC 6258; 95.08 ± 1.170, *C. albicans* STD 1128; 89.13 ± 0.876, *C. guilliermondii* STD 1685; 90.12 ± 1.167.

Table 4. Percent viability of H9c2 rat cardiac myoblast cells when treated with different concentrations of cinnamaldehyde, ligand [L], Co (II) complex and reference drug fluconazole after 48 h pre-treatment.

Test agent (µg.ml)	Cinnamaldehyde	Ligand [L]	Co (II) complex	Fluconazole	p- value
6.25	99.67 ± 0.27	99.68 ± 0.18	98.30 ± 0.78	90.49 ± 1.70	< 0.001
12.5	99.60 ± 0.42	99.58 ± 0.33	96.45 ± 1.75	86.18 ± 2.10	< 0.001
25	98.93 ± 1.50	98.17 ± 1.08	90.21 ± 2.39	79.56 ± 2.47	< 0.001
50	96.22 ± 1.01	87.85 ± 1.40	85.82 ± 1.58	69.35 ± 1.94	< 0.001
100	94.47 ± 2.60	90.54 ± 1.11	84.90 ± 1.31	63.42 ± 2.30	< 0.001
200	92.08 ± 1.43	89.30 ± 1.69	80.05 ± 1.60	45.31 ± 2.05	< 0.001

L- [$C_{20}H_{20}N_2$]. The data represents (Mean ± S.D) of three experiments. ANOVA – was used to compare among four test agents for each of the concentrations used and a significant difference was found ($p < 0.001$).

Research Highlights:

- In liquid medium ligand [L] and Co(II) complex were 4.55 and 21.0 fold more effective than cinnamaldehyde.
- MIC_{80} of Co(II) complex correlated well with ergosterol inhibition.
- Microscopic analysis of treated cells indicated disruptions of cell wall and membrane.
- Fungicidal activity of Co(II) complex originates from loss of membrane integrity and decrease in ergosterol content.
- In comparison to fluconazole Co(II) complex exhibited limited toxicity.