

# Antifungal activity of $\alpha$ -methyl trans cinnamaldehyde, its ligand and metal complexes: promising growth and ergosterol inhibitors

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**Abstract** Antifungal effectivity and utility of cinnamaldehyde is limited because of its high MIC and skin sensitivity. In this study,  $\alpha$ -methyl trans cinnamaldehyde, a less irritating derivative, have been self coupled and complexed with Co(II) and Ni(II) to generate *N, N'*-Bis ( $\alpha$ -methyl trans cinnamaldehyde) ethylenediimine [ $C_{22}H_{24}N_2$ ], [ $Co(C_{44}H_{48}N_4)Cl_2$ ] and [ $Ni(C_{44}H_{48}N_4)Cl_2$ ]. Ligand and complexes were characterized on the basis of FTIR, ESI-MS, IR and  $^1H$ NMR techniques. Synthesized ligand [L] and complexes were investigated for their MICs, inhibition of ergosterol biosynthesis and  $H^+$  extrusion against three strains of *Candida*: *C. albicans* 44829, *C. tropicalis* 750 and *C. krusei* 6258. Average of three species MIC of methyl cinnamaldehyde is 317  $\mu$ g/ml (2168  $\mu$ M). Compared to methyl cinnamaldehyde ligand [L], Co(II) and Ni(II) complex are found to be 4.48, 17.78 and 21.46 times more effective in liquid

medium and 2.73, 8.93 and 10.38 times more effective in solid medium. At their respective MIC<sub>90</sub> average inhibition of ergosterol biosynthesis caused by methyl cinnamaldehyde, ligand [L], Co(II) and Ni(II) complex, respectively was 80, 78, 90 and 93%.  $H^+$  extrusion was also significantly inhibited but did not co-relate well with MIC<sub>90</sub>. Results indicate ergosterol biosynthesis as site of action of  $\alpha$ -methyl cinnamaldehyde, synthesized ligand and complexes.  $\alpha$ -methyl cinnamaldehyde and ligand did not show any toxicity against H9c2 rat cardiac myoblast cell, whereas Co(II) and Ni(II) complexes on an average produced 19% cellular toxicity.

**Keywords** *Candida* ·  $\alpha$ -methyl trans cinnamaldehyde · Metal complexes · PM-ATPase · Ergosterol

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## Introduction

Primary and opportunistic fungal infections continue to increase rapidly because of prolonged use of antibiotics, cancer chemotherapy regimes and HIV infections. *Candida albicans* is causative agent of most candidiasis; but other emerging *Candida* species, including *C. glabrata* and *C. krusei*, are now posing serious threat (Nguyen et al. 1996; Pfaller et al. 1998). Existing classes of antifungals: azoles and polyenes

have severe hepatotoxicity, nephrotoxicity and are being increasingly resisted by infecting fungi (Odds et al. 2003; Gupta and Thomas 2003). Natural remedies may offer safe alternatives for less severe infections.

Cinnamaldehyde is effective in inhibiting growth of bacteria, yeast and filamentous molds and is reported to interfere with F-type mitochondrial ATPase and lowering of intracellular ATP (Gill and Holley 2004). Antimicrobial activity of cinnamaldehyde is attributed to its membrane action (Bang et al. 2000). It has been tentatively established that aromatic ring and hydrocarbon chain affect antifungal properties (Sheikh et al. 2010). Antifungal MICs of cinnamaldehyde is high, its use is further limited by its irritant effect on skin which is attributed to aldehyde function (Wang et al. 2005).  $\alpha$ -substituted cinnamaldehyde are known to be less skin sensitizer because they react slowly with amine (Vimala and Raymonds 1997).  $\alpha$ -methyl cinnamaldehyde is one such derivative, which is also expected to be more effective because of its increased hydrophobicity.

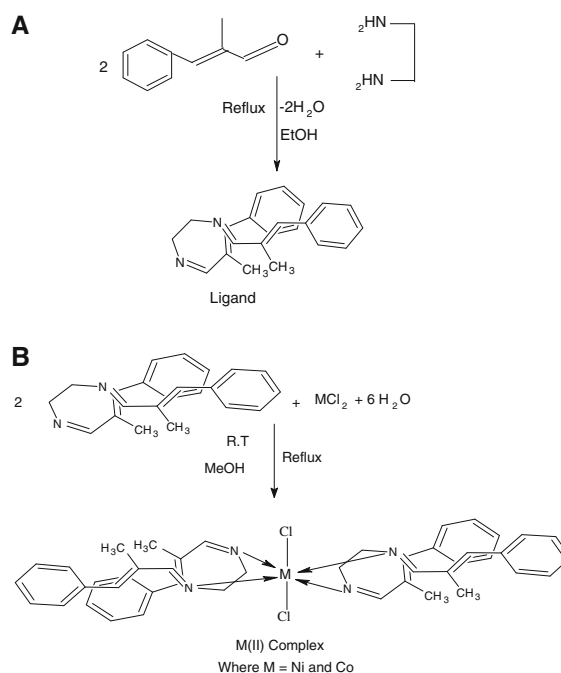
In this study we report synthesis of self coupled ligand *N, N'*-Bis ( $\alpha$ -methyl trans cinnamaldehyde) ethylenediimine [ $C_{22}H_{24}N_2$ ] and its Ni(II) and Co(II) complexes of the type [ $Co(C_{44}H_{48}N_4)Cl_2$ ] and [ $Ni(C_{44}H_{48}N_4)Cl_2$ ]. Synthesized compound have been characterized employing standard chemical techniques. Antifungal efficacy of synthesized compounds have been tested against three standard *Candida* species: *C. albicans* ATCC 44829, *C. tropicalis* ATCC 750 and *C. krusei* ATCC 6258 by determining MICs, sensitivity and investigating membrane associated properties of PM-ATPase function and ergosterol biosynthesis.  $\alpha$ -methyl cinnamaldehyde and its ligand and complexes display markedly improved anticandidal activity, compared to cinnamaldehyde and its complexes (Sheikh et al. 2010) and showed very limited toxicity against H9c2 rat cardiac myoblast cells.

## Materials and methods

### Materials

The ligand was prepared by condensing the  $\alpha$ -methyl trans cinnamaldehyde and the diamine (ethylene diamine) in presence of the few drops of hydrochloric

acid (Scheme 1a). The ligand [L] was then refluxed at room temperature with nickel chloride to form its metal complex [ $NiLCl_2$ ] and cobalt chloride to form its metal complex [ $CoLCl_2$ ] (Scheme 1b). Elemental analyses were performed by a Perkin Elmer 2400 CHNSO Elemental Analyser. IR spectra were recorded as KBr pellets using a Perkin Elmer 1620 FT IR spectrophotometer. Far IR spectra were recorded as CsI pellets in the region  $650$ – $100\text{ cm}^{-1}$  using a JASCO FT IR spectrophotometer.  $^1\text{H}$  NMR spectra were recorded using a Bruker DPX-300 MHz spectrophotometer operating at room temperature with  $\text{DMSO-}d_6$  as solvent. The chemical shift ( $\delta$ ) is reported in parts per million (ppm) using tetramethylsilane as internal standard. Positive and negative ESI mass spectra were measured by Bruker (esquire 3000–00037) instrument. Magnetic susceptibility measurements were carried out from a microanalysis laboratory by Gouy method at room temperature. Electronic spectra were recorded on a spectro-UV–Vis Dual Beam 8 auto cell UVS-2700 LABOMED, INC, US spectrophotometer using DMSO as solvent. Melting point was recorded on a Metrex melting point apparatus. Synthesis of ligand *N, N'*-Bis ( $\alpha$ -methyl trans cinnamaldehyde) ethylenediimine, its Ni(II) and Co(II) complexes was carried out broadly



**Scheme 1** a Synthesis of ligand [L] b Metal complexes

by procedure detailed in reference (Khalaji and Weil 2007; Sheikh et al. 2010). The compounds were recovered in solid state and were recrystallized from methanol. Yield 65% Ni (II) and 62% Co (II) complexes.

#### Growth conditions and media

*C. albicans* ATCC 44829, *C. tropicalis* ATCC 750 and *C. krusei* ATCC 6258 used in this study were kind gifts from I.A Khan (Clinical microbiology unit, IIIM Jammu, India). All of the strains were grown on Yeast extract Peptone Dextrose (YPD) medium containing 2% (w/v) glucose, 2% peptone, and 1% yeast extract (Hi Media, India). YPD agar plates containing 2.5% Agar (Hi Media) in addition were used to maintain the culture.  $\alpha$ -methyl trans cinnamaldehyde was purchased from ALDRICH chemicals (Germany), whereas all inorganic chemicals were of analytical grade and procured from E. Merck (India). Fluconazole was purchased from Hi Media, India.

## Methods

#### Determination of minimum inhibitory concentration (MIC<sub>90</sub>)

##### *Microtiter assay*

Cells were grown for 48 h at 30°C to obtain single colonies, which were resuspended in a 0.9% normal saline solution to give an optical density at 600 nm (OD<sub>600</sub>) of 0.1. The cells were then diluted 100-fold in YNB medium containing 2% glucose and the respective auxotrophic supplements. The diluted cell suspensions were added to the wells of round-bottomed 96-well microtiter plates (100  $\mu$ l/well) containing equal volumes of medium and different concentrations of test agents (Kohli et al. 2002). Fluconazole was included as positive control. In addition to this a drug-free control was also included. The plates were incubated at 30°C for 24 h. The MIC test end point was evaluated both visually and by observing the OD<sub>620</sub> in a microplate reader (BIO-RAD, iMark, US) and is defined as the lowest compound concentration that gave  $\geq 90\%$  inhibition of growth compared to the controls.

##### *Filter disk assay*

Strains were inoculated into liquid YPD medium and grown overnight at 37°C. The cells were then pelleted and washed three times with distilled water. Approximately 10<sup>5</sup> cells/ml were inoculated in molten agar media at 40°C and poured into 100 mm diameter petriplates. Filter discs were kept on solid agar and test compound was spotted on the disc as described previously (Ahmad et al. 2010). Test compounds (ten-fold more than MIC) dissolved in 10% DMSO, or solvent control (10% DMSO) were pipetted onto 4-mm-diameter filter disc. 200  $\mu$ g/ml of fluconazole was also on the discs to serve as positive control. The diameter of zone of inhibition was recorded in millimeters as difference in zone diameter from the centre of disc for test compound with that of solvent control, after 48 h and was compared with that of control. The results are presented as Mean  $\pm$  standard deviation of at least three replicate experiments performed on separate days.

##### *Ergosterol extraction and estimation assay*

Total intracellular sterols were extracted after (Arthington et al. 1999). Briefly, a single *Candida* colony from an overnight Sabouraud dextrose agar plate culture was used to inoculate 50 ml of Sabouraud dextrose broth (Hi Media) containing various concentrations of the test compounds along with positive control fluconazole. The cultures were incubated for 16 h with shaking at 35°C. The stationary-phase cells were harvested by centrifugation at 2,700 rpm (Sigma 3K30) for 5 min and washed once with sterile distilled water. The net wet weight of the cell pellet was determined. Three milliliters of 25% alcoholic potassium hydroxide solution was added to each pellet and vortex mixed for 1 min. Cell suspensions were transferred to sterile borosilicate glass screw-cap tubes and were incubated in an 85°C water bath for 1 h. Following incubation, tubes were allowed to cool to room temperature. Sterols were then extracted by addition of a mixture of 1 ml of sterile distilled water and 3 ml of *n*-heptane followed by vigorous vortex mixing for 3 min. Prior to analysis, a 1 ml aliquot of sterol extract was diluted fivefold in 100% ethanol and scanned spectrophotometrically between 240 and 300 nm using LABOMED, INC Spectrophotometer (USA). The presence of ergosterol

and the late sterol intermediate 24(28) DHE in the extracted sample resulted in a characteristic four-peaked curve. The absence of detectable ergosterol in extracts was indicated by a flat line. Ergosterol content was calculated as a percentage of the wet weight of the cell.

### *Proton efflux measurements*

Mid-log phase cells harvested from YEPD medium were washed twice with distilled water and routinely 100 mg cells were suspended in 5 ml solution containing 0.1 M KCl and 0.1 mM CaCl<sub>2</sub>. Suspension was kept in a double-jacketed glass container with constant stirring. Test compounds were added to achieve the desired concentration in this 5 ml solution. For glucose stimulation experiments, 100  $\mu$ l of glucose was added to achieve a final concentration of 5 mM. The container was connected to a water circulator at 25°C. H<sup>+</sup> extrusion rate was calculated from the volume of 0.01 N NaOH consumed as reported earlier (Rashid et al. 2004).

### 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  $\mu$ g/ml streptomycin, and 2.5  $\mu$ g/ml Amphotericin B, at 37°C in humidified incubator with 5% CO<sub>2</sub> (Gupta et al. 2006). For treatments, 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°C in 5% CO<sub>2</sub> humidified incubator together with untreated control sample. After incubation cells were washed in PBS and incubated with MTT solution for 45 min at 37°C. After discarding the supernatant MTT crystals were dissolved with acid isopropanol and the absorbance was measured at 570 nm. All assays were performed in triplicate. Percent viability was defined as the relative absorbance of treated versus untreated control cells. Plates were analysed in an ELISA plate reader (Labsystems Multiskan RC, Helsinki, Finland) at 570 nm with a reference wavelength of 655 nm.

Direct reduction or interaction of MTT observed with ligand and its complexes varied from 5 to 20% depending upon concentration used in the study. Given the low magnitude of direct reduction involved, any reduction by compounds accumulated by cells would be very minimal. No correction has been made for this reduction.

## Results

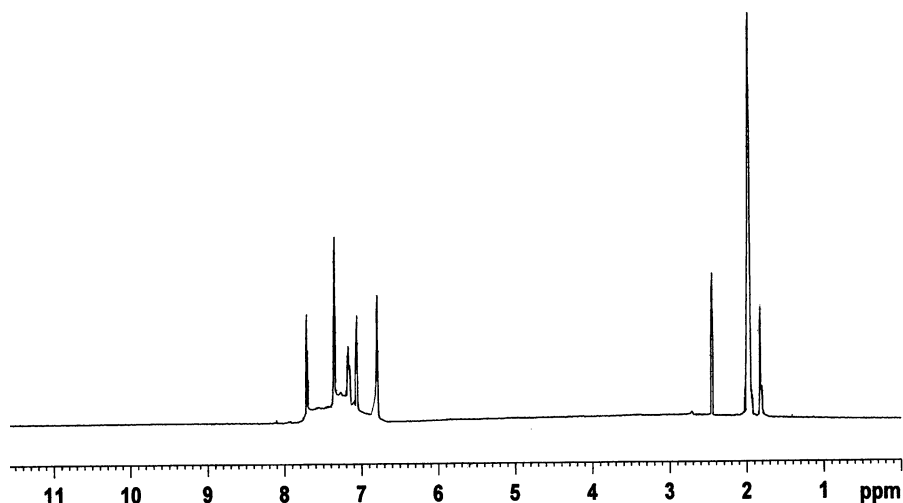
### Physical properties and IR Spectra

Physical properties and analytical data of the ligand and its metal complexes support its proposed structure. The molar conductivity ( $\Lambda_m$ ) of the Ni(II) and Co(II) complexes measured in  $1 \times 10^{-3}$  mol l<sup>-1</sup> DMSO at room temperature shows values of 38, 32  $\Omega^{-1}$  cm<sup>2</sup> mol<sup>-1</sup> respectively. Elemental analysis results show that the composition of the complexes is in agreement with its formulation given in (Scheme 1a, b). No band corresponding to free primary diamine and hydroxyl group was detected similarly in the IR spectrum of ligand absence of a broad absorption band in the region 1660–1765 cm<sup>-1</sup> characteristic for carbonyl group of (HC=O) in  $\alpha$ -methyl trans cinnamaldehyde, indicates that the Oxygen was detached from the HC=O. Appearance of a strong signal at 1445 cm<sup>-1</sup> may be attributed to the C–N bond (Nakamoto 1970). The shifting in the band of  $\nu$ (C–N) towards the lower wave number in the metal complex indicated that the coordination takes place through the nitrogen of the  $\nu$ (C–NH) group, hence implying that the ligand [L] is tetradentate. This was finally established through far IR spectra by the appearance of new signals seen at 450, 455, 340, and 345 cm<sup>-1</sup> in the spectra of metal complexes.

### <sup>1</sup>H NMR Spectra

<sup>1</sup>H NMR also supports the formation of ligand by showing the absence of an aldehydic peak (H–C=O) at 10 ppm (Fig. 1). The aromatic region of the ligand shows two doublet signals in the range 7.271–7.301 ppm (Ar–CH, 4H), 7.412–7.450 ppm (Ar–CH, 4H) and one sharp singlet at 7.18 ppm (Ar–CH, 2H), because these aromatic protons are in different chemical environment. Another sharp singlet at 2.46 ppm

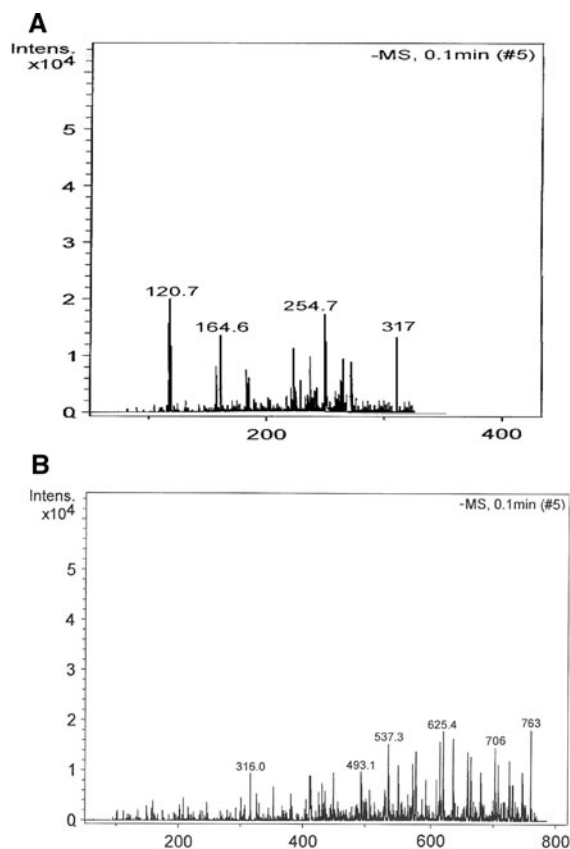
**Fig. 1** A region of the  $^1\text{H}$  NMR spectrum of the ligand [L]



( $\text{C}=\text{N}-\text{CH}_2$ , 4H) has been ascribed to methylene protons. Two more sharp signals one in the pseudo aromatic region at 6.85 ppm (2H,  $\text{C}=\text{C}-\text{CH}$ ) and another at 7.71 ppm (2H,  $\text{C}-\text{N}=\text{C}-\text{H}$ ) have been ascribed to methene protons. The signals of  $\text{C}-\text{N}=\text{C}-\text{H}$  has been shifted to down field due to the deshielding effect of nitrogen group attached to it. At 1.95 ppm ( $\text{C}-\text{CH}_3$ , 6H) sharp singlet has been ascribed to methyl protons. These proton signals undergo down field shifting in the metal complexes of the ligand, because of the paramagnetic effect of metal (II) ion, hence it supports the coordination of the ligand towards the metal ion (Chaudhary and Singh 2004; Kemp 1975).

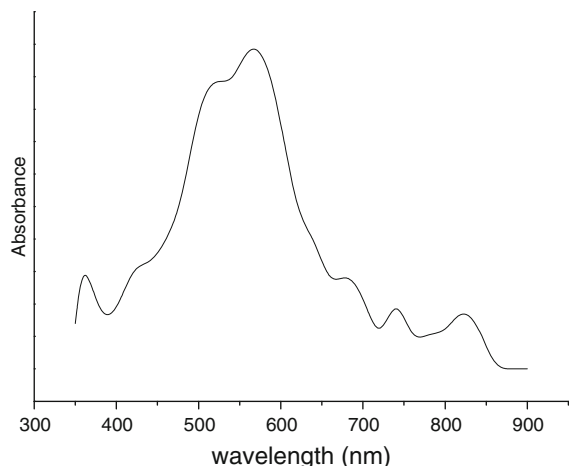
#### Electro spray ionization mass spectra (ESI MS)

The electro spray ionization mass spectrum (ESI MS) of the ligand was studied in DMSO solution (Fig. 2a). A negative ion ESI mass spectrum of ligand confirms the proposed formula by showing a peak at  $m/z$  317 corresponding to the moiety [ $\text{C}_{22}\text{H}_{24}\text{N}_2$ ] atomic mass  $m/z$  316.23]. The series of peaks in the range  $m/z$  120.7, 164.6, 254.7 etc., may be assigned to various fragments. This data suggests the 2 + 1 condensation of  $\alpha$ -methyl trans cinnamaldehyde and ethylenediamine. Their intensity gives an idea of stability of fragments. Similarly negative ion ESI-MS of the nickel and cobalt complexes shows a peak at  $m/z$  763 (Fig. 2b) and  $m/z$  763.5 respectively, which is consistent with the molecular ion fragment and it supports the proposed structure of the complex.



**Fig. 2** Electro spray ionization mass spectrum **a** ligand [L], **b** Ni(II) complex

Electronic spectra and magnetic measurements of Cobalt (II) and Nickel (II) complex of  $\alpha$ -methyl trans cinnamaldehyde.



**Fig. 3** Electronic spectrum of the Co(II) complex

The electronic spectra of the Co(II) complex (Fig. 3) consist of transition bands at 520, 565 nm which are assigned to the transition  ${}^4T_{1g} \rightarrow {}^4T_{1g}(P)$ , 682 nm which is assigned to the transition  ${}^4T_{1g} \rightarrow {}^2T_{1g}$ , and 739, 824 nm which are assigned to the transition  ${}^4T_{1g} \rightarrow {}^4T_{2g}$ . The Co(II) complex has an effective magnetic moment value ( $\mu_{\text{eff}}$ ) of 4.82 BM. These values are indicative of an octahedral geometry around the Co(II) ion. Electronic spectra of the Ni(II) complex showed transition bands at 415, 530 nm which are assigned to the transition  ${}^3A_{2g} \rightarrow {}^3T_{1g}(F)$ , 690, 750 and 860 nm which are assigned to the transition  ${}^3A_{2g} \rightarrow {}^3T_{2g}$ . An indication of the most probable geometric configuration of the synthesized metal complex is their magnetic moment values. The Ni(II) complex possesses

magnetic moment at  $\mu_{\text{eff}} = 3.29$  BM, which is in agreement with an octahedral geometry around the Ni(II) ion.

#### Minimum inhibitory concentration (MIC<sub>90</sub>) and sensitivity assay

Table 1 summarizes the minimum inhibitory concentration (MIC<sub>90</sub>) values, in microgram/ml ( $\mu\text{g/ml}$ ) and microMolar ( $\mu\text{M}$ ) of methyl cinnamaldehyde, ligand [L], Co(II) and Ni(II) complex against three different *Candida* species. On concentration basis average MIC<sub>90</sub> of methyl cinnamaldehyde was 2168  $\mu\text{M}$ . Average MIC<sub>90</sub> of ligand [L], Co(II) and Ni(II) complex is 483.8, 122 and 101  $\mu\text{M}$ , respectively. Fluconazole had a mean MIC<sub>90</sub> of 120  $\mu\text{M}$ . Compared to methyl cinnamaldehyde, thus ligand [L] is 4.48 times more effective whereas Co(II) and Ni(II) complex is 17.78 and 21.46 times more effective. Antifungal activity on solid media examined by disc diffusion assay yields results along similar lines. Yeasts were found to be more sensitive to Ni(II) and Co(II) complex, compared to ligand [L] and original compound (Table 2). Average value of zone of inhibition of three species at 3 mg/ml methyl cinnamaldehyde, ligand [L], [Co(L)Cl<sub>2</sub>] and [Ni(L)Cl<sub>2</sub>] was 8.3, 10.6, 14.3 and 16.6 mm. For fluconazole (200  $\mu\text{g/ml}$ ) the average value was 15.3 mm. On concentration basis thus ligand [L] was 2.73 times more effective compared to methyl cinnamaldehyde. Average fold effectiveness of [Co(L)Cl<sub>2</sub>], [Ni(L)Cl<sub>2</sub>] and fluconazole was determined to be 8.93, 10.38 and 57.58 times compared to methyl cinnamaldehyde.

**Table 1** Minimum inhibitory concentrations (MIC<sub>90</sub>) of four bioactive compounds and standard drug tested against different *Candida spp*

Bioactive compound	MIC <sub>90</sub> ( $\mu\text{g/ml}$ )		
	<i>C. albicans</i> ATCC 44829	<i>C. tropicalis</i> ATCC 750	<i>C. krusei</i> ATCC 6258
Methyl trans cinnamaldehyde	250	300	400
Ligand [L]	110	160	190
[Co(L)Cl <sub>2</sub> ]	60	100	120
[Ni(L)Cl <sub>2</sub> ]	50	80	100
Fluconazole <sup>a</sup>	16	32	64

<sup>a</sup> Positive control L- [C<sub>22</sub>H<sub>24</sub>N<sub>2</sub>]. Average of three species: methyl trans cinnamaldehyde; 2168  $\mu\text{M}$ , ligand [L]; 483.8  $\mu\text{M}$ , [Co(L)Cl<sub>2</sub>]; 122  $\mu\text{M}$ , [Ni(L)Cl<sub>2</sub>]; 101  $\mu\text{M}$ , fluconazole; 120  $\mu\text{M}$

**Table 2** Disc diffusion assay of methyl cinnamaldehyde its ligand [L], Co(II) and Ni(II) complexes against different *Candida spp*

Zone of inhibition (mm)					
	Methyl cinnamaldehyde	Ligand [L]	Co(II) complex	Ni(II) complex	Fluconazole <sup>a</sup>
<i>Candida albicans</i>					
3 mg/ml	11 (±0.9)	14 (±1.0)	17 (±0.9)	19 (±0.0)	17 (±0.5)
1 mg/ml	02 (±0.5)	06 (±1.5)	09 (±0.5)	10 (±0.3)	
<i>Candida tropicalis</i>					
3 mg/ml	08 (±0.4)	10 (±1.0)	14 (±1.0)	16 (±0.5)	15 (±1.0)
1 mg/ml	02 (±0.5)	05 (±1.5)	09 (±0.5)	11 (±0.8)	
<i>Candida krusei</i>					
3 mg/ml	06 (±1.5)	08 (±0.6)	12 (±1.0)	15 (±0.5)	14 (±0.5)
1 mg/ml	01 (±0.8)	04 (±0.5)	07 (±1.0)	08 (±0.6)	

The data represents (Mean ± S.D) of three experiments

<sup>a</sup> Positive control (200 µg/ml). Average value of three species at 3 mg/ml: methyl trans cinnamaldehyde; 8.3 mm, ligand [L]; 10.6 mm, [Co(L)Cl<sub>2</sub>]; 14.3, [Ni(L)Cl<sub>2</sub>]; 16.6 and fluconazole (200 µg/ml); 15.3 mm

### Ergosterol extraction and estimation assay

Table 3 summarizes the effect of  $\alpha$ -methyl cinnamaldehyde its ligand [L], Co(II) and Ni(II) complex on ergosterol biosynthesis in three *Candida* strains. The total ergosterol content was determined for each isolate grown in presence of varying concentrations of test compounds. As shown in (Table 3), the average decrease in total cellular ergosterol content for different *Candida* isolates after exposure to their respective MIC<sub>90</sub> of methyl cinnamaldehyde was 80%. Average decrease in total cellular ergosterol after exposure to MIC<sub>90</sub> of ligand [L], Co(II) and Ni(II) complex was 78, 90 and 93%, respectively. Inhibition at MIC<sub>90</sub> of fluconazole was 91%. From the results, it is clear that with increase in test compound concentrations, %ergosterol inhibition increases and finally at MIC<sub>90</sub> value close to 90% reduction in ergosterol content is obtained. MIC<sub>90</sub> values of methyl cinnamaldehyde its ligand [L], Co(II) complex, Ni(II) complex and fluconazole corresponds very well with percent inhibition of sterol biosynthesis. It would thus appear that major mode of action of methyl cinnamaldehyde, its ligand and complexes is through inhibition of ergosterol biosynthesis.

### H<sup>+</sup> extrusion studies

Primary determinant or source of acidification in yeast is Pma1p, both in absence and presence of

glucose (Serrano 1980; Ben-Josef et al. 2000; Elias et al. 2001). Proton-pumping ability of fungi mediated by the Pma1p at the expense of energy is crucial for the regulation of internal pH and growth regulation of fungal cell. Table 4 gives rate of H<sup>+</sup> extrusion by *Candida* species in presence and absence of glucose. No significant variation was observed between various organisms. Untreated *Candida* cells (control) in presence of 0.1 mM CaCl<sub>2</sub> and 100 mM KCl showed acidification starting from pH 7.0. Acidification decreased in the presence of methyl cinnamaldehyde its ligand [L], Co(II) and Ni(II) complex. In the absence of glucose, average reduction in H<sup>+</sup> extrusion at MIC<sub>90</sub> by methyl cinnamaldehyde was 62.11%. Ligand [L], Co(II) and Ni(II) complex caused average inhibition of 70.21, 82.89 and 90.96% respectively. Presence of glucose (5 mM) stimulated H<sup>+</sup>-efflux by 3.88, 5.22 and 3.86 fold with respect to control in *C. albicans*, *C. tropicalis* and *C. krusei* respectively. Average inhibition of glucose stimulated H<sup>+</sup> efflux was 30.09, 44.32, 50.32 and 55.75% for methyl cinnamaldehyde its ligand [L], Co(II) and Ni(II) complex respectively. H<sup>+</sup> efflux in the presence of glucose therefore did not correspond with MIC<sub>90</sub> of methyl cinnamaldehyde or synthesized ligand and complexes.

### Toxicity profile

Synthesized compounds were tested against H9c2 cardiac myoblasts for toxicity. Subconfluent populations

**Table 3** % Decrease in ergosterol content of control cells and treated samples

<i>C.albicans</i> ATCC 44829		<i>C.tropicalis</i> ATCC 750		<i>C.krusei</i> ATCC 6258	
Sample	% Decrease in Ergosterol content	Sample (µg/ml)	% Decrease in Ergosterol content	Sample (µg/ml)	%Decrease in Ergosterol content
Methyl cinnamaldehyde					
Control	0	Control	0	Control	0
100 µg/ml	21.13 ± 0.297	100	24.09 ± 1.423	100	21.11 ± 0.678
200 µg/ml	41.12 ± 0.189	200	63.22 ± 1.234	300	57.23 ± 2.213
250 µg/ml	55.01 ± 0.104	300	94.08 ± 0.213	400	91.09 ± 0.189
Ligand [L]					
Control	0	Control	0	Control	0
40 µg/ml	18.13 ± 0.799	50	29.08 ± 1.324	50	20.00 ± 0.231
80 µg/ml	39.04 ± 0.436	100	59.00 ± 0.876	100	51.09 ± 0.098
110 µg/ml	66.00 ± 1.234	160	86.00 ± 0.289	190	81.12 ± 0.678
Co(II) complex					
Control	0	Control	0	Control	0
20 µg/ml	23.17 ± 1.530	30	34.00 ± 2.123	40	29.00 ± 0.987
40 µg/ml	43.12 ± 0.209	60	66.08 ± 0.456	80	62.21 ± 1.103
60 µg/ml	77.00 ± 0.085	100	97.19 ± 1.324	120	96.18 ± 0.099
Ni(II) complex					
Control	0	Control	0	Control	0
20 µg/ml	28.24 ± 1.423	25	40.00 ± 0.987	30	37.09 ± 0.719
40 µg/ml	59.20 ± 0.111	50	71.08 ± 0.056	60	69.00 ± 1.170
50 µg/ml	80.21 ± 0.324	80	100 ± 0.178	100	100 ± 1.167
Fluconazole <sup>a</sup>					
Control	0	Control	0	Control	0
16 µg/ml (MIC)	81.00 ± 1.423	32 (MIC)	93.12 ± 1.423	64 (MIC)	100 ± 1.423

The data represents (Mean ± S.D) of three experiments

<sup>a</sup> Positive control. Average ergosterol inhibition of three species at their respective MIC<sub>90</sub>: methyl trans cinnamaldehyde; 80%, ligand [L]; 78%, [Co(L)Cl<sub>2</sub>]; 90%, [Ni(L)Cl<sub>2</sub>]; 93%, Fluconazole; 91%

of H9c2 cells were treated with increasing concentrations of these compounds and the measure of viable cells was obtained after 48 h by measuring optical density. Figure 4 depicts that all the compounds including the reference compound fluconazole showed a viability of 100% at the concentration range of 3.13 µg/ml. At 100 µg/ml methyl cinnamaldehyde, its ligand [L], Co(II) and Ni(II) complex offered a remarkable viability of 95, 90, 86 and 85%. At 200 µg/ml methyl cinnamaldehyde, ligand [L], Co(II) and Ni(II) complex showed 7, 11, 18 and 20% toxicity, respectively. Toxicity of reference drug fluconazole at 200 µg/ml was 55%. It was noticed that MIC<sub>90</sub> concentration of synthesized compounds, which had profound effect on growth, ergosterol

biosynthesis and H<sup>+</sup> ATPase activity of *Candida*, showed significant measure of viable cells. This indicates that the synthesized compounds have low cytotoxic activity.

## Discussion

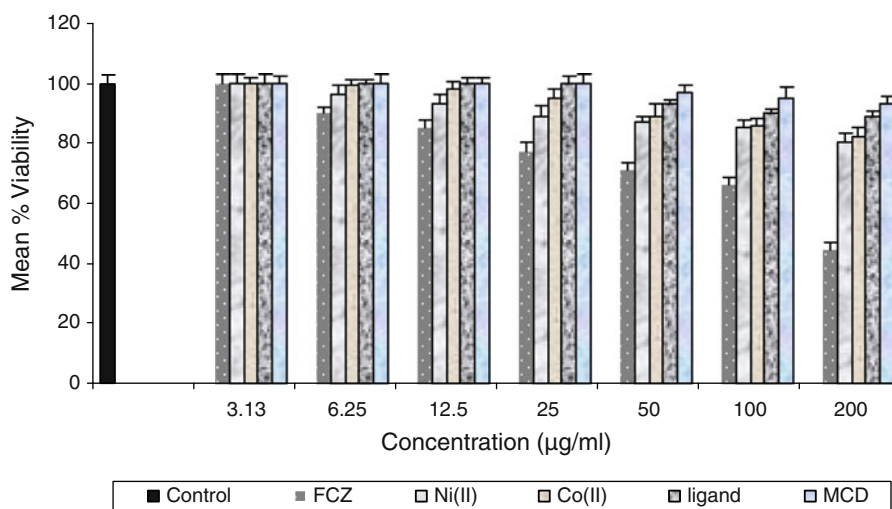
Results derived from structure-antifungal activity relationship of cinnamaldehyde suggest that aromatic ring, length of hydrocarbon chain outside the ring and lipophilicity affect antifungal properties of cinnamaldehyde (Ooi et al. 2006; Cheng et al. 2008). We have previously reported increase in antifungal activity of cinnamaldehyde following its coupling and metal



**Table 4** Effect of test compounds on rate of H<sup>+</sup>- efflux in different *Candida spp* at pH 7.0: Control stands for cells without any compound, incubated in 0.1 mM CaCl<sub>2</sub> and 0.1 M KCl at 25°C

Incubation with	Relative H <sup>+</sup> - efflux rate (n mol/mg yeast cells/min)			
	<i>C. albicans</i> ATCC 44829	<i>C. tropicalis</i> ATCC 750	<i>C. krusei</i> ATCC 6258	Average inhibition
Control	1 <sup>a</sup>	1 <sup>a</sup>	1 <sup>a</sup>	–
Control + methyl cinnamaldehyde (MIC <sub>90</sub> )	0.35	0.38	0.40	62.11
Control + ligand [L] (MIC <sub>90</sub> )	0.24	0.28	0.37	70.21
Control + [Co[L] Cl <sub>2</sub> ] (MIC <sub>90</sub> )	0.11	0.18	0.23	82.39
Control + [Ni[L] Cl <sub>2</sub> ] (MIC <sub>90</sub> )	0.05	0.08	0.13	90.96
Control + Glucose (5 mM)	3.88	5.22	3.86	–
Control + methyl cinnamaldehyde + Glucose	2.31	3.20	2.50	38.09
Control + ligand [L] + Glucose	2.09	2.82	2.28	44.32
Control + [Co(L)Cl <sub>2</sub> ] + Glucose	1.83	2.48	2.10	50.32
Control + [Ni(L)Cl <sub>2</sub> ] + Glucose	1.61	2.18	1.91	55.75

<sup>a</sup> The control had an average proton efflux rate (n moles/mg yeast cells/min) of 5.4, 5.0 and 5.9 respectively for *C. albicans* ATCC 44829, *C. tropicalis* ATCC 750 and *C. krusei* ATCC 6258. The data represents mean of at-least three experiments (variation was less than ± 5%)



**Fig. 4** Percentage of viable cells after 48 h pre-treatment of H9c2 myoblasts with fluconazole, Ni(II) complex, Co(II) complex, ligand [L] and methyl cinnamaldehyde evaluated by MTT assay

complexing (Sheikh et al. 2010). As compared to cinnamaldehyde,  $\alpha$ -methyl trans cinnamaldehyde with an additional carbon in chain shows increased MIC<sub>90</sub> of 250, 300 and 400 µg/ml for *C. albicans*, *C. tropicalis* and *C. krusei*, respectively. Self coupling of methyl cinnamaldehyde and further complexing with Co(II) and Ni(II) increases lipophilicity, which in turn may increase their permeation through

lipid membranes (Chohan et al. 2004). This is reflected in significant drop in MIC<sub>90</sub> of ligand [L] as 110, 160 and 190 µg/ml, respectively, for three *Candida* species. Complexation of two molecules of ligand [L] employing Co(II) and Ni(II) ions leading to further multiplication of methyl cinnamaldehyde structure and increased lipophilicity leading to fold effectiveness of 17.78 and 21.46, respectively.

Compared to methyl cinnamaldehyde fold effectiveness of fluconazole was 18.06 which is in the same range as of synthesized complex. Antifungal activity on solid media examined by filter disc assay reinforce MIC<sub>90</sub> results that coupling and complexation leads to increase in anticandidal effectivity of methyl cinnamaldehyde against all the three strains. On solid media, however, fold effectiveness of synthesized ligand and complexes was 2.73, 8.93 and 10.38 as compared to original molecule. Less fold effectiveness of synthesized ligand and complexes on solid media as compared to liquid medium indicates that increase in structure leads to restricted diffusion on solid media.

Mechanism of antimicrobial activity of cinnamaldehyde is not well established, available literature supports both membrane action and specific cellular processes (Kwon et al. 2003; Walsh et al. 2003). Two key membrane associated properties have been explored: PM-ATPase activity and ergosterol biosynthesis. In the absence of glucose methyl cinnamaldehyde, its ligand [L], Co(II) and Ni(II) complex cause mean inhibition in H<sup>+</sup>-extrusion of 62, 70, 82 and 91%. Presence of 5 mM glucose activated H<sup>+</sup>-extrusion. Glucose stimulated H<sup>+</sup>-extrusion however, was inhibited to a significantly lesser extent by all tested compounds. Mean inhibition of three species was only 38, 44, 50 and 56% by methyl cinnamaldehyde, ligand, Co(II) and Ni(II) complex at their respective MIC<sub>90</sub> values. Glucose modified form of H<sup>+</sup>ATPase thus appears to be less sensitive. Inhibition values do not correspond well with MIC<sub>90</sub> values suggesting that inhibition of PM-ATPase may not be the primary effect of methyl cinnamaldehyde and its complexes.

Ergosterol biosynthesis is important target of antifungals because it contributes to membrane integrity, fluidity and proper functioning of membrane bound enzymes. MIC<sub>90</sub> and ergosterol content correspond well for agents whose primary target is ergosterol biosynthesis pathway (Shimokawa and Nakayama 1992). Same is evident from positive control result, fluconazole at its MIC<sub>90</sub> value caused mean decrease in ergosterol content by 91%. At their respective MIC<sub>90</sub>, methyl cinnamaldehyde, ligand [L], Co(II) and Ni(II) complex cause mean ergosterol reduction by 80, 78, 90 and 93%. This correlation suggests that ergosterol biosynthesis is primary site of action of methyl cinnamaldehyde, its ligand and

complexes. Sub confluent population of H9c2 rat cardiac myoblast cells was employed for in vitro MTT cell viability. No toxicity was displayed by methyl cinnamaldehyde and ligand [L]. Co(II) and Ni(II) complexes showed an average 19% cellular toxicity.

To conclude  $\alpha$ -methyl trans cinnamaldehyde, a less skin sensitive derivative of cinnamaldehyde was self coupled and complexed with Co(II) and Ni(II). Synthesized ligand and complexes showed increased anticandidal activity and have a very limited toxicity towards vertebrate cells.

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