Synthesis and synergistic studies of isatin based mixed ligand complexes as potential antifungal therapeutic agents

Ovas Ahmad Dar,a Shabir Ahmad Lone,b Manzoor Ahmad Malik,a Faisal Mohammed Aqlan,c Mohmmad Younus Wanic,d Athar Adil Hashmia,*, Aijaz Ahmadd,**

a Department of Chemistry, Jamia Millia Islamia, New Delhi 110025, India
b Clinical Microbiology and Infectious Diseases, School of Pathology, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, 2193, South Africa
c Chemistry Department, Faculty of Science, University of Jeddah, P.O. Box 80327, Jeddah 21589, Kingdom of Saudi Arabia
d Infection Control, Charlotte Maxeke Johannesburg Academic Hospital, National Health Laboratory Service, Johannesburg, 2193, South Africa

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ABSTRACT

Metal based drugs are important class of chemotherapeutic agents that have the potential to circumvent drug resistance. Increasing drug resistance, treatment failures and limited treatment options necessitates the development of new therapeutic drugs with different mechanisms of action. Towards this direction, we synthesized a series of isatin based mixed ligand complexes of [Cu(dbm)LClH2O] (mlc1), [Co(dbm)LCl2] (mlc2) and [Ni(dbm) LClH2O] (mlc3) and evaluated their antifungal activity alone and in combination with fluconazole (FLC) against seven different Candida albicans isolates. The insight mechanism of antifungal action was revealed by studying apoptosis via terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay. The study revealed that all these compounds showed antifungal activity at varying concentrations with mlc3 as the most potent compound with minimum inhibitory concentration ranging from 0.5–8 μg/mL and minimum fungicidal concentration ranging from 4–16 μg/mL. Upon combination with FLC, most of the interactions were either synergistic (54 %) or additive (32 %) with no antagonistic combination against any of the tested isolate. The study on their mechanism of action revealed that these compounds show apoptotic effect on C. albicans at sub-inhibitory concentrations, suggesting that strategies to target this process may augment the current antifungal treatment modalities.

1. Introduction

Fungal infections take more than 1.3 million lives each year worldwide, nearly as many as tuberculosis. There is an urgent need for the development of new antifungal agents or strategies that could add to the current armament against fungi and augment the treatment options which have been challenged by increasing multi-drug resistance. A good strategy to design new antifungal drugs has been the complexation of bioactive compounds with transition metals. Metal complexes of sulphonamide drugs, fluoro quinolones and penicillin have already been synthesized and it was observed that metalation enhances the efficacy of the already established drugs [1, 2, 3]. Pd(II) complex of tetracycline has been reported to have potency sixteen times more than the parent compound against E.coli HB101/pBR322, a bacterial strain resistant to tetracycline whereas Pd(II) complex of doxycycline is two times more potent than doxycycline against resistant strain [2]. In another study Copper(II) norfloxacin complex sharply decreased the viability and proliferation rate of HL-60 cells, leading to cell death through apoptosis in a time-dependent manner [1].

Isatin possess an indole ring structure, common to many pharmaceuticals and heterocyclic natural products of biological interest [4]. Its derivatives have shown important biological activities such as antimicrobial, anticonvulsant, cysticidal, antimalarial, herbicidal, antimycobacterial, anticancer, anti-inflammatory and antiviral [5, 6]. They also exhibit anti-HIV, antihelmintic and antiprotozoal activities [7]. Dibenzoylmethane (DBM) on the other hand belongs to a small group of flavonoids known as β-hydroxychalcones [8] and is rarely found in nature. DBM and its derivatives have attracted considerable attention because of their promising biological activities [9, 10]. DBM has also been found as a possible candidate for dementia treatment [11]. Based on
our previous studies it is our premise that complexation of bioactive ligands with transition metals and combination therapy greatly improves the treatment efficacy and provides a broader treatment window that greatly helps to circumvent drug resistance [12, 13, 14]. In this study, we proposed to prepare some mixed ligand complexes of a ligand derived from isatin and DBM as a co-ligand and tested them against different FLC susceptible and resistant C. albicans isolates alone and in combination with FLC to obtain new antifungal agents or potentiators that could be used in combination therapy. The mechanism of action was studied by TUNEL assay.

2. Results and discussion

The mixed ligand metal complexes (mlc1-mlc3) were successfully synthesized by reacting equimolar amounts of Schiff base ligand (L) with the corresponding metal salts and dibenzoylmethane in presence of NaOH as shown in Scheme 1. The synthesized metal complexes were colored, air stable and insoluble in most of the organic solvents except DMF and DMSO. The ligand (L) and metal complexes (mlc1-mlc3) were analyzed using various physico-chemical properties like melting point, yield, color, elemental analysis and conductivity measurements. The data is shown in Table 1. It was concluded from the results that mlc1 and mlc3 complexes had molar conductance values of the 21.25 and 24.72 Ω⁻¹cm²mol⁻¹ respectively, indicating their non-electrolytic nature. While mlc2 complex had the molar conductance of 86.43 Ω⁻¹cm²mol⁻¹ indicated the electrolytic nature of this complex. All the compounds were further characterized by various spectral techniques like FT-IR, NMR, UV-Vis spectra, mass spectrometry, magnetic moments and thermal studies. The analytical data of the ligand and metal complexes are in good agreement with their proposed formula.

2.1. FT-IR spectral studies

The infrared spectrum of the synthesized Schiff base ligand and metal complexes were taken in the range of 4500–400 cm⁻¹ to interpret the nature of the ligands to metal ion bonding. Upon chelation the intensities of the peaks are expected to change as compared to that of the ligands and also some new peaks appeared due to chelation. The IR spectra of the Schiff base ligand, DBM and mlc1 metal complex as an example is shown in Fig. 1.

The IR spectrum of the ligand (L) displayed a strong band at 1600 cm⁻¹ corresponding to υ(C=≡N) of imine group. In complexes (mlc1-mlc3) the imine υ(C=≡N) of the ligand shifted to lower wavenumbers 1543-1586 cm⁻¹ upon coordination to metal [15]. A broad band of medium intensity at 3130-3205 cm⁻¹ assigned to υ(N–H) was also observed. The strong band observed in the ligand at 1727 cm⁻¹ is characteristic of υ(C=O) amide[5]. In complexes this band was shifted to lower frequencies (10-12 cm⁻¹), indicating the coordination of carbonyl oxygen of the ligand to metal ions. In DBM the band present at 1665 cm⁻¹ correspond to υ(C=O) and in complexes this band is shifted to lower frequencies signifying the coordination of carbonyl oxygen of DBM to metal ions [16]. In DBM the band present at 1282 cm⁻¹ was assigned to the phenolic C–O stretching mode. In case of complexes (mlc1-mlc3) this band was found at higher wavenumbers in the range of 1287–1290 cm⁻¹ confirming the involvement of the enolic oxygen in coordination. The mlc1 and mlc3 complexes show a broad band at 3500 and 3468 cm⁻¹ characteristic of υ(OH) coordinated water molecule. The IR spectra of the mlc2 and mlc3 complexes are shown in supplementary data.
Table 1

| Comp. | Colour          | Mol. formula | Mol. Wt. | (m/z) ratio | Yield (%) | $\lambda$$_{max}$ (\$\Omega$$^{-1}$ cm$^{2}$ mol$^{-1}$) | Mp (°C) | $\mu$_{eff} (BM) |
|-------|----------------|--------------|----------|------------|-----------|----------------------------------------------------------|---------|-----------------|
| L     | Orange         | C$_{9}$H$_{10}$N$_{2}$O   | 222.2    | 223.0      | 80        | -                                                       | 162     | -               |
| mlc1  | Yellow Green   | [C$_{20}$H$_{22}$ClCuN$_{2}$O$_{4}$] | 558.7   | 559.2      | 68        | 86.43                                                   | 221     | 4.60            |
| mlc2  | Brown          | [C$_{20}$H$_{22}$Cl(CuN$_{2}$O$_{3}$)Na] | 598.3   | 599.2      | 68        | 86.43                                                   | 221     | 4.60            |
| mlc3  | Reddish Brown | [C$_{20}$H$_{22}$Cl(N$_{2}$NiO$_{4}$)] | 557.6   | 558.7      | 72        | 24.72                                                   | 215     | 3.10            |

The low frequency region of the spectra in complexes (mlc1-mlc3) showed the presence of new medium and weak intensity bands in the region of 400–600 cm$^{-1}$, assigned to v(M-O) and v(M-N) stretching vibrations. The above results revealed that the two ligands coordinated to metal ions via the carbonyl oxygen of dibenzoylmethane moiety in enolic or ketonic form and the azomethine nitrogen and carbonyl oxygen of ligand (L).

2.2. Mass spectrometry

The mass spectra of the ligand (L) and metal complexes (mlc1-mlc3) were recorded and were found in close agreement with the molecular weights of these compounds. The mass spectra [M + H]$^+$ of the ligand showed the molecular ion peak at m/z 223.0 and the mixed ligand complexes (mlc1-mlc3) showed molecular ion peaks at 563.5, 599.2 and 558.7 respectively. The mass spectrum is given in supplementary data.

2.3. Magnetic susceptibility and electronic spectral studies

The electronic spectra were recorded in 10$^{-3}$ M DMSO solution in the range of 200–800 nm at room temperature using same solvent as blank. Two bands were observed in the ligand at 255 and 305 nm. The band at 305 nm might be attributed to the n-$\pi^{*}$ transitions of the aromatic rings and the band at 305 nm might be attributed to the n-$\pi^{*}$ transitions of the azomethine group. In complexes the n-$\pi^{*}$ transitions of the aromatic rings and the azomethine nitrogen and carbonyl oxygen of dibenzoylmethane moiety in enolic or ketonic form and the azomethine nitrogen and carbonyl oxygen of ligand (L).

The electronic spectra and magnetic moment suggest an octahedral geometry around the Ni(II) ion [15].

The 1H NMR of the ligand (L) and complexes (mlc1-mlc3) was recorded in CDCl$_{3}$ and DMSO-d$_{6}$ respectively using tetramethylsilane (TMS) as internal standard. The 1H NMR of the ligand (L) gave characteristic signal at 9.46 ppm (s, 1H) which was assigned to the NH proton [17]. In addition, the aromatic protons of the ligand appeared as multiplets in the range of 6.65–7.49 ppm (m, 9H). The 1H NMR of the complexes (mlc1-mlc3) was not clear probably due to the paramagnetic nature of the complexes. The 1H NMR and 13C NMR of the ligand and complexes obtained as such is given in the supporting information (see Figs. S7-S14).

2.4. $^{1}$H NMR and $^{13}$C NMR spectra

The 1H NMR of the ligand (L) and complexes (mlc1-mlc3) was recorded in CDCl$_{3}$ and DMSO-d$_{6}$ respectively using tetramethylsilane (TMS) as internal standard. The 1H NMR of the ligand (L) gave characteristic signal at 9.46 ppm (s, 1H) which was assigned to the NH proton [17]. In addition, the aromatic protons of the ligand appeared as multiplets in the range of 6.65–7.49 ppm (m, 9H). The 1H NMR of the complexes (mlc1-mlc3) was not clear probably due to the paramagnetic nature of the complexes. The 1H NMR and 13C NMR of the ligand and complexes obtained as such is given in the supporting information (see Figs. S7-S14).

2.5. Thermogravimetric studies (TG and DTG)

The thermal analysis of the complexes was studied by using thermo gravimetric techniques within a temperature range from room temperature to 1000 °C at a heating rate of 10 °C/min. As a representative case the TG/DTG curve of complex mlc3 is depicted in Fig. 3. The temperature intervals and the percentage loss of masses of complexes is summarized in Table 2. The thermal decomposition of the complexes mlc1-mlc3 undergoes in three stages. The first stage is a degradation step where weight loss of 3% (Calcd. 3.2%) in the temperature range of 50–200 °C with DTG peaks at 120 °C was observed in mlc1 complex. This weight loss is due to the liberation of one coordinated water. The mlc1 complex in the second stage degrade at the temperature range of 200–400 °C by
with DTG peak observed at 590 °C. The overall weight loss observed in this complex was found to be 87% (Calcd. 88.7%). The metallic residue left has the observed weight of 13% (Calcd. 11.3%).

The mlc2 complex undergoes decomposition in three stages. The first stage occurred at the temperature range of 100–270 °C with the weight loss of 12% (Calcd. 12.28%). This weight loss corresponds to the loss of Cl2 molecule with DTG peak observed at 242 °C. The second stage of degradation occurred at the temperature range of 270–590 °C with the weight loss of 38% (Calcd. 38.49%). This weight loss corresponds to the loss of C14H10N2O fragment with DTG peaks observed at 378 °C. The third stage in this complex proceeded with one degradation step. This degradation step occurred within the temperature range of 590–860 °C with the weight loss of 39% (Calcd. 39.84%), which corresponds to the loss of C15H12O2 fragment with DTG peak observed at 700 °C. The total weight loss observed was found to be 89% as against the calculated value of 89.61%. The metallic residue finally left has the observed mass of 11% as against the calculated value of 10.39%.

In mlc3 complex the first stage of degradation occurred at the temperature range of 100–300 °C with the weight loss of 9% (Calcd. 9.5%). This weight loss corresponds to the simultaneous loss of one coordinated water molecule and one coordinated chlorine atom. The DTG peaks were observed at 152 and 241 °C. The second stage of degradation occurred at the temperature range of 300–515 °C with the weight loss of 39% (Calcd. 39.77%). This weight loss corresponds to the loss of C14H10N2O fragment with DTG peaks observed at 405 °C. The third stage proceeded with one degradation step and occurred within the temperature range of 590–860 °C with the weight loss of 40% (Calcd. 40.12%), which corresponds to the loss of C15H12O2 fragment with DTG peak observed at 617 °C. The total weight loss observed was found to be 88% as against the calculated value of 89.39%. The metallic residue finally left has the observed mass of 12% as against the calculated value of 10.61%.

From all the physical measurements and spectroscopic techniques, the metal complex mlc1 appears to have a distorted octahedral geometry and the complexes mlc2 and mlc3 have octahedral geometry. The structures of these complexes was drawn in chemdraw ultra 12.0 and energy minimized (MM2) in chem3D pro using the set functions. The energy minimized structures are shown in Fig. 4.

2.6. Biological studies

2.6.1. Minimum inhibitory concentrations and minimum fungicidal concentrations

The ligand (L) and its mixed ligand complexes (mlc1-mlc3) were evaluated in vitro against seven different isolates of C. albicans by microbroth dilution assay. All the MIC and MFC results are summarized in Table 3. The MIC values range from 0.5 μg/mL to 500 μg/mL while as MFC values vary from 4 μg/mL to 1000 μg/mL. The complex mlc3 exerted highest inhibitory activity against all the tested fungal isolates with an MIC values ranging from 0.5–8 μg/mL while as the ligand has shown the least inhibitory activity ranging from 125–500 μg/mL. We did not evaluate dibenzoylmethane (DBM) because it is known to be inactive. As a positive control, FLC was used because of its common use in the treatment Candida infections. As expected, the MIC values for FLC against susceptible and standard laboratory strain was ranging from 0.12 μg/mL to 0.25 μg/mL while as these values range from 16 μg/mL to 32 μg/mL against FLC resistant isolates. These results are congruent with the CLSI Interpretive Guidelines for In vitro Susceptibility Testing of Candida species [18]. Furthermore, all the test compounds were prepared to different concentrations using 1% DMSO and therefore DMSO was used as negative vehicle control and was observed to have no inhibitory activity against any of the tested isolates. Based on the MIC results, order of potency of these compounds was mlc3-mlc2-mlc1>L. The MIC data revealed that the structural changes from ligand to its metal complexes produced the marked enhancement in their potency as antifungal agents. Unlike FLC, all the test compounds showed MFC values indicating their potential to kill the fungal pathogen within varying concentration ranges.

2.6.2. In vitro combination antifungal activities

Having established the individual MIC values for L, mlc1, mlc2, mlc3 and FLC, the MIC and FICI values of these compounds in combination with the FLC were determined in 1:1 combination by microbroth dilution assay against seven C. albicans isolates (See supporting information Table S1). When combined with FLC (n = 28), most of the combinations were either synergistic (54%; n = 15) or additive (32%; n = 9) and only few were indifferent (14%; n = 4) with no antagonistic interaction. Ligand (L) when combined with FLC showed strong synergistic inhibitory

![Fig. 3. TGA and DTG curves of mlc3 complex.](Image)

The simultaneous loss of coordinated chloride atom and C14H10N2O moiety with the weight loss of 45% (Calcd. 45.8%) with DTG peaks observed at 210 and 396 °C. The third stage of decomposition in this complex occurred at the temperature range of 400–700 °C with the weight loss of 39% (Calcd. 39.7%) due to the loss of C15H12O2 fragment with DTG peak observed at 590 °C. This weight loss corresponds to the degradation step and occurred within the temperature range of 590 °C.

The weight loss observed was found to be 88% as against the calculated value of 88.7%. The metallic residue finally left has the observed mass of 13% (Calcd. 11.3%).

The mlc2 complex underwent decomposition in three stages. The first stage occurred at the temperature range of 100–270 °C with the weight loss of 12% (Calcd. 12.28%). This weight loss corresponds to the loss of Cl2 molecule with DTG peak observed at 242 °C. The second stage of degradation occurred at the temperature range of 270–590 °C with the weight loss of 38% (Calcd. 38.49%). This weight loss corresponds to the loss of C14H10N2O fragment with DTG peaks observed at 378 °C. The third stage in this complex proceeded with one degradation step. This degradation step occurred within the temperature range of 590–860 °C with the weight loss of 39% (Calcd. 39.84%), which corresponds to the loss of C15H12O2 fragment with DTG peak observed at 700 °C. The total weight loss observed was found to be 89% as against the calculated value of 89.61%. The metallic residue finally left has the observed mass of 11% as against the calculated value of 10.39%.

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Table 2

| Complex                                      | TG range(°C) | DTG_{max}(°C) | n° | Found (calcd. %) | Assignment                                      | Metallic residue |
|----------------------------------------------|--------------|---------------|----|-----------------|------------------------------------------------|------------------|
| (C29H23ClCuN2O4)                             | 50–200       | 120           | 1  | 3.0(3.2)        | Loss of one coordinated H2O molecule             |Cd               |
|                                              | 200–500      | 310,390       | 2  | 45(45.8)        | Loss of 1/2Cl2 and C14H10N2O                      |Cu               |
| (C29H23ClN2NiO4)                             | 500–800      | 590           | 1  | 39(39.7)        | Loss of C15H12O2                                 |Co               |
|                                              | 100–270      | 242           | 1  | 12(12.28)       | Loss of coordinated Cl2 molecule                 |Ni               |
| (C29H21Cl2CoN2O3)Na                            | 270–590      | 378           | 1  | 38(38.49)       | Loss of C15H12O2                                |Cu               |
| (C29H21CNaN2O4)                             | 590–860      | 700           | 1  | 39(38.84)       | Loss of coordinated Cl2 molecule                 |Ni               |
|                                              | 100–300      | 152,241       | 2  | 9(9.5)          | Loss of one coordinated H2O and 1/2Cl2 molecule  |Ni               |
|                                              | 300–515      | 405           | 1  | 39(39.77)       | Loss of and C15H12O2                             |Ni               |
|                                              | 515–765      | 617           | 1  | 40(40.12)       | Loss of C15H12O2                                |Ni               |

n° = number of decomposition.
effects against the majority of the tested *C. albicans* strains (6 out of 7) with FICI values ranging from 0.146–0.380 (See supporting information Table S1). Likewise, the combination of mlc1 with FLC also exhibited good synergy against the majority of the tested *C. albicans* strains (5 out of 7), with FICI values ranging from 0.266–0.500. Interestingly, mlc3 which was the most active compound showed only one synergistic activity against one of the resistant isolates while as all other activities are which was the most active compound showed only one synergistic activity against one of the resistant isolates while as all other activities are

Table 3
Minimum inhibitory concentrations (μg/mL) of ligand (L) and mixed ligand complexes (mlc1-mlc3) against FLC susceptible and resistant isolates.

| Isolates                | Ligand (L) | mlc1 | mlc2 | mlc3 | FLC |
|-------------------------|------------|------|------|------|-----|
|                         | MIC        | MFC  | MIC  | MFC  | MIC | MFC  | MIC  | MFC  | MIC  | MFC  |
| Lab strain              |            |      |      |      |     |      |      |      |      |      |
| *C. albicans* SC5314    | 125        | 500  | 125  | 250  | 32  | 125  | 0.5  | 8    | 0.25 |
| FLC Susceptible strains |            |      |      |      |     |      |      |      |      |      |
| 4175                    | 250        | 500  | 250  | 500  | 64  | 500  | 2    | 8    | 0.12 |
| 4179                    | 250        | 1000 | 125  | 500  | 64  | 125  | 1    | 4    | 0.25 |
| 4180                    | 125        | 500  | 125  | 500  | 64  | 125  | 1    | 4    | 0.25 |
| FLC Resistant strains   |            |      |      |      |     |      |      |      |      |      |
| 4085                    | 500        | 1000 | 250  | 500  | 125 | 500  | 8    | 16   | 16   |
| 4122                    | 500        | 1000 | 250  | 500  | 125 | 500  | 4    | 8    | 32   |
| 4135                    | 500        | 1000 | 250  | 500  | 125 | 500  | 8    | 16   | 32   |

2.6.3. TUNEL assay: FITC labeling
Numerous molecular level apoptotic regulators have been identified and characterized [21]. DNA damage is a well-established hallmark of late apoptosis. TUNEL assay was used as primary screening tool for apoptosis as it targets fundamental apoptotic event in yeast cells. To investigate whether the newly synthesized compounds display features of the late stages of apoptosis in *C. albicans*, we evaluated DNA fragmentation and visualized it with the help of a TUNEL assay by labeling 3’-OH ends of nicked DNA with fluorescent dUTP (TUNEL-FITC). Fig. 5 represents *C. albicans* cells (SC5314) exposed to MIC and ½ MIC values of mlc3 for 1 h, exhibiting a significant amount of changes in nuclear DNA and that of the ligand (L) and complexes mlc1 and mlc2 are shown in Fig. S17. In cells exposed to low concentrations of the test compounds (MIC/2), the proportion of TUNEL-positive nuclei (green fluorescence) was significantly increased compared to that of the untreated control population. The analyses of results revealed remarkable apoptosis for mlc3 treated Candida cells with >95% apoptotic cells when treated with ½ MIC values. The other compounds also showed apoptotic activity on *C. albicans* at different ratios when cells were treated with sub-MIC values. At MIC values very, less number (<10%) of apoptotic cells were observed. Cells exposed to positive controls (2 μg/ml amphotericin B) revealed an increase in TUNEL-positive nuclei identified as green fluorescence spots.

Compounds inducing apoptosis in *Candida* at sub-MIC values is of dominant value as these compounds may then be used for the design of new drugs with fungicidal activity at higher concentrations and programmed cell death at lower concentrations. The most used polyene - amphotericin B, is known to be fungicidal and induces apoptosis in *C. albicans* [22, 23] which could be a possible reason for low resistance levels against this drug. In contrast FLC, which is a fungistatic drug aids *Candida* to develop resistance at lower concentrations [24].

Many metal-based drugs have been demonstrated to show mechanism of action distant from conventional drugs which make them ideal candidates where resistance to conventional drugs has already emerged. The different mechanism of action of metal-based drugs could also be utilized by using them in combination with conventional drugs to target multiple pathways with limited resistance. Some previous studies have shown that treatment of *Candida* with Silver(I) complexes resulted in many morphological features of programmed cell death which was shown to be due to reduction in the ergosterol biosynthesis, a sterol essential to maintain membrane integrity [25]. Altered susceptibility to miconazole and amphotericin B mediated through alterations in the respiration rate has been observed when *Candida* cells were treated with Cu and Ag complexes [26].

In a previous study we observed that treatment of *Candida* with metal complexes disrupts their membrane integrity [20]. In this study we observed that all the metal complexes show synergistic interaction with fluconazole and one of the complexes (mlc3) showed
remarkable apoptosis. The different behavior of these otherwise structurally similar complexes could be due to the different nature of the metal ions in their complexes. The most ideal complex (mlc3) could be further studied for its detailed mechanism of action by studying the other markers of early and late apoptosis, such as Annexin V-FITC and PI labeling, cytochrome C oxidase activity, membrane potential and ergosterol biosynthesis assay. Furthermore, this complex will also be tested against other species of Candida including C. auris, which are known multidrug resistant fungal species responsible for several outbreaks worldwide.

3. Conclusions

Isatin and DBM based mixed ligand complexes were synthesized, characterized and evaluated for their antifungal properties. mlc2 and mlc3 complexes possess octahedral geometry whereas a distorted octahedral geometry was assigned to the complex mlc1, based on various physical and spectroscopic techniques. The biological results revealed that these compounds, with special emphasis to mlc3, have a potential to be used as antifungal drugs and significant potentiators with known antifungal azole drug, Fluconazole. The mechanism of action appears to be due to apoptosis in C. albicans and therefore this study paves the way for the study and role of metal based drugs as potential antifungal agents and potentiators in mediating fungal cell death by inducing apoptosis. Further investigations would result in a strategy that would lead to the development of novel antifungal agents that switch on endogenous cell suicide mechanisms and therefore bypasses the development of drug resistance.

4. Experimental

4.1. Materials and physical methods

Isatin, aniline and metal chlorides were analytical grade products from Merck. Dibenzoylmethane was purchased from Aldrich and all chemicals were used as received. Solvents used for the synthesis and analysis was reagent grade chemicals and used without further purification.

The percentages of carbon, hydrogen, nitrogen in Schiff base ligand and metal complexes were determined using Flash EA 1112 elemental analyzer (Thermo Scientific). Electrothermal melting point apparatus was used to determine the melting point of the synthesized compounds. The absorption spectra of the synthesized compounds were recorded between 200-600 nm(cm⁻¹) using UV/visible spectrophotometer (UV-260 Shimadzu, with 1cm quartz cuvettes). IR spectra of the ligand and complexes were recorded in KBr pellets on a Perkin-Elmer 283 spectrophotometer. For ¹H NMR and ¹³C NMR measurements Brucker WH 300 (200MHz) and Brucker WH 270(67.93 MHz) were used using CDCl₃ or DMSO-d₆ as a solvent and tetramethylsilane as an internal standard. ESI-MS (AB-Sciex 2000, Applied Biosystem) was used to record mass spectra.

![Ambient Light Channel](image1)
![FITC Channel](image2)

\[ \text{mlc3} - \frac{1}{2} \text{MIC} \]

![Ambient Light Channel](image3)
![FITC Channel](image4)

\[ \text{mlc3} - \text{MIC} \]

Fig. 5. Fluorescent images of C. albicans SC5314 cells treated with MIC and \( \frac{1}{2} \) MIC values of mlc3 complex. Green fluorescence emitting cells are TUNEL-FITC positive, indicating active DNA fragmentation, in cells undergoing apoptosis.
of the synthesized ligand and complexes. Magnetic moments were measured using Sherwood scientific magnetic susceptibility balance at 25° C. The thermogravimetric analysis (TGA/DTG) was carried out in the temperature range of 20–1000° C in a dynamic nitrogen atmosphere with a heating rate of 10° C/min using NETZSCH STA 449F3 thermal analyzer.

4.2. Synthesis of Schiff base ligand and its mixed ligand complexes

4.2.1. Synthesis of (phenylimino)indolin-2-one ligand (L)

To a hot ethanolic solution (20 mL) of aniline (1.47 g, 10 mmol) a 10 mL ethanolic solution of alnine (0.93 g, 10 mmol) was added in the stoichiometric ratio of 1:1 respectively. The reaction mixture was stirred for 5 h at 50° C and monitored by taking TLC at regular intervals. The resulting clear solution was then reduced to half the volume and then allowed to stay at room temperature. The orange colored crystalline product was then filtered off, washed with least amount of ethanol and diethyl ether then dried in vacuum over fused calcium chloride for further use.

Colour (Orange) Yield: 80%. Elemental Anal. Calc. for C29H21Cl2CoN2O3: C, 60.44; H, 3.85; N, 4.86; Found: C, 60.31; H, 3.87; N, 4.78. IR (KBr Pellet, cm⁻¹): 1161 (O=C), 1730 (C=O amide), 2932 (CH of DBM), 1868 (C=O). 1H NMR (DMSO-d6, δ, ppm): 4.96 (s, 1H, NH), 6.65–7.49 (m, 9H, ArH). 13C NMR (DMSO-d6, δ, ppm): 117.35 (C=O), 128.70 (CH of DBM), 183.8 (C=O). Colour (Yellow) Yield: 70%. Elemental Anal. Calc. for C29H21Cl2CoN2O3: C, 61.92; H, 4.12; N, 4.86; Found: C, 60.62; H, 3.97; N, 4.75; IR (KBr Pellet, cm⁻¹): 1161 (O=C), 1722 (C=O amide), 2928 (CH of DBM), 1868 (C=O). 1H NMR (DMSO-d6, δ, ppm): 4.96 (s, 1H, NH), 6.65–7.49 (m, 9H, ArH). 13C NMR (DMSO-d6, δ, ppm): 117.35 (C=O), 128.70 (CH of DBM), 183.8 (C=O). Mass spectrum (ESI) [M + H]+ = 563.50.

4.2.2. Synthesis of mixed ligand complexes

To a 20 mL hot ethanolic solution of dibenzoylmethane (0.45 g, 2 mmol), NaOH (2 mmol) was added. After 15 minutes to the flask was added an ethanolic solution of Schiff base ligand (0.44 g, 2 mmol) and then ethanolic solution of [MCl₄]₂NHzH₂O (2 mmol) was added drop wise with constant stirring. The reaction mixture was refluxed for 5–6 h at 80° C under nitrogen atmosphere. The reaction mixture was then cooled at room temperature. The product was filtered off, washed several times with ethanol and diethyl ether then dried in vacuum over fused calcium chloride.

| [Cu(dlm)]LCl₅H₂O | (mlc1) |
|------------------|--------|
| Colour (Yellow)  | Yield: 70%. Elemental Anal. Calc. for [Cu₂(C₂O₄)Cl₃]: C, 60.44; H, 4.35; N, 4.86; Found: C, 60.35; H, 3.97; N, 4.84; IR (KBr Pellet, cm⁻¹): 1161 (O=C), 1722 (C=O amide), 2928 (CH of DBM), 183.8 (C=O). 1H NMR (DMSO-d6, δ, ppm): 4.96 (s, 1H, NH), 6.65–7.49 (m, 9H, ArH). 13C NMR (DMSO-d6, δ, ppm): 117.35 (C=O), 128.70 (CH of DBM), 183.8 (C=O). Mass spectrum (ESI) [M + H]+ = 563.50. |

| [Co(dlm)]LCl₅H₂O | (mlc2) |
|------------------|--------|
| Colour (Yellow)  | Yield: 68%. Elemental Anal. Calc. for [Co₂(C₂O₄)Cl₃(NH₃)]: C, 60.44; H, 4.35; N, 4.86; Found: C, 60.35; H, 3.97; N, 4.84; IR (KBr Pellet, cm⁻¹): 1161 (O=C), 1722 (C=O amide), 2928 (CH of DBM), 183.8 (C=O). 1H NMR (DMSO-d6, δ, ppm): 10.28 (s, 1H, NH), 6.65 (1H, CH), 6.28–7.52 (m, 9H, ArH). 13C NMR (DMSO-d6, δ, ppm): 151.15 (C=O), 163.52 (C=O), 138.8 (C=O, C=O of DBM), 183.8 (C=O of DBM), 112.0–133.4 (Ar C's), Mass spectrum (ESI) [M + H]+ = 599.2. |

| [Ni(dlm)]LCl₅H₂O | (mlc3) |
|------------------|--------|
| Colour (Reddish Brown) Yield: 72%. Elemental Anal. Calc. for [Ni₂(C₂O₄)Cl₃]: C, 62.46; H, 4.15; N, 5.02; Found: C, 61.39; H, 3.98; N, 4.96; IR (KBr Pellet, cm⁻¹): 1580 (s, C=O amide), 1648 (s, C=O). 3130 (s, N-H), 3468 (s, O-H), 1348 (s, N=C), 1287 (s, C=O). 565 (s, Ni-O), 488 (s, Ni-N). 1H NMR (DMSO-d₆, δ, ppm): 10.97 (s, 1H, NH), 6.68 (1H, CH), 3.62 (s, 2H, H₂O), 6.32–7.60 (m, 19H, ArH). 13C NMR (DMSO-d₆, δ, ppm): 151.43 (C=O), 164.92 (C=N), 143.4 (C-N=C), 93.1 (CH of DBM), 185.2 (C=O of DBM), 182.1 (C=O of DBM), 111.2–130.0 (Ar C's), Mass spectrum (ESI) [M + H]+ = 558.70. |

4.3. Microbiological analysis

4.3.1. Strains, media and chemicals

In this study, along with one laboratory strain (Candida albicans SC5314), three fluconazole (FLC) susceptible (4175; 4179; 4180) and three FLC resistant (4085; 4122; 4135) clinical strains of C. albicans were used. Clinical Candida strains were isolated from either HIV positive patients or patients with other immunocompromised conditions that were attending clinics at the Charlotte Maxeke Johannesburg Academic Hospital, Johannesburg, South Africa. All the strains were maintained on Sabouraud Dextrose (SD) Agar (Sigma Aldrich, USA) and prior to experiments; cells were grown in fresh SD broth. Candida albicans used in this study were isolated from patients under the ethical clearance number M10102 obtained from the Human Research Ethics Committee, University of the Witwatersrand. Fluconazole was purchased from Sigma Fluke (USA) and stock solution of 2 mg/mL was prepared in sterile distilled water. All other chemicals and media were purchased from Sigma Aldrich and Merck.

4.3.2. Determination of Minimum Inhibitory Concentrations and Minimum Fungicidal Concentrations

Minimum Inhibitory Concentrations (MIC) and Minimum Fungidal Concentrations (MFC) of all the newly synthesized Schiff base ligand and its derivative complexes against all the tested C. albicans were determined by a serial dilution technique using Clinical and Laboratory Standards Institute (CLSI) guidelines M27-A3 [27]. Briefly, 100 μL volumes of the test compounds with a final concentration of 2000 μg/mL were added in the first row and were serially diluted. In addition, negative control (1% DMSO), sterility control (media only) and positive control (FLC) were also included in each test. After proper sealing, plates were incubated at 37° C for 24 h. After incubation, 400 μL/mL of p-iodonitrotetrazolium violet solution (INT) was added to each well (40 μL). Viable microorganisms interact with INT to create a colour change from clear to a red-purple colour. Thus, the lowest dilution with no colour change was considered as the MIC for that test compound. Further, MFC was determined by sub-culturing the test dilutions from each well without colour change on SD agar plates and incubated for 24 h. The lowest concentration that showed no fungal growth was defined as the MFC value. All the experiments were done in duplicate and all the results were expressed in μg/mL.

4.3.3. Combination of test compounds with FLC

Based on the MIC values, ligand and its mixed ligand complexes (mlc1-mlc3) were examined for the type of combination interaction with FLC by determining the Fractional Inhibitory Concentration Index (FICI), following the method described previous[28]. In each combination set, test compounds and FLC were added into a well in 1:1 ratio. Interactions were assessed on the basis of zero-interaction theory of Loewe additivity and FICI values were calculated as follows:

\[
\text{FICI} = \frac{\text{MICa tested alone} + \text{MICb tested alone}}{\text{MIC in combination}}
\]

where MICa is the MIC of the Schiff base derivatives and MICb is the MIC of FLC (FLC). Interpretations of FICI values were done as synergy when ≤0.5, additive between 0.5 and 1.0, indifferent between 1.0 and 4.0 and antagonistic when FICI values were >4.0.

4.3.4. Apoptosis study

To study the apoptotic effect of ligand and its mixed ligand complexes (mlc1-mlc3) against C. albicans, Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was...
performed by using In Situ Cell Death Detection Kit, Fluorescein (Roche Applied Science), as described previously [29]. Briefly, cells were exposed to MIC and ½ MIC values of all the test compounds followed by washing 3 times in PBS. Cells are then fixed with a fixative solution (4% paraformaldehyde in PBS pH 7.4) for 1 hour at 25 C. After fixation, protoplasts were prepared by digesting cell wall with lyticase in different washing steps in protoplast buffers pH 7.4 (buffer I - containing 1M sorbitol, 50 mM tris base, 10 mM MgCl2 and 30 mM DTT; buffer II – 1M sorbitol, 50 mM tris base, 10 mM MgCl2 and 1 mM DTT; buffer III – 1M sorbitol, 50 mM tris base and 10 mM MgCl2). Cells were harvested and washed thrice for 5 min each with buffer I (3 mL/g cells). Cells that were incubated in buffer II (5 mL/g cells; supplemented with lyticase) for 2 h at 25 C were then centrifuged and discarded the supernatant then incubated with buffer III (5 mL/g cells) for 20 min. Again, centrifuge to remove buffer III, then washed the protoplasts once with PBS and remove buffer III, then washed the protoplasts once with PBS and harvested and washed thrice for 5 min each with buffer I (3 mL/g cells).

Declarations

Author contribution statement

Mohammad Younus Wani: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.
Ovas Dar, Faisal Mohammed Aqlan: Performed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.
Mohammad Younus Wani: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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The authors declare no conflict of interest.

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