Synthesis, Characterization and Antimicrobial Studies of 3-Methoxy-4-Hydroxy Benzaldehyde & O-Phenylene Diamine Schiff Base Ligand and Its Mixed Ligand Metal Complex Synthesized by Microwave Assisted Method

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Abstract **- Benzimidazole Schiff base ligand of 3-methoxy-4-hydroxy benzaldehyde and o-phenylene diamine, acts as anti-bacterial and anti-fungal agent. Recent studies reveal their ability to exhibit anti-proliferative, anticancer, antioxidant properties in drug chemistry. In this work, 4-[1-(4-Hydroxy-3-methoxybenzyl)-2, 3-dihydro-1H-benzimidazol-2-yl]-2-methoxyphenol ligand and the chelating mixed acac complex of 4-[1-(4-Hydroxy-3-methoxybenzyl)-2,3-dihydro-1H-benzimidazol-2-yl]-2-methoxyphenol were prepared by microwave assisted, are reported. All the isolated Schiff base ligand and mixed acac metal complex were characterized by using IR, 1H NMR, UV-VIS and TGA/DTA analysis. The biological activities of all the isolated ligands and their corresponding mixed acac metal complexes have been used to screening against the microorganisms both gram positive and gram negative and the results were compared with standard.**

Index Terms **- Benzimidazole, Schiff base mixed ligand, 3-methoxy-4-hydroxy benzaldehyde, o-phenylene diamine, acetylacetonate (acac), antibacterial, antifungal and Gentamycin and Nystatin.**

INTRODUCTION

Schiff's base have been found to act as neutral chelating agents as well as negatively charged species during the complexation reactions. Schiff's base can bind with different metal centers involving coordination sites and allows successful synthesis of metallic complexes with interesting stereochemistry[1].

Benimidazole and its derivatives possess a wide variety of useful biological properties due to their structural similarities with the common nucleobases[2]. The imine group present in many natural and synthetic compounds have been reported to be crucial for their biological activities such as antibacterial and antifungal[3].

Vanillin (3-methoxy-4-hydroxybenzaldehdye) is a salicyaldehyde derivative with high biological and physiological effect in living organism. It is a weak inhibitor of tyrosinase[3] and displays both antimutagenic and comutagenic properties in E.Coli[4].

Literature reveals the intense study of 3-methoxy-4-hydroxybenzaldehyde and *o*-phenylenediamine with transition metals but with limited scope of biological studies. The ligand was synthesized in a microwave assisted method with limited solvent especially recycled solvents, following green chemistry. Condensation of vanillin (aldehyde) and *o*-phenylene diamine (amine) group was carried out using glacial acetic acid. Further, with metal acetylacetonates (*acac*), $M(acac)_x$ [M = Cu(II) x= 2] as precursor complex of the type ML_2, was prepared using this ligand and has been characterized. The Mixed acac-ligand complexes of Cu(II) containing vanillin-*o*-phenylene diamine Schiff base and metal acetylacetonate (*acac*) have been prepared by the ligand exchange reactions. Its spectroscopic studies included IR, ^{1}H NMR, UV and TGA which was correlated with literature data[4].

In our present study, we synthesized a single crystal of Schiff base compound and its purity was increased by conducting a repeated recrystallized process and the crystal growth was obtained through the method of slow evaporation of solution with ethanol as solvent medium at room temperature. In this paper, we focus on the microwave assisted synthesis, characterization and biological studies of ligand and its complex. All the synthesized compounds were then tested for determining their *invitro* antibacterial and antifungal activities against E.Coli and A.Niger respectively.

EXPERIMENTAL

2.1.1 Material and methods:

All the chemicals used in this project were of AR grade were obtained from Sigma-Aldrich private limited, Nice chemicals and SD-fine chemicals. Melting points were determined using a Gallenkamp melting point apparatus. IR Spectra are recorded using KBr disc on a FTIR Perkin Elmer spectrometer within the range of 4000-400 cm^{-1} and Shimadzu Japan (FTIR, 8400). The solid reflectance spectra of the compounds were recorded in UV-VIS spectrophotometer Perkin Elmer USA-model Lambsa 35, ^1H NMR with DMSO-d^6 was recorded on Bruker 400 MHz high resolution multinuclear FT-NMR. TGA studies using Perkin Elmer USA and Elemental analysis using Variomicro select. XRD data was collected.

2.1.2: Synthesis of ligand 4-[1-(4-Hydroxy-3-methoxybenzyl)-2, 3-dihydro-1 H-benzimidazol-2-yl]-2-methoxyphenol (HL):

O-phenylene diamine (1.08g, 0.01 mol) was taken in 100 mL round bottom flasks, to which 3-Methoxy-4-hydroxy benzaldehyde (3.04g, 0.02 mol) was added with ethanol as medium. The above mixture was placed in microwave oven to reflux for 5 min. The resulting Schiff base was separated as light brown crystals. The product obtained was filtered, washed and recrystallized using ethanol. 80% yield was obtained with melting point 180^0 C. Elemental analysis (CHN) showed % of elements closer value to theoretical value. %C 70.3(70.24), %H 5.77(5.32) and %N 7.33(7.45).

The above mixture was also refluxed by conventional method for 2 hours and the product obtained was filtered, washed and recrystallized.

2.1.3: *Preparation of Metal $(acac)_2$:*

Metal acetylacetonate was prepared by known method[5]. Copper sulphate (1.2g, 0.02 mol) was dissolved in 50 mL distilled water. A solution of sodium acetylacetonate was prepared by adding drop-wise sodium hydroxide (1N) solution of acetylacetone (10 mL, 10 g, 0.10 mol) until the oily emulsion formed dissolves. The copper salt solutions were added to this solution with stirring when light blue coloured crystals of copper acetylacetonate was separated, which was suction filtered and dried (M.P. 210^0 C yield 80%).

2.1.4: *Preparation of Copper (II) HL mixed ligand-acac complex:*

To an ethanolic solution (2.32 g, 0.01 mol) of 4-[1-(4-ydroxy-3-methoxybenzyl)-2, 3-dihydro-1H-benzimidazol-2-yl]-2-methoxyphenol (HL) was added (2.64 g , 0.01 mol) of copper acetylacetonate dissolved in 20 mL of ethanol, drop-wise, with constant stirring and continue for 20 minutes and the resulting mixture was refluxed in microwave oven for 5 minutes. The obtained copper metal complexes (dark brown crystals) were filtered, washed with small amount of ethanol and dried over calcium chloride (M.P. 250^0 C, yield 80%). Elemental analysis (CHN) showed % of elements closer value to theoretical value. %C 54.0 (53.6), %H 4.5 (4.88) and %N 4.8 (4.6).

The Cu(II) complex had a non-ionic character according to the molar conductivity measurments.

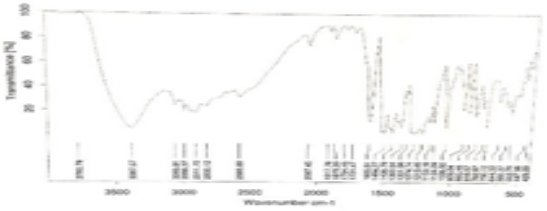
2.1.5: Biological activities:

The pharmacological activity of all the isolated Schiff base ligand and mixed ligand *acac* compounds were studied by screening were done *in vitro* cup diffusion methods.[6] The pharmacological activity, of the all isolated ligand and their metal complexes are molds were grown on sabouraud dextrose agar (SDA) at 25^0 C for 48 hours and determined by using agar well diffusion method and fungal growth were sub cultured on nutrient broth for their *in vitro* testing. 15 mL of molten SDA (45^0 C) was added to 100 µL volume of each compound having concentration of 100 µL/mL in the DMSO and poured into a sterile Petri plate. The solid appeared at the petri plate which poisoned agar plates were inoculated at the center with bacterial and fungal plugs (8 mm) obtained from activity growing colony and incubated at 25^0C for 48 hours. Diameter of the bacterial and fungal colonies was measured and expressed as present zone of inhibition.[7]

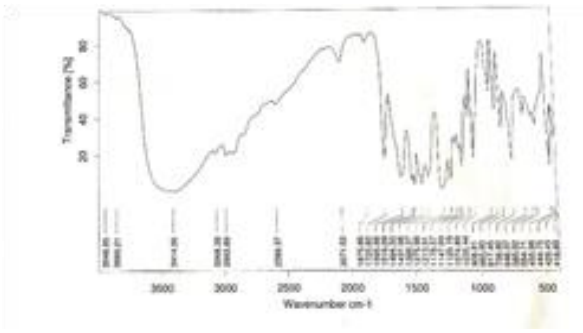
All the isolated ligands and their metal complexes were tested against the microorganisms such as, E.coli, S.Aureus for antibacterial and against A.niger and C.albicans for antifungal behaviors. These biological activities of all the compounds were compared with standard (Gentamycin and Nystatin) and control (DMSO). The minimum inhibitory concentration, *MIC*, value was defined as the lowest concentration of the chemical agent giving complete inhibition of visible growth.

RESULTS AND DISCUSSION

FT-IR spectra: FT-IR spectral data of the ligand and the complex are given in Table 2. The weak broad bands in the region 3300-3423 cm^{-1} due to hydrogen bonded OH group. This indicates that the phenolic oxygen atoms present in the Schiff bases are coordinated to the metal centres. The –OH band in HL changed significantly upon metal complexation, indicating deprotonation and subsequent involvement of the phenoxyl group in metal coordination as observed in Figure 1. The coordination of the phenolic oxygen atom could be supported by the appearance of medium- to-strong bands at a lower frequency region, 500 cm^{-1}, assignable to frequency (M-OC) vibration. The strong m(C=N) bands occurring in the range of 1794 cm^{-1} are shifted slightly toward lower frequency 1725 cm^{-1} compared to the free Schiff bases indicating the co-ordinated azomethine nitrogen atom to the metal centre. The strong band (N-H) occurring in the range of 3780 cm^{-1} are shifted towards lower frequency 3414 cm^{-1} compared to the free Schiff bases indicating the co-ordinated nitrogen to the metal centre. The appearance of a strong broad band at 3400 cm^{-1} in the Copper complex strongly supports the presence of coordinated water molecule. The coordination of oxygen atom could be supported by the appearance of medium-to-strong bands at a lower frequency region, 429 cm^{-1}, assignable to M-OC vibration. Data table 2 related to FTIR attached in supplementary file.



HL($C_{22}H_{20}N_2O_4$) IR



($C_{22}H_{20}N_2O_4$-Cu) IR

Figure 1: FTIR spectra of ligand and its complex

Electronic spectrum:

The electronic absorption spectra of metal complexes are recorded in DMF in the range 200 – 800 nm. The electronic spectrum of free Schiff base revealed three bands around 241, 350 and 450 nm characteristic of π-π * and n-π* transitions. In the metal complexes, this band is shifted to a longer wave length with increasing intensity. This shift may be attributed to the donation of lone pair of electrons of oxygen of Schiff base to metal ion[17]. The copper complexes exhibits bands around 255-300 nm, 350- 355 nm and 477-498 nm. The broad intense and poorly resolved bands around 350-355 nm may be assigned to LMCT or MLCT. The high intensity band around 250 nm is of ligand cause assignable to n-π* or π-π* transition. The complexes exposed shoulder broad bands in the range of 300-325 nm may be assigned to the d–d transition. The UV-Vis spectral data of the ligand and its complex are attached in supplementary file.

^{1}H NMR spectrum of ligand and its copper complex:

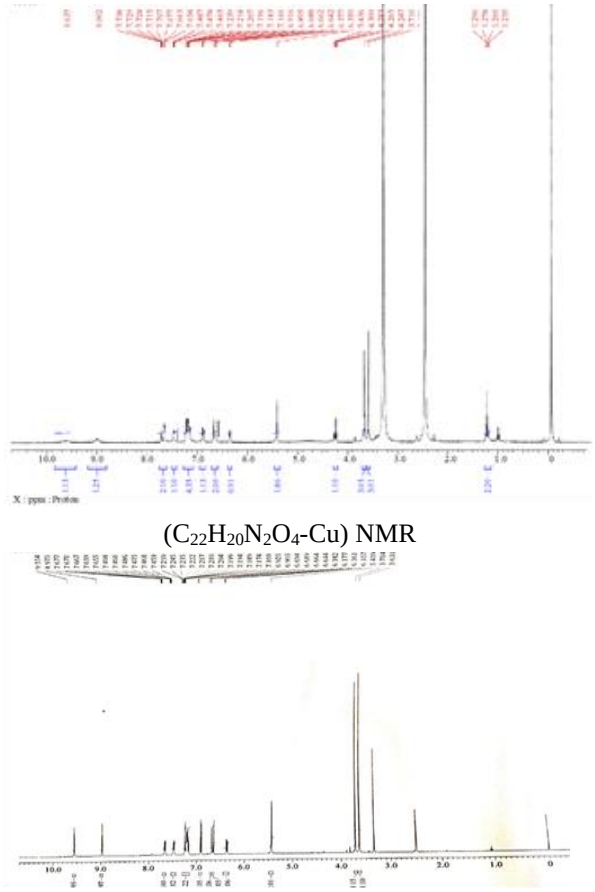
The ^{1}H NMR spectra of a representative Schiff base ligands and its mixed ligand acac-complexes are reported in the table 4. The ligand shows a resonance signal are about 7.75 δ corresponding to the resonance absorption of protons of the amide -NH group. The observed signals at about 8.4 δ corresponds to the azomethine protons of =CH group and signals at 10.8 δ corresponds to the hydrogens of the -OH groups of the ligand as observed in Figure 2. The multiplets centres at about 6.9 δ and 7.5 δ are attributed to aromatic protons.

In the proton NMR spectra of the metal acac-complex, the azomethine =CH signal is shifted to downfield, as expected, and appears at about 9.6 δ. However, the

resonance signals of the protons of the -NH group does not appear, has been shifted significantly. Whereas, the signals due to the protons of -OH group of the ligand have diminished in the spectrum of the metal complex indicating the deprotonated form of the ligand and enolization. The observed broad signals of the metal complex indicate the paramagnetic nature of the copper complex.

The ^1H NMR data provided in supplementary file.

HL ($C_{22}H_{20}N_2O_4$) NMR:



($C_{22}H_{20}N_2O_4$-Cu) NMR

Figure 2: ^1H NMR spectra of ligand and its complex

XRD studies:

The powder X-ray diffraction study was carried out, ligand is crystalline in nature. The Miller indices (hkl) along with observed and calculated dangles, 2θ values and relative intensities. Space group found to be P 21/c.

Ligand with reference number:XRD28/09/20200 31240.

The average crystalline sizes of the complexes dxrd were calculated using Debye Scherrer equation ($D = K\lambda/\beta Cos\theta$), where D=Particle size, K =Dimensionless shape factor, ƛ=X-ray wavelength (0.15406 Å), βfull width at half maximum of the diffraction peak, θ=Diffraction angle. In the XRD plot, values determined to be 2 θ= 13.63, FWHM = 0.06224, Particle size (D) = 126.74, hkl max observed to be 9, 20, 17 and HKL index values are 1.45, 7.69, 21.46 respectively.

Antimicrobial activity:

The results concerning the in vitro antimicrobial activity of the ligand and copper complex, together with the inhibition zone (mm) are presented in Table 5.

Procedure of the biological activities has been briefed in the section 2.1.5.

Table 5: Biological Activity of the Ligand and copper complex:

Sl No	Name	Antibacterial		Antifungal	
		E.Coli	S.Aureus	C.Albicans	A.Niger
1.	HL($C_{22}H_{20}N_2O_4$)	14 mm	13 mm	16 mm	15 mm
2	Cu(HL-acac).$2H_2O$ $C_{27}H_{29}CuN_2O_{10}$	19 mm	18 mm	19 mm	17 mm
3.	Control	08 mm	08 mm	08 mm	08 mm
4.	Standard	20 mm	20 mm	20 mm	20 mm

The pharmacological activity of all the isolated Schiff base ligand and mixed ligand *acac* compounds were studied by screening were done *in vitro* cup diffusion methods. The zone of inhibition around the bore were measured after 24 hr. The antimicrobial activities is classified as standards (=20 mm), highly active results were observed for complex both for antibacterial and antifungal activities.

Biological docking studies:

In silico docking analysis was performed between ligand HL and Gentamicin with APH(2″)-Ia of *Staphylococcus aureus*. The protein crystal structure was retrieved from the RCSB-PDB with the PDB id 5IQG in .pdb format. The protein was loaded to AutoDock vina(Trott & Olson, 2010) of the PyRx software for docking analysis. The structure of the ligands L_9 were drawn in Marvin sketch and saved in the .pdb format. Energy minimization was performed using the Open Babel (O'Boyle et al., 2011) in PyRx0.8. The grid box was set to the XYZ coordinates

of 35.69, -1.15 and 64.11 respectively and box dimensions were 18.62, 22.70 and 14.30 along the XYZ axis, respectively to cover the entire protein. The protein-ligand interaction of the conformation complex with the lowest AutoDock vina score was visualized using PyMOL 2.4 and interaction analyzed using LIGPLOT+ software (Laskowski & Swindells, 2011). These results are summarized in this paper. HL forms three hydrogen bonds with Asn378 (2.89& 3.21Å), Glu445 (2.98 Å) and Tyr448 (3.23 Å) as depicted. Gentamicin, the reference ligand forms two hydrogen bonds with Asp326 (3.30Å) and Tyr408 (3.31Å) as in Figure 3. Along with hydrogen bonding, ligands formed hydrophobic interactions where residues Asn373, Asp396, Val444, Tyr447, and Tyr448 are highly conserved in all interactions. Higher the number of hydrogen bonds betters the specificity of the ligand with the target protein (Klebe, 2013).

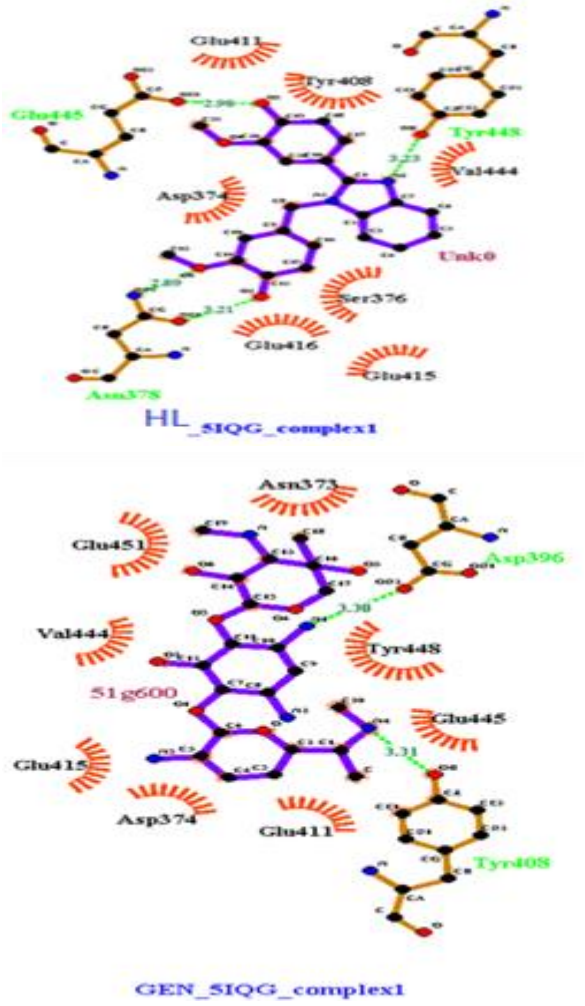


Figure 3: 2D schematic representation of interaction of 5IQG with Ligand. The hydrophobic interactions are represented as red semi-circles with spokes. Hydrogen bonds are represented by dotted lines.

Single crystal study of HL:

Bond precision: C-C = 0.0025 A

Wavelength=0.71073

Cell: a=7.9699(9) b=16.4422(18) c=14.3772(18) alpha=90 beta=95.222(4) gamma=90

Temperature:297 K

	Calculated	Reported
Volume	1876.2(4)	1876.2(4)
Space group	P 21/c	P 21/c
Hall group	-P 2ybc	-P 2ybc
Moiety formula	C_{22} H_{20} N_2 O_4	C_{22} H_{20} N_2 O_4
Sum formula	C_{22} H_{20} N_2 O_4	C_{22} H_{20} N_2 O_4
Mr	376.40	376.40
Dx,g cm-3	1.333	1.333
Z	4	4
Mu (mm-1)	0.093	0.093
F000	792.0	792.0
F000’	792.38	
h,k,lmax	9,20,17	9,20,17
Nref	3681	3659
Tmin,Tmax	0.983,0.987	0.614,0.747
Tmin’	0.982	

Correction method= # Reported T Limits: Tmin=0.614 Tmax=0.747

AbsCorr = MULTI-SCAN

Data completeness= 0.994 Theta(max)= 26.000

R(reflections)= 0.0488(3145)

wR2(reflections)= 0.1277(3659)

Moiety formula C_{22} H_{20} N_2 O_4

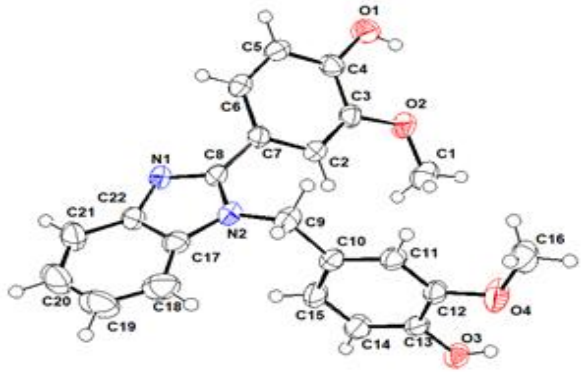


Figure 4

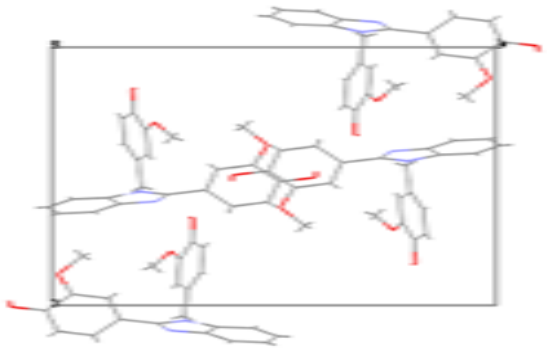


Figure 5

Figure 4: The molecular structure of the title compound showing 50% probability displacement ellipsoids and the atom-numbering scheme.

Figure 5: The crystal packing of the title compound, hydrogen bonds are shown as dashed lines.

In the title molecule, $C_{22}H_{20}N_2O_4$, the substituted benzene ring forms a dihedral angle of 7.6 (1) with the benzimidazole ring system. The second substituted benzene ring forms a dihedral angle of 92.9 (2) with the benzimidazole ring[18]. An intramolecular O—H---O hydrogen bond generates an S(6) ring motif. In the solid state, molecules are linked into chains along the [102] via intermolecular bifurcated O—H ---O hydrogen bonds, which generate R1 2 (5) ring motifs. The crystal packing is also consolidated by C—H interactions, and π– π stacking interactions between the imidazole and substituted benzene rings.

The synthesis of benzimidazole has received much attention owing to varies biological activities such as antidiabetic (Minoura et al., 2004), antimicrobial, antifungal (Pawar et al., 2004)[10], antiviral (Tomei et al., 2003)[11], antiHIV (Rao et al., 2003)[12] and anticancer (Demirayak et al., 2002)[13] properties exhibited by a number of derivaties of these compounds. Owing to the biological importance of the attached benzimidazole ring system, we report here the single-crystal X-ray diffraction study of 4-[1-(4-Hydroxy-3-methoxybenzyl)-2, 3-dihydro-1H-benzimidazol-2-yl]-2-methoxyphenol.

The bond lengths (Allen et al., 1987)[14] and angles in the title molecule (Fig. 2) are normal and are comparable to those observed in a closely related structure (Yeap et al., 2009)[15]. The dihedral angle between C9-N2-C8-C7 rings is 7.6.

The molecular structure is stabilized by an intramolecular O3-H3---O4 hydrogen bond which generates an S(6) ring motif (Bernstein et al., 1995)[16].

In the solid state, the molecules are linked via O3-H3---N1 and O1-H1---O3 bifurcated donor bonds into chains along the [102]. These hydrogen bonds form an $R_1^2(5)$ ring motif. The crystal packing is consolidated by C-H---π interactions involving the C7-C6 benzene ring, π-π stacking interactions between the C7-C6 (centroid C_g1) ring at (x+1, -y+3/2, z+1/2) and the N1/C8/N2/C17/C22 (centroid C_g2) ring at (x, y, z).

Hirshfeld Surface analysis report

Dnorm

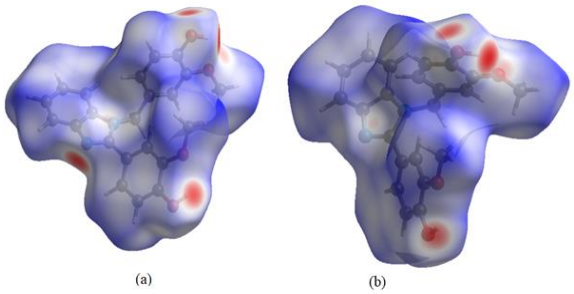


Fig. 6: Views of the Hirshfeld surface for (I) mapped with dnorm in the range -0.5985 to 1.4695 a.u.

The intermolecular interactions responsible for the crystal structure was investigated by performing three dimensional Hirshfeld surface analysis mapped over dnorm, shape-index and two-dimensional fingerprint plots using Crystal Explorer 17 (Turner et al., 2017). Bright red spots represented closer interatomic N-H…O and O-H…O interactions and has a negative *dnorm* value (figure 2) ; blue regions represented longer C…H, H…H contacts and a positive *dnorm* value (figure6(b)); while the white shades demonstrated the weaker C…C interactions equal to vdW separation with a *dnorm* value of zero (Journal of Taibah University for Science, Volume 11, Issue 1, January 2017, Pages 141-150, https://doi.org /10.1016/j.jtusci.2016.01.001).

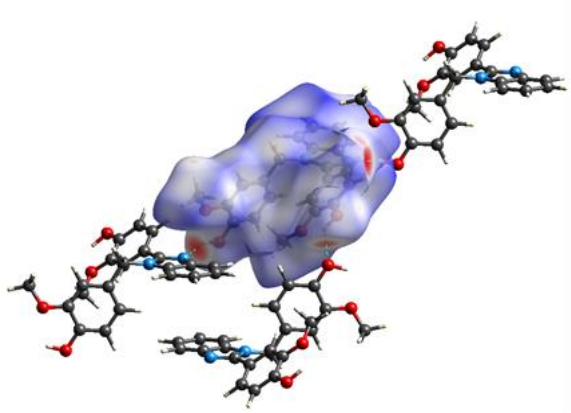


Fig 7: Crystal field surface mapped over *dnrom* showing close O-H…O and N…H-O interactions as bright red spots.

Shape index and Curvedness

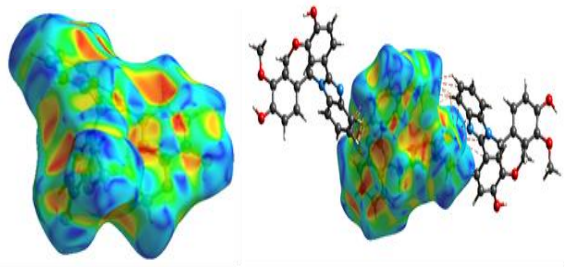


Figure 8: Hirshfeld surfaces mapped over the shape index in the range -1.0 to 1.0.

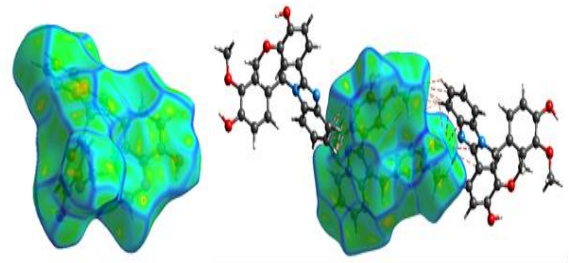
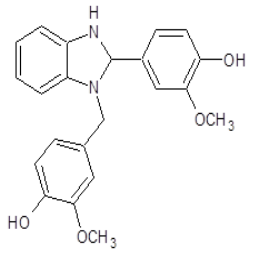


Figure 9: Hirshfeld surfaces mapped over the *Curvedness* in the range -4.0 to 4.0.

The *shape index* is sensitive to slight changes in the surface shape; the information conveyed by the *shape index* are consistent with 2D fingerprint plots. π—π interaction is indicated by adjacent red and blue triangles in the shape-index map and high planarity zone in the curvedness. The flat areas of the surface correspond to low values of *curvedness*, whereas sharp curvature areas correspond to high values of *curvedness* and usually tend to divide the surface into patches, indicating interactions between neighbouring molecules (*Crystal Structure, Hirshfeld Surface Analysis, Spectroscopic and Biological Studies on Sulfamethazine and Sulfaquinoxaline Ternary Complexes with 2,2'-Biquinoline. C. Villa-Pérez ; J.F. Cadavid-Vargas ; A. L. Di Virgilio ; G. Echeverría ; G.E. Camí and D.B. Soria, DOI: 10.1039/C7NJ03624H) , (Shamsuzzaman et al. / Journal of Taibah University for Science 11 (2017) 141–150, http://dx.doi.org/10.1016/j.jtusci.2016.01.001)*

Stoichiometry of the ligand is observed to be 2:1 ratio.



4-[1-(4-hydroxy-3-methoxybenzyl)-2,3-dihydro-1*H*-benzimidazol-2-yl]-2-methoxyphenol

Thermal Studies:

Thermal analysis (TGA and DTA) techniques are used to find out the decomposition of the metal complex. The complexes were heated in the temperature ranges room temperature to 1000 0C. The temperature range and the experimental peak shows that the weight loss with the decomposition reactions is discussed below.

The TG curve of both Cu(acac)2 shows a three-step decomposition pattern. The first step occurring at 120 0C is endothermic and corresponds to weight loss of 11% and is attributed to the loss of water of hydration. The second step, occurring at 260 0C, is exothermic and corresponds to weight loss of 60% is attributed to the loss of a more volatile acac ligand. The third step occurring between 420-430 0C is also exothermic and corresponds to weight loss of 80% and is attributed to the loss of remaining acac ligand to form the final product CuO.

CONCLUSIONS

In the present research study, we synthesized new ligand and complex of Cu(II) are characterized by various physicochemical and spectral analyses. The results exhibit that the synthesized ligand binds with metal ions in tetra dentate through N donor sites of Ortho- phenelyne diamines well as O atom of the vanillin group. IR, UV, 1H NMR, TGA and XRD studies of the complexes also helped to characterize the complexes. On the basis of all the above spectral studies, a six coordinated octahedral geometry for this complex has been proposed. The antibacterial data show that the metal complex has biological activity and cytotoxic properties. The Schiff base ligand and its complex were also tested for their antibacterial activity to assess their inhibiting potential against, Escherichia. Coli (as gram negative bacteria) and Staphylococcus aureus (as gram positive bacteria) using two different concentrations (5 and 10 mM). The

results showed the Cu(II) complex have the better rate in anti-bacterial activity with gentamicin as standard drug. Compared to ligands copper complexes are shown very good activity in both antibacterial and antifungal on comparing to the standard and control. Higher the number of hydrogen bonds betters the specificity of the ligand with the target protein. Conventional method of preparation of Schiff base observed to less efficient. The yield obtained was less with just 70% purity. Microwave assisted reflux proved to be faster, efficient, eco-friendly and better method for preparation of Schiff base. Mixed ligand complex of HL-acac copper showed better chelate effect than HL-Cu complex.

Supplementary Data

FTIR, UV-vis, XRD, TGA, NMR spectral data of both ligand and complex, docking values of ligand and hirsfeld images figures S3 to S7 are attached as supplementary data.

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