A COMPARATIVE STUDY ON THE ANTIMICROBIAL ACTIVITY OF MIXED LIGAND COMPLEXES OF SCHIFF BASES AND AMINO ACIDS WITH DIVALENT COPPER.

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Abstract
The mixed ligand Cu(II) complexes involving Schiff bases (L) and amino acids (AH):4-benzylimino-2,3-dimethyl-1-phenylpyrazal-5-one(L_1) and L-lysine (Lys); 4-N-dimethylaminobenzylimino-2,3-dimethyl-1-phenylpyrazal-5-one(L_2) and L-asparagine (Asn); benzyliminothiosemicarbarzide (L_3) and L-phenylalanine (Phe); and the three Schiff bases stated above, each with D-phenylalanine (D-Phe),have been synthesized and characterized by analytical and spectroscopic methods. All the complexes were screened for their in-vitro antimicrobial activity against bacteria viz. *E. coli, P. aeroginosa, S. aureus*, *B. subtilis* and fungi viz. *C. albicans* and *A. niger;* by agar-well diffusion method. The minimum inhibitory concentrations (MICs) of the mixed ligand complexes were determined by agar plate serial dilution method. The Cu(II) complexes possessed great antimicrobial activity with CuL_1Lys being the greatest antimicrobial. The MICs of the complexes was 0.25-0.50mg/mL. The mixed ligand complexes should find use in pharmaceutical formulations.

Keywords: Antimicrobial, comparative, Schiff bases, amino acids, mixed ligand, complexes

Introduction
There is evidence on the environmental origin of some clinically relevant resistant genes [1]. The existing resistance problem compelled researchers to look into possible modification of the existing arsenal that could confer improved activity [2].
Schiff bases have been used as fine chemicals and medical substrates [3,4]. Benzaldehyde and its derivatives have been reported to form a wide range of Schiff bases and metal complexes with a high degree of antimicrobial properties including antibacterial, antifungal, antiviral and anti-inflammatory activities. Also, complexes having pyrazolone derivatives have been found to possess enhanced antimicrobial activity when compared with the free ligand [5]. Generally, complexes of Cu have been found to show markedly increased biological properties [6].
Amino acids are used for a variety of applications in industry but they are used mainly as additives to animal feed. Lysine, methionine, threomine and tryptophan are most important in the production of these feeds [7]. In the industry, amino acids are also used to chelate metal cations in order to improve the absorption of minerals from supplements, which may be required to improve the health or productivity of these animals [8].The chelating ability of amino acids has been used in fertilizers for agriculture to facilitate the delivery of minerals to plants in order to correct mineral

deficiencies such as iron chlorosis. These fertilizers are also used to prevent deficiencies from occurring and improving the overall health of the plants [9]. The remaining production of amino acids is used in the synthesis of drugs and cosmetics [7]. Amino acids are important as low-cost feed stocks and are used in chiral pool synthesis as enantiometrically-pure building blocks [10] and have been investigated as precursor chiral catalysts for asymmetric hydrogenation reactions although no commercial applications exist [11].

There are lysine conjugates that show promise in the treatment of cancer, by causing cancerous cells to destroy themselves when the drug is combined with the use of phototherapy, while leaving non-cancerous cells unharmed [12]. Asparagine is required for development and function of the brain. It also plays an important role in the synthesis and metabolism of ammonia, which is toxic to the human body. The nervous system needs asparagine to maintain the equilibrium as well as in amino acid transformation. Deficiency of asparagine causes congenital microcephaly and a progressive form of encephalopathy [13]. The biological functions of D-amino acids remain unclear although it is generally known that some such as D-phenylalanine may have pharmacological activity.

The refuted analytical activity of DL-phenylalanine may be explained by the possible blockage by D-phenylalanine of enkephalin degradation by the enzyme carboxypeptidase A [14].

In the present study, the synergetic effect of two biologically important ligands viz. Schiff bases and amino acids; with divalent copper, for use in pharmaceutical formulations, is of great interest.

Materials and Methods

All chemicals were of AR grade and were used without further purification except ethanol which was re-distilled. 4-aminoantipyrine(99%), benzaldehyde (99%), 4-N-dimethylaminobenzaldehyde(99%), thiosemicarbazide (99%), L-lysine (98%), L-asparagine (98%), L-phenylalanine (99.9%), D-phenylalanine(99%), d_6-DMSO, d-chloroform and NaOH were purchased from Sigma Aldrich Inc.(U.S.A). $CuCl_2$ (99%), n-hexane, anhydrous $CaCl_2$, chloroform and dichloromethane were purchased from Merck AG (Germany).

Nutrient agar was procured from Tulip Diagnostics (P) Ltd (Belgium), sabouraud dextrose agar from Lab M Ltd (U.K), pure ciprofloxacin and fluconazole from Oxoide^{TM}, the test organisms: *E. coli,P. aeroginosa, S. aureus, B. subtilis, A, niger* and *C. albicans* were clinical isolates from University of Benin Teaching Hospital, Benin City, Edo State, Nigeria.

The melting point and molar conductivity of the Schiff base ligands and mixed ligand Cu(II) complexes were determined using Stuart scientific model SMPS melting point apparatus and Inolab digital conductivity meter Cond 720 WTW Series, respectively. The elemental composition of the complexes were determined using LECO CHNS-932 elemental analyzer and Buck scientific model 210 VGP atomic absorption spectrometer. Perkin Elmer Universal ATR Spectrum 100 FT-IR, Water Synapt GR Electrospray Positive Mass Spectrometer and TGA/DSC Q600 were used to determine the structures.

Synthesis of Schiff base ligands

The Schiff base ligands were synthesized using solid phase, green chemistry approach by grinding 1:1.2 moles of the respective aldehyde and amine in a mortar. The reactions were monitored by IR

spectroscopy and the products were crystallized and dried in vacuum [15-17].

Synthesis of mixed ligand metal complexes.

The mixed ligand metal complexes were synthesized by refluxing/stirring equimolar 1-phenylpyrazal-5-one(L_2) and L-asparagine(Asn); benzyliminothiosemicarbazide (L_3) and L-phenylalanine (Phe); and the three Schiff bases stated above, each with D-phenylalanine (D-Phe); with equimolar amount of NaOH, in an inert atmosphere of argon. The resultant precipitates were washed with water, dried in vacuum and stored in sample containers over anhydrous $CaCl_2$ in a dessicator [18-20].

Antimicrobial activity

The antimicrobial activities of the free ligands and mixed ligand metal complexes were tested on four bacteria: *E. coli*, *P. aeroginosa*, *S. aureus*, *B. subtilis* and two fungi*: A. niger and C. albicans*, using agar-well diffusion method. Ciprofloxacin, an antibacterial agent and fluconazole, an antifungal agent were used as control. Nutrient agar and sabouraud agar were prepared according to manufacturer's recommendation (28g/L) while bacteria and fungi concentrations were diluted according to Macfarland's standard [21].The resultant zones of inhibition were measured in millimeters. The minimum inhibitory concentrations (MICs) of the ligands and mixed ligand Cu complexes were determined using the agar plate serial dilution method in concentration range of 0.25 to 1.0mg/L [22].

Results and Discussion

CuL_1Lys: Colour: Marine blue. Yield: 84%. Mol. Wt. 499Decomp. Temp. 110^{o}C. Molar cond.($\Omega^{-1}cm^2mol^{-1}$): 36.50 Selected FTIR (cm^{-1}): 3214 $\nu(NH_{AH})$, 3149$\nu(CH_{Ar})$, 1601ν(CN), 1621 $\nu(CO_L)$, 1573$\nu(COO^-)$, 639ν(M-O), 399 ν(M-N).Anal. Calc. for CuL_1Lys (%): C, 57.66; H, 6.01; N, 14.01. O, 9.61, Cu, 12.71; Found: C, 57.54; H,6.02; N, 13.99 O, 9.72, Cu, 12.73$\Delta H_{decomp.}$(J/g) = +479.09

CuL_1D-Phe: Colour: Brown. Yield: 37%. Mol. Wt. 518 Decomp. Temp. 115^{o}C. Molar cond.($\Omega^{-1}cm^2mol^{-1}$): 48.10 Selected FTIR (cm^{-1}): 3332 $\nu(NH_{AH})$, 3032 $\nu(CH_{Ar})$, 1614ν(CN), 1655 $\nu(CO_L)$, 1593$\nu(COO^-)$, 466ν(M-O), 402ν(M-N). Anal. Calc. for CuL_1D-Phe (%): C, 62.49; H, 5.21; N, 10.80. O, 9.26, Cu, 12.25; Found: C, 62.38; H, 5.33; N, 10.81 O, 9.25, Cu, 12.23$\Delta H_{decomp.}$(J/g) = +795.18

CuL_2Asn: Colour: Light brown. Yield: 56%. Mol. Wt. 528 Decomp. Temp. 100^{o}C. Molar cond.($\Omega^{-1}cm^2mol^{-1}$): 27.80 Selected FTIR (cm^{-1}): 3380 $\nu(NH_{AH})$, 2795$\nu(CH_{Ar})$, 1586ν(CN), 1673 $\nu(CO_L)$, 1586$\nu(COO^-)$, 457ν(M-O), 391ν(M-N). Anal. Calc. for CuL_2Asn (%): C, 54.49; H, 5.49; N, 15.89. O, 12.11, Cu, 12.02; Found: C, 54.30; H, 5.75; N, 16.06 O, 11.88, Cu, 12.01$\Delta H_{decomp.}$(J/g) = +364.62

CuL_2D-Phe: Colour: Dark green. Yield: 80%. Mol. Wt. 561 Decomp. Temp. 95^{o}C. Molar cond.($\Omega^{-1}cm^2mol^{-1}$): 35.30 Selected FTIR (cm^{-1}): 3333 $\nu(NH_{AH})$, 2867$\nu(CH_{Ar})$, 1496ν(CN), 1614$\nu(COO^-)$, 1614 $\nu(CO_L)$, 515 ν(M-O), 402ν(M-N). Anal. Calc. for CuL_2D-Phe (%): C, 61.98; H, 5.70; N, 12.47; O, 8.55;Cu, 11.31; Found: C, 61.80;

H, 5.97; N, 12.48; O, 8.53;Cu, 11.22$\Delta H_{decomp.}$(J/g) = +120.95

CuL_3Phe: Colour: Dark green. Yield: 97%. Mol. Wt. 406 Decomp. Temp. 40^{o}C. Molar cond.($\Omega^{-1}cm^2mol^{-1}$): 64.10 Selected FTIR (cm^{-1}):3419ν(NH_L), 3145 ν(NH_{AH}), 1731ν(CN), 1602ν(COO^-), 1279ν(C=S) 800ν(M-O),421ν(M-N). Anal. Calc. for CuL_3Phe (%): C, 50.18; H, 4.67; N, 13.78. S, 7.87, O, 7.87, Cu, 15.62; Found: C, 49.99; H, 4.93; N, 13.84; S, 7.97; O, 7.64;Cu, 15.63$\Delta H_{decomp.}$(J/g) = +282.03

CuL_3D-Phe: Colour: Green. Yield: 85%. Mol. Wt. 406 Decomp. Temp. 40^{o}C. Molar cond. ($\Omega^{-1}cm^2mol^{-1}$): 56.00 Selected FTIR (cm^{-1}): 3244ν(NH_L), 3141 ν(NH_{AH}),1601ν(CN), 1601ν(COO^-), 1271ν(C=S) 751ν(M-O), 421ν(M-N). Anal. Calc. for CuL_3D-Phe (%): C, 50.18; H, 4.67; N, 13.78; S, 7.87; O, 7.87;Cu, 15.62; Found: C, 49.97; H, 4.94; N, 13.83 S, 7.95, O, 7.66, Cu, 15.65$\Delta H_{decomp.}$(J/g) = +208.60

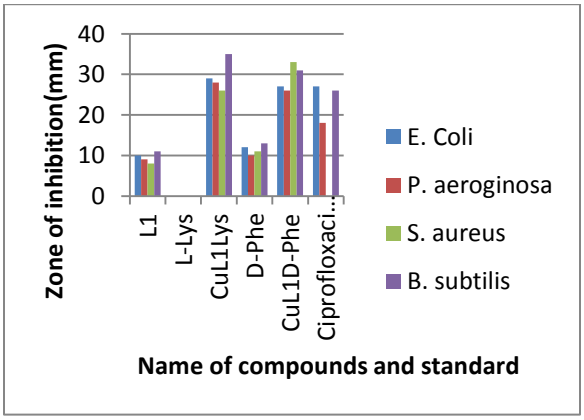

Fig. 1: Antibacterial activity of CuL_1Lys/D-Phe complexes.

The analytical data showed that the mixed ligand Cu(II) complexes were of the composition $[M(L)(AH)]^+$. All the synthesized complexes were coloured. They were insoluble in water and ethanol. The complexes did not decompose up to 110^{o}C for CuL_1Lys, 115^{o}C for CuL_1D-Phe; 100^{o}C for CuL_2Asn, 95^{o}C for CuL_2D-Phe and 40^{o}C for CuL_3Phe and CuL_3D-Phe. This result showed that CuL_1D-Phe was the most thermally stable of the complexes. In the mixed ligand complexes, Lys behaved as a bidentate ligand coordinating through the α-amino N and carboxylate O. Also the Schiff bases acted as bidentate ligands in the complexes, coordinating through the imine N and the carbonyl O, for L_1 and L_2 moieties and amine N for L_3 moiety. The decomposition pattern of the Cu(II) complexes were in good agreement with the results of elemental analysis. The molar conductance values 27.80-64.10 $\Omega^{-1}cm^2mol^{-1}$ of the complexes showed that they were non electrolytes [19,23]. The results of the antimicrobial properties of the complexes are shown in Figures 1-6 and Table 1 below.

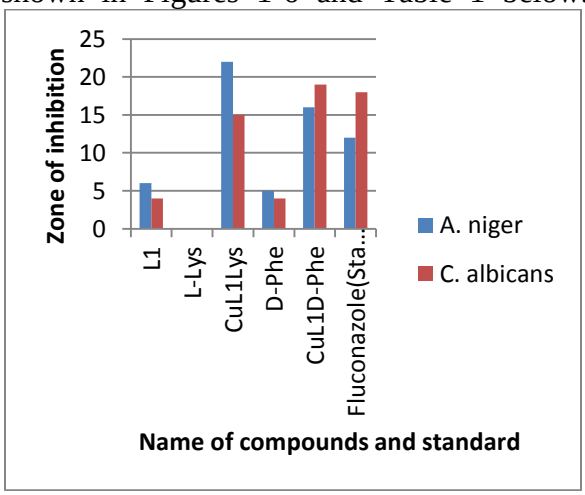

Fig. 2: Antifungal activity of CuL_1Lys/D-Phe complexes.

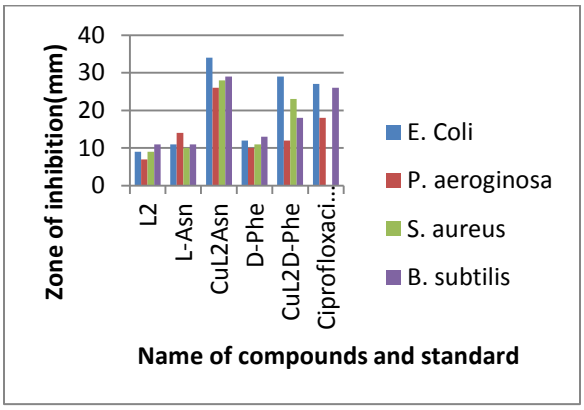

Fig 3: Antibacterial activity of CuL_2Asn/D-Phe complexes.

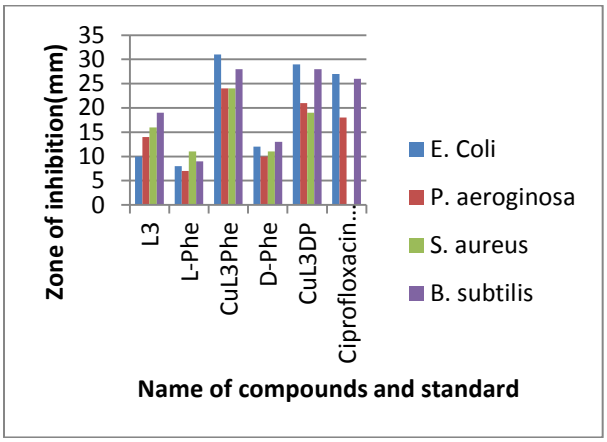

Fig.5: Antibacterial activity of CuL_3Phe/D-Phe complexes.

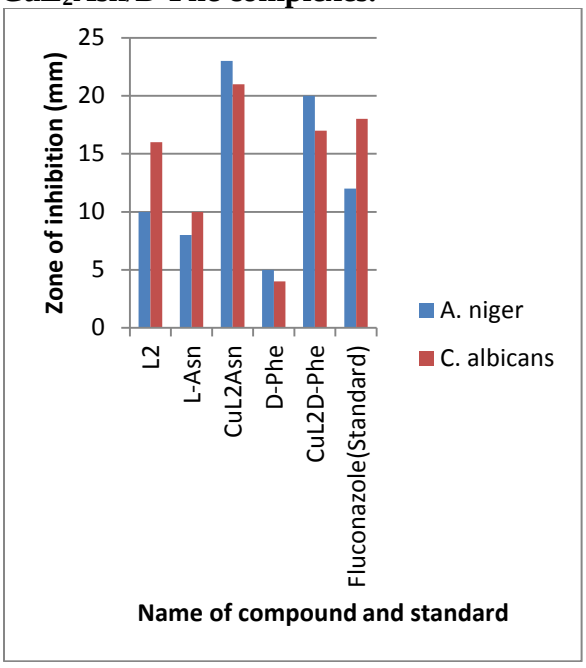

Fig.4: Antifungal activity of CuL_2Asn/D-Phe complexes.

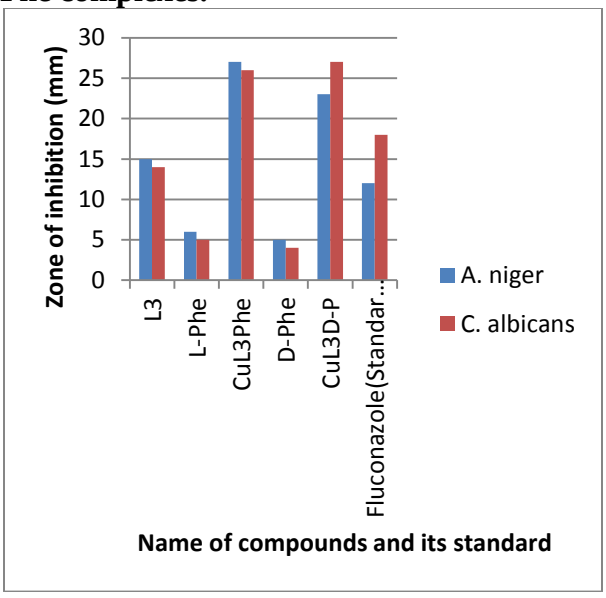

Fig.6: Antifungal activity of CuL_3Phe/D-Phe complexes.

Table 1: Minimum inhibitory concentrations of the Cu(II) complexes (mg/mL).

Complexes	*E. Coli*	*P. aeroginosa*	*S. aureus*	*B. subtilis*	*A. niger*	*C. albicans*
CuL_1Lys	0.25	0.25	0.25	0.50	0.50	0.50
CuL_1D-Phe	0.25	0.50	0.25	0.25	0.50	0.50
CuL_2Asn	0.25	0.50	0.25	0.25	0.50	0.50
CuL_2D-Phe	0.25	0.50	0.50	0.25	0.50	0.50
CuL_3Phe	0.25	0.50	0.50	0.50	0.50	0.50
CuL_3D-Phe	0.50	0.50	0.50	0.50	0.50	0.50

The results showed that the CuL_1Lys/D-Phe complexes possessed greater antibacterial characteristics than the free ligands [24] and their activities were comparably greater than ciprofloxacin, the standard drug, as shown in Fig. 1. It was observed that L-lys, which was inactive [25] was enhanced upon complexation and that CuL_1Lys was more active against *E. coli*, *P. aeroginosa* and *B. subtilis* while CuL_1D-Phe was more active against *S. aureus*. The comparable activities of the two Cu complexes of L_1 may be due to the electron donating effect of the respective side chains of both L-lys and D-Phe.

The antifungal result of CuL_1Lys/D-Phe complexes showed that CuL_1Lys was more active against *A. niger* as compared to CuL_1D-Phe, which was more active against *C. albicans*(as shown in Fig. 2). In general, the antifungal activities of the L_1Cu mixed ligand complexes were comparable to fluconazole and the activities of the free ligands were enhanced upon complexation.

The antibacterial result of CuL_2Asn/D-Phe complexes showed that the activities of the free ligands were enhanced upon complexation with CuL_2Asn possessing greater activities against all the test bacteria, as shown in Fig. 3. It should be noted that ciprofloxacin did not indicate any activity against *S. aureus* [26].

From the antifungal result (Fig. 4), though the activities of the free ligands were markedly enhanced upon complexation, CuL_2Asn was more active against *A. niger* and *C. albicans*, than CuL_2D-Phe which in turn was more active against *A. niger* when compared to the control drug.

The antibacterial activities of CuL_3Phe/D-Phe complexes (Fig.5) were comparable, however, CuL_3Phe was slightly more active against *E. coli*, *P. aeroginosa* and *S. aureus*. Also, the antifungal result of CuL_3Phe/D-Phe (Fig.6) showed that CuL_3Phe was slightly more active against *A. niger* while CuL_3D-Phe was more active against *C. albicans*.

A comparative study of the ligands and their complexes showed that the complexes exhibited higher antibacterial and antifungal activities than the free ligands [19]. The increased activities of the complexes can be considered in light of Tweedy's chelation theory [27] which emphasizes that chelation considerably reduced the polarity of the metal ion because of the partial sharing of its positive charge with the donor groups and the π-electron delocalization over the whole chelate ring. Such chelation could enhance the lipophilic character of the central metal atom, which subsequently favours its permeation through the lipid layer of the cell membrane. The nature of the substituents on the compounds and their positions equally played a vital role in evaluating the antimicrobial activities of the complexes. Also, it is obvious that the inhibitory action in L_3 could be attributed to the electron donating effect of the amine group and sulphur group. Also, it could be due to the presence of uncoordinated N-atom on the L_3 complexes, which may bind with the trace elements present in the microorganisms and inhibit their growth. The complexes penetrate the cell wall of the organisms, denaturing their lipoproteins thereby causing impairment in their normal cellular processes and leading to death. The slight differences in the activities of CuL_3Phe/D-Phe complexes may be attributed to their optical activities.

The MICs of all the complexes as shown on Table 1,ranged from 0.25 to 0.5mg/mL MICs are used by diagnostic laboratories mainly to confirm resistance but most often as a research tool to determine the in-vitro activity of antimicrobials [28] and data from such studies have been used to determine MIC breakpoints [29].

Conclusion

The Cu complexes were comparable in activity to the control drugs and the activities of the free ligands were markedly enhanced upon complexation. CuL_3Phe was most active against *B. subtilis* and *A. niger* while CuL_3DPhe was most active against *S. aureus* and *C. albicans*. In general, the complexes were good antibacterial and antifungal agents which should find use in pharmaceutical formulations.

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