ISSN 2063-5346

Section A-Research paper

Modulatory Role of Mixed Ligand Copper Complexes with Amino Acids, Coumarin-3-Carboxylic Acid and Nano Composite Formulation Against the Human Cancer Cell Line Mcf-7 Paradigms

Santosh Payghan[*1],Vaishali Kate[1], Atul Deshmukh[1],Sagar Satpute[2], Sanjeevani Desai[3],Pradip Deshmukh[4], Prashant Jain[5], Mahesh Bhat[5]

[1]Rajeshbhaiyya Tope College of B. Pharmacy, Aurangabad, Maharashtra

[2]Tatyasaheb Kore College of Pharmacy, Kolhapur, Maharashtra

[3] Krishna Institute of Pharmacy, Karad.

[4]Appasaheb Rajale College of Pharmacy, Ahmednagar

[5]Nuper Therapeutics, A division of Jain Pharmaceuticals, Pune

***Corresponding Author: Dr. Santosh Payghan**

Rajeshbhaiyya Tope College of B. Pharmacy,

Aurangabad, Maharashtra

E-mail: santos14july@gmail.com

ABSTRACT:

Copper mixed ligand complexes were created with the goal of using them as anticancer agents. Different physicochemical approaches were used to characterise the complexes, including elemental analysis, TG-DTA, 1H NMR, IR, and uv-visible spectroscopy. All compounds designated by physicochemical techniques have the molecular formula [Cu (Ala) (CCA)].2H2O, [Cu (Gly)(CCA)]. 2H2O [Cu (Ser)(CCA)].2H2O [Cu (Cys)(CCA)].2H2O [Cu (Ser)(CCA)].2H2O [Cu (Cys)(CCA)].2H2O [Cu (Ser)(CCA)].2H2O The particle size of all complexes was measured using NTA and found to be between 56 and 83 nanometres. The cytotoxic activity of all complexes on the MCF-7 cell line was determined using the MTT test technique, revealing that they are effective anti-breast cancer drugs. In comparison to alanine and glycine, the mixed ligand combination of serine and glycine was reported to have greater cytotoxicity against the MCF-7 cell line. It might be due to the free –OH and –SH groups found in serine and cysteine, which indicate appropriate DNA binding. We concluded that freshly manufactured mixed ligand complexes comprising amino acids had a larger cytotoxic impact than the CCA complex based on cytotoxic assessment results. It's possible that this is because the amino acids in the complexes have a high affinity for DNA.

Keywords: MCF-7 cell line, MTT, cytotoxicity, DNA, CCA and amino acids.

INTRODUCTION:

Cancer may begin practically anywhere in the billions of cells that make up the human body. Human cells normally expand and multiply (via a process known as cell division) to generate new cells as needed by the body. Cells die as they get old or injured, and new cells replace them. Low blood oxygen supply to the tumour and brief, constant, or fast malignant development may precede protracted spontaneous remissions of cancer. The main tumor's continuous or fast development, not the spread of additional metastases.[1]When a group of cells begins to grow abnormally and ignore the division principles of typical cells, cancer develops. These cancer cells are uncontrollable in their development and multiplication. Assemble a degree of self-contained tumor tissue.[2] However; the clinical treatment strategy is the most complex in terms of pharmaceutical distribution for therapeutic therapy. A pre-programmed delivery of drug at certain amounts is appropriate for a variety of therapeutic procedures, including cancer therapy. [3] In 2020, an estimated 19.3 million new cancer cases (18.1 million excluding

nonmelanoma skin cancer) would be diagnosed worldwide, with around 10.0 million cancer deaths (9.9 million excluding nonmelanoma skin cancer).[4] With an anticipated 2.3 million new cases (11.7 percent), female breast cancer has surpassed lung cancer as the most often diagnosed malignancy, followed by lung (11.4 percent), colorectal (10.0 percent), prostate (7.3 percent), and stomach (5.6 percent). With a projected 1.8 million fatalities (18 percent), lung cancer remained the top cause of cancer mortality, followed by colorectal (9.4%), liver (8.3%), stomach (7.7%), and female breast (6.9%) cancers. In transitioned vs transitional nations, overall incidence was 2- to 3-fold greater for both sexes, although mortality varied 2-fold for males and slightly for women. Female breast and cervical cancer death rates, on the other hand, were much higher in transitional nations than in transitioned countries (15.0 vs 12.8 per 100,000 and 12.4 vs 5.2 per 100,000, respectively). In 2040, there are estimated to be 28.4 million cancer cases worldwide.[5]The number of cancer patients has risen dramatically in recent decades across the world. The globe is predicted to experience roughly 20 million cancer cases in the next two decades. It is the second leading cause of mortality in industrialised nations, and the third major cause of death in developing countries. Cancer is a major health issue that our society is dealing with. Cisplatin is one of the anticancer moiety options available. Following the discovery of the coordination chemical cisplatin (cis-diaminedichloroplatinum (II)) and its anticancer effect.[6]A literature review found that platinum complexes interact with DNA via covalent bonding. Platinum complexes exhibit a variety of adverse effects, including acquired resistance and cytotoxicity in normal cells, due to the creation of covalent bonding. To address these issues with platinum complexes, researchers have turned their attention to the creation of novel non-platinum complexes as anticancer medicines, with the mechanism of such medications' interactions with DNA still being investigated. [7,8]According to the literature, compounds that engage with DNA by intercalation or partly intercalation rather than covalent bonding have greater anticancer activity, less side effects, and anticancer cell resistance.[9] Copper is a bio essential and biorelevant element that functions as a pharmacological agent as well as in biological systems. According to the literature review, copper (II)-based complexes are prospective anticancer and cancer inhibitory drugs. A variety of copper complexes have been proven to be effective anticancer medicines in vitro and in vivo. The copper complex [Cu (II) (pyrimol) Cl] was effective at DNA cleavage and had lethal effects on the L1210 murine leukaemia and A2780 human ovarian cancer cell lines. [10] A recent study of mixed ligand copper (II) complexes of diamines found that they had anticancer activity that is superior thancisplatin. According to the literature review, mixed ligand palladium (II) and platinum (II) complexes including amino acids have been shown to have anticancer properties. [11,12]Coumarin and its derivatives are well-known natural products with a diverse spectrum of biological properties. Anticancer activity has been seen in lanthanide complexes of coumarin derivatives such as bis(4-hydroxy-3-coumarinyl)-acetic acid, N,N0-bis(8-aceto-7-hydroxy-4-methylcoumarin)-ethylenediamine, and coumarin-3-carboxylic acid.[12]

MATERIAL AND METHODS:

Preliminary Investigation

Visual evaluation of organoleptic properties such as physical condition and colour of mixed ligand copper complexes was performed, as well as identification tests such as infrared spectra, ultraviolet spectra, XRD, and a few other physicochemical tests on samples of mixed ligand copper complexes to determine their identity, purity, and physicochemical character. [13] Melting point via capillary method is one of the most essential features for assessing a medicine's purity. The melting point of CCA was determined using the open capillary tube method. The Fourier Transform Infrared (FTIR) technology is effective for assessing pharmaceutical quality. The FTIR Spectrum's

principal peaks are related to the medication's chemical makeup. The FTIR spectra of mixed ligand complexes were recorded using FTIR (Bruker, Germany). Between wave numbers 4500 and 500 Cm-1, the spectrum was obtained.

Preparation of Mixed ligand copper complexes: [14]

The different ratios of ligands and copper complexes were prepared. **Copper acetate** *(1g Copper acetate was dissolved in 25ml hot water and diluted to 25 ml with ethyl alcohol to make a Copper acetate Solution.),* **Coumarin-3-carboxylic acid** *(0.950g coumarin-3-carboxylic acid was dissolved in warm ethyl alcohol and diluted to 100 mL with ethyl alcohol to make a ligand solution.),***L-Alanine** *(0.4404gL-Alanine was dissolved in 25mL boiling water to make AL-Alanine Solution.),* **L-Glycine** *(0.375gL Glycine was dissolved in 25mL hot water to make AL-Glycine Solution.),* **L-Cysteine** *(0.6057gL-Cysteinein 25mL hot water was used to make AL-Cysteine Solution.)* **L-Serine** *(0.5255gL Serine was dissolved in 25mL boiling water to make AL-Serine Solution.)*

Mixed metal-ligand complexes:[14, 15]

Copper acetate, amino acids, and Coumarin-3-carboxylic acid were mixed together to make the complexes. The complexes were synthesised in varied ratios (1:1:1, 1:1:2, 1:2:1,). The metal, amino acids, and coumarine -3- carboxylic acid were found to form complexes in the molar ratio 1:1:1 correspondingly.

Identification of mixes ligand complexes:

Fourier Transform Infrared (FTIR) Spectroscopy: [16]

The fundamental peaks relating to the chemical composition of the conjugate are seen in the FTIR spectrum. The FTIR spectrum was captured using an FTIR camera (Bruker, Germany). The spectrum was captured between wave numbers 4500 and 500 Cm-1.

UV Visible Spectroscopy: [17]

With matching quartz cells of 1 cm in width, a single beam UV-Visible spectrophotometer (Shimadzu, 1601) was employed. 2 mg of mixed ligand copper complexes were accurately weighed and diluted in 2 ml DMSO to make stock solutions. 1 ml of sample was taken from these stock solutions and diluted up to 10 ml to get concentration (100g/ml) solutions. The spectra of this stock solution (100g/ml) were obtained in a 1.0 cm cell in the region of 400-200 nm against a blank solvent DMSO. For subsequent research, the wavelength of maximum absorption was investigated.

Elemental Analysis: [18]

Thermos Finnigan Elemental Analyzer was used to determine the amounts of C, H, N, S, and (O) in the complexes (Model-FLASH EA-1112). The metal ion content of the complexes was determined using a gravimetric method. Carbon, hydrogen, nitrogen, sulphur, and oxygen percentages are calculated. This may be used to calculate the structure of mixed ligand complexes.

Thermal studies: [18]

The Perkin-Elmer Diamond TG-DTA equipment was used to perform simultaneous thermos gravimetric and differential thermal analysis (TG - DTA) of complexes. The 1 mg sample is transferred to a pan and heated at a rate of 10^{o} C per minute in a nitrogen environment using a programmed furnace. The weight loss and complicated decomposition are estimated.

Nuclear Magnetic Resonance (NMR): [16]

NMR was used to determine the NMR spectra of mixed ligand copper complexes (Mercury Plus 300MHz). It was decided to use a dry NMR tube that was devoid of cracks and chips. Based on the solubility of produced mixed ligand complexes, the solvent (DMSO) was chosen. In DMSO, a particular amount of mixed ligand complexes was dissolved. The clear sample solution was put into the NMR tube, which was then placed in the tube holder and examined.

X-Ray Diffraction: [19]
XRD was used to determine the XRD spectra of mixed ligand complexes (Riguku, miniflex). The quartz slide that was chosen was clean and dry. It is used to transfer a sample of complexes. In the XRD chamber, the slide was put in a slide holder and evaluated.

Nanoparticle tracking analysis: [19]
Using the Nano Sight LM10, the NTs of mixed ligand complexes were determined. In DMSO, a 10mg sample of complexes is dissolved. The sample was put in a cuvette with the appropriate dilutions and then kept in an analyser to measure particle size and distribution.

Solubility: [20]
In eppendorf tubes, 0.5 ml of DMSO was collected. The amount of mixed ligand complexes added to this solution was increased until the solution became saturated. The eppendorf tubes were then sealed and inverted for 24 hours in a rotary shaker. The sample's absorbance was measured, and the solubility was calculated. Water, methanol, ethanol, acetone, ethyl acetate, and DMSO were used to test the solubility of the compound.

Conductivity: [21]
The LT26 delux conductivity metre was used to measure the conductivity of mixed ligand complexes. A ten-milligram sample of complexes is dissolved in ten millilitres of DMSO. The instrument was set to 20 mhos and the readings were taken.

Anti-cancer activity: [8]
MTT assay was used to test the anti-cancer activity of the compound in MCF-7 cells (TATA MEMORIAL CENTRE, Advanced Centre for Treatment, Research and Education in Cancer (ACTREC), Kharghar, Navi Mumbai). In DMSO, the mixed ligand complexes dissolve. Different varieties of conc. are created, such as 10, 20, 40, and 80(g/ml). Three trials are carried out, with the average value being used as the final conclusion.

RESULT AND DISCUSSION

Preliminary Investigation:
Organoleptic tests were performed in order to determine the physical condition, and it was discovered that it was a solid state with a white color.The identification tests of CCA were performed by the capillary method. Melting point was observed in 189-192^{0}C range. From the results it is clear that the CCA is in the pure form.

Mixed ligand copper complexes:
A mixed ligand copper complexes were prepared by the different ratios (1:1:1, 1:1:2, 1:2:1,) of copper acetate Solution, Coumarin-3-carboxylic acid Solution, L-Alanine Solution, L-Glycine Solution, L-Cysteine Solution, L-Serine Solution. Among these ratios the 1:1:1 shows the desirable results and structures of formulated complexes were shows in figure 1.

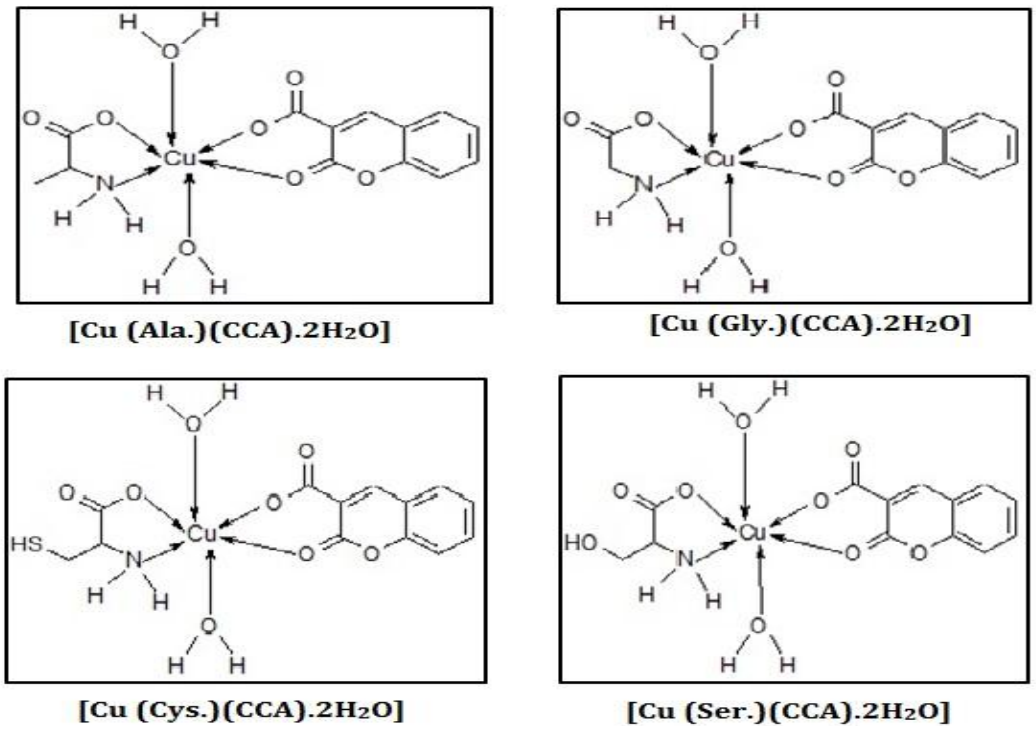

Figure 1 Structure of mixed ligand copper complexes

Identification of mixed ligand copper complexes

The synthesized complexes of copper, coumarin-3-carboxylic acid and amino acids viz. alanine, glycine, serine was bluish colored whereas the complex of cysteine was grey color. All the complexes were crystalline and thermally stable solid, signifying a possibility of strong metal ligand bond. The complexes were found to be soluble in DMSO and insoluble in methanol, ethanol, acetone and water. The complex was characterized by, elemental analysis, simultaneous Thermogravimetry- Differential Thermal Analysis (TG-DTA), Infra-red spectroscopy, Nuclear Magnetic Resonance (NMR spectroscopy), X-Ray diffraction (XRD), UV-visible spectroscopy and Nano particles Tracking Analysis (NTA) etc. The amount metal ion present in the complexes determined by gravimetrically.

Fourier Transform Infrared (FTIR):

The FTIR spectra of the metal complexes (Figures 2 to 5) recorded in KBr discs over the range 4000-400 cm^{-1}, were quite complex due to presence of numerous bands with varying intensities, making the task quite difficult. However, an attempt has been made to assign some of the important bands on the basis of reported infrared spectra of several N and/or O donor ligands, coumarin-3-carboxylic acid and their metal complexes. [13-17]

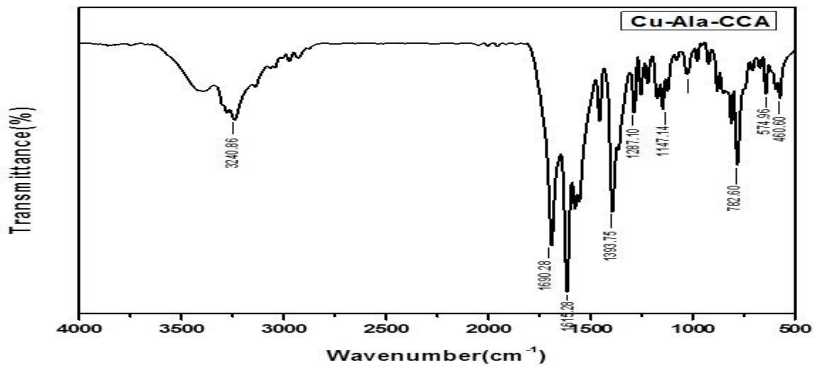

Figure 2: Experimental IR spectra of [Cu (Ala.) (CCA).2H$_2$O] complex

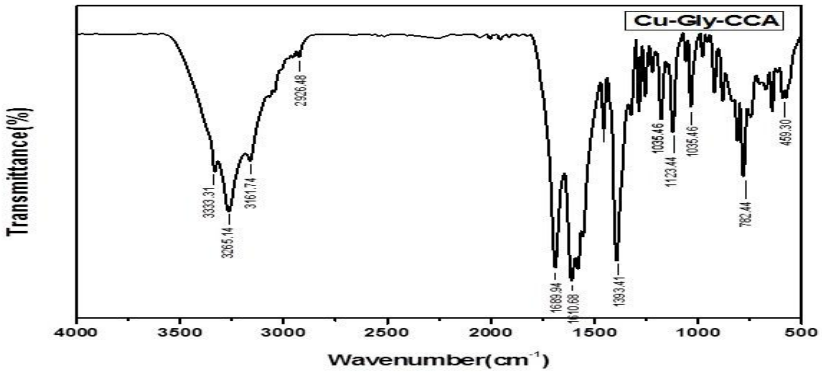

Figure 3: Experimental IR spectra of [Cu (Gly.) (CCA).2H_2O] complex

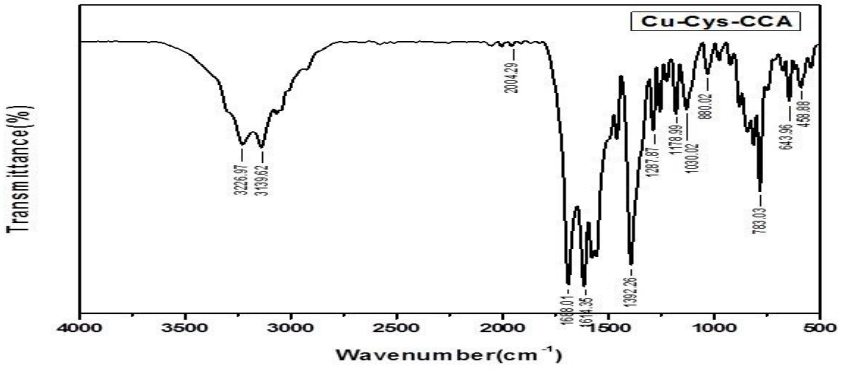

Figure 4: Experimental IR spectra of [Cu (Cys.) (CCA).2H_2O] complex

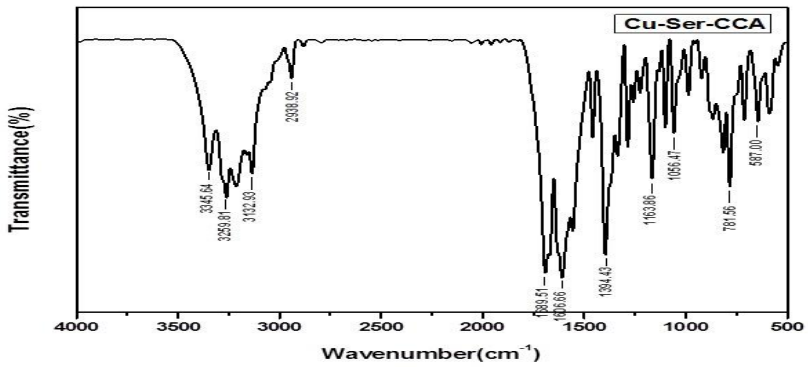

Figure 5: Experimental IR spectra of [Cu (Ser.) (CCA).2H_2O] complex

The observed differences between the spectra of free ligands and the metal complexes fall into three categories,

i. Spitting of single peak, I the free ligand into several closely spaced bands in the complex, which may be due to coordination to the metal ion.
ii. Change in the band intensities and appearance of new bands of relatively weak intensities. This may be due to reduction of the effective symmetry of the coordination. As a consequence, vibrations which were infrared inactive in the isolated ligands may become active in the coordinated ligand.
iii. Change in band positions of some vibrational bands of the ligands on the coordination indicates these bands are associated with the stretching vibrations of bonds involving the coordinated atoms.

The mode of bonding of the mixed ligand to Cu complexes was elucidated by the experimental IR spectra of the complexes as compared with the spectra of the free ligand (Table 1).

Table 1: IR spectral data of the copper complexes

Complex	[Cu (Ala) (CCA).2H_2O]	[Cu (Gly)(CCA). 2H2O]	[Cu (Cys)(CCA). 2H2O]	[Cu (Ser)(CCA). 2H2O]
(O-H)	3394	3333	3226	3345
(N-H)asym.(A. a)	3240	3265	3226	3211
(N-H)Sym.(A. a)	3160	3161	3139	3132
(C=O)xylic	1615	1610	1614	1606
(C=O)nylic	1690	1689	1688	1689
(H-O-H)H_2O	1556	1555	1555	1556
(C=O)F (A.a) asym	1456	1456	1458	1455
(C=O)F (A.a)sym	1393	1393	1392	1394
(C-C)	1147	1123	1129	1163
(C-O)	1287	1285	1287	1283
(C-N)(A.a)	1028	1035	1030	1056
(S-H)	-	-	616	-
(M-N)CCA &A. a	782	782	783	781

An important feature of infrared spectra of the metal complexes is the absence of band due to O-H stretching vibrations of either the O-H group of coumarin-3-carboxylic acid (CCA) or of the –COOH group of the amino acid.This observation leads to the conclusion that the complex formation takes place by deprotonating of hydroxyl group of CCA and carboxylic group of the amino acid moiety.A broad band observed at3400 -3300 cm^{-1} due to asymmetric and symmetric O-H stretching modes and a weak band in the range 1615-1555 cm^{-1} due to H-O-H bending vibrations indicating presence of a coordinated water molecule. Further it was confirmed by TG-DTA studies (Kostova et al, 2004) reported IR spectra of several metal complexes with CCA. The weak band at 3176 cm–1 in the IR spectrum of HCCA was assigned to the υ(O-H) vibration. Due to the intramolecular O-H-O hydrogen bond in HCCA, the υ(O-H) band is broad and shifted to lower frequency in comparison with free O-H frequency. Kostova et al [14] explained the binding mode of CCA to metal on the basis of detailed study of the IR frequencies of carboxylic C=O and carbonylic C=O group in the metal complexes. According to our DFT calculations of HCCA, the strong IR (medium in Raman) bands at 1746 and1685 cm^{-1}were assigned to the carboxylic υ(C=O) and carbonylic υ(C=O) modes, respectively, whereas the strongIR band at 1208 cm^{-1} was attributed to the carboxylic υ(C–O) mode. In the IR spectrum of mixed ligand Cu (II) complex, the carboxylic υ(C=O) modes are shifted to lower frequencies (1606, 1610, 1614, 1615cm^{-1}), while the carbonylic υ(C=O) band shows insignificant change (1690,1689, 1688, cm^{-1}).The observed carboxylic υ(C=O) shift to lower frequencies indicates that the bond length of longer C=O bond is longer in the mixed ligand complexes. The strong IR band observed at 1394, 1393 cm^{-1}in the IR spectra of all the mixed ligand complexes indicates that the C-C bonding in the CCA. The strong IR bands at 1287cm^{-1}were assigned to the carboxylic υ(C–O) modes. The IR spectra of the carboxylic C=O and the carbonylic C=O groups of HCCA and Cu in Cu-Amino acid-CCA

complexes showed that the bidentate coordination of CCA to Cu (II) through the carboxylic Oxygen and the carbonylic oxygen atoms whereas the C=O in the lactone ring do not take in the binding with Cu.Literature survey indicates that stability of amino acid complexes increases with the decrease in the N-H stretching vibrational frequency. [19-21] The Broad bands between at 3240 - 3130 cm^{-1} due to N-H (asymmetric) and N-H (symmetric) vibrations of free amino acid. The IR bands are shifted to higher frequency in the spectra of mixed ligand metal complexes suggesting co – ordination of the amino group through nitrogen with the Cu ion. The asymmetric vibrational frequency of υ(C=O) band of free amino acid was shifted to lower frequency in the range 1553– 1540 cm^{-1} and the symmetric mode of υ(C=O) in the free amino acids is also shifted to lower wave numbers 1393, 1396 cm^{-1} in the spectra of complexes indicating the coordination of carboxylic acid group via oxygen with metal ion. The strong band observed in the IR spectra at 1030 cm^{-1}due to the symmetric C-N stretching frequency of amino acids of complexes confirming co-ordination through the amino group of the amino acids.TheBroadband's observed at 3345 cm^{-1} due to the –OH stretching vibration in the spectra of Cu–Serine complexes indicates hydroxyl group of serine does not take part in the co-ordination. Similarly weak band observed at 2700 cm^{-1} and 670 $cm^{-1}$for the metal complexes of cysteine shows that Sulphur of S-H bond of the cysteine does not take part in the coordination. The spectra of copper complexes with amino acids and CCA show strong absorption bands in the range 990 – 773 cm^{-1}assigned to O-Cu-O and the M-N bond of the amino acids and CCA.

UV-Visible Spectroscopy:

The electronic data of mixed ligand copper complexes in DMSO solution recorded in the ultraviolet and visible region are summarized in Tables 2.

Table 2: UV-Visible spectral data of the copper complexes

Complex	λ nm	Proposed Assignments
[Cu (CCA)(Ala.).2H_2O]	282	Π ⟶ Π*
	320	n ⟶ Π*
[Cu (CCA)(Gly.).2H_2O]	267	Π ⟶ Π*
	320	n ⟶ Π*
[Cu (CCA)(Cys.).2H_2O]	286	Π ⟶ Π*
	318	n ⟶ Π*
[Cu (CCA)(Ser.).2H_2O]	283	Π ⟶ Π*
	318	n ⟶ Π*

The data indicates that the absorption peaks are bear close resemblance to those of the ligands. Therefore, it is concluded that during the formation of the complexes, there was not much structural changes observe in the ligand as shown in figures 6 to 9.

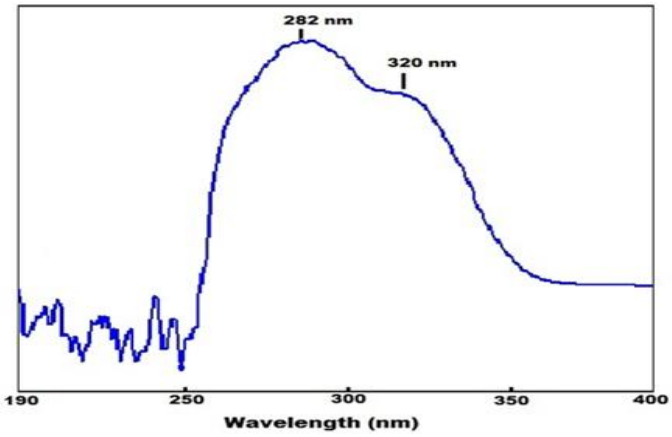

Figure 6: UV-visible spectra of [Cu (Ala.) (CCA).2H_2O] complex

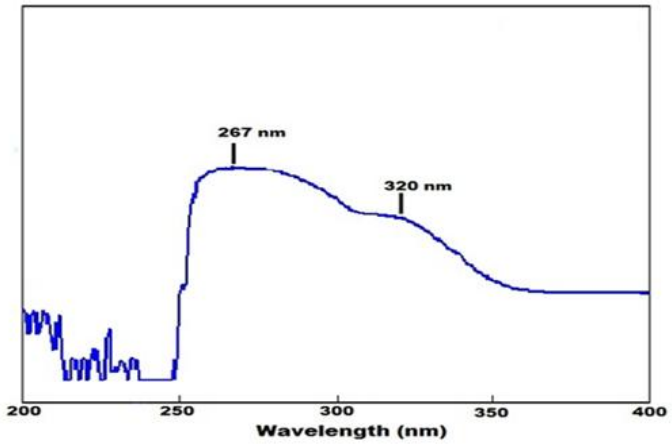

Figure 7: UV-visible spectra of [Cu (Gly.) (CCA).2H_2O] complex

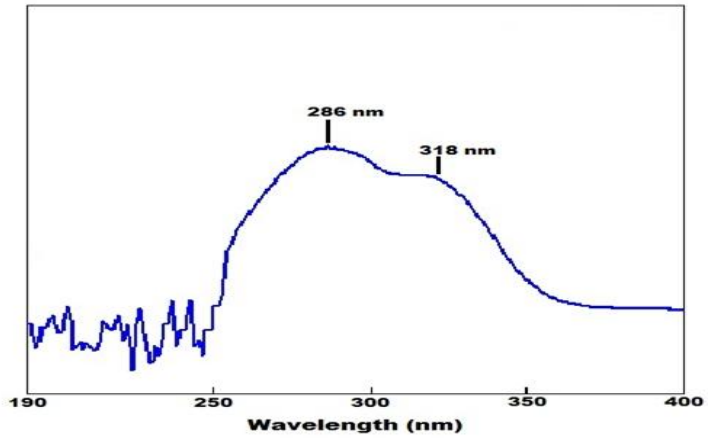

Figure 8: UV-visible spectra of [Cu(Cys.)(CCA).2H_2O] complex

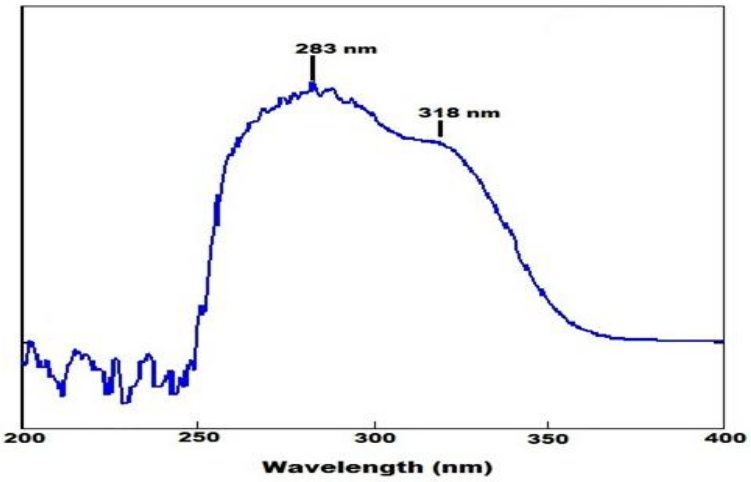

Figure.9: UV-visible spectra of [Cu (Ser.) (CCA).2H_2O] complex

The Π⟶Π* transitions in the metal complexes are observed at different positions suggesting that the Π electron system of the ligands undergo alteration to a varying extend on coordination with metal ion. The bands observed in the range 282-283 nm of the alanine, cysteine and serine complexes are assigned to the Π⟶Π* transitions of the electron of the unshared electron pair on hetero atoms present in the ligands. The band observed in the range of the 267 nm of the glycine complex for Π⟶Π*. The absorbance wavelength for the Π⟶Π* transition in the glycine complex decreases due to the strong bonding between glycine and Cu metal ion in the complex. In addition, the electronic spectra of the complexes also show charge n⟶Π* transition in the range 320 nm. These bands show lower wavelength may be assigned to the charge transfer.

Elemental analysis:

The complexes were analyzed for C, H, N, S, and (O) contents using Thermo Finnigan Elemental Analyzer (Model-FLASH EA-1112).

Table 3 Elemental analysis data of the copper complexes

Complexes	Elemental Analysis Observed (Calculated)					
	% H	% C	% N	% O	% Cu	% S
[Cu (Ala.) (CCA).2H_2O]	4.12 (3.98)	41.15 (41.40)	3.81 (3.71)	33.92 (33.97)	17.00 (16.85)	-
[Cu (Gly.)(CCA).2H_2O]	3.60 (3.58)	39.68 (39.70)	3.86 (3.85)	35.31 (35.29)	17.40 (17.50)	-
[Cu (Cys.)(CCA).2H_2O]	3.70 (3.66)	37.98 (38.16)	3.49 (3.42)	31.50 (31.31)	15.53 (15.54)	7.80 (7.82)
[Cu (Ser.) (CCA).2H_2O]	3.86 (3.82)	40.00 (39.72)	3.44 (3.57)	36.50 (36.67)	16.20 (16.16)	-

The data of the elemental analysis of the complexes obtained are presented in Table 3. The percentage composition of elements present in the complexes was also calculated using empirical formula of the complexes. The data indicates that there was good agreement between the calculated and the observed values.

Thermal analysis:

The simultaneous TG-DTA curve of mixed ligand complex of copper, alanine and coumarin-3-carboxylic acid (Cu-Ala-CCA) was recorded in the nitrogen atmosphere at a heating rate 10^{o}C/min is shown in figure 10.

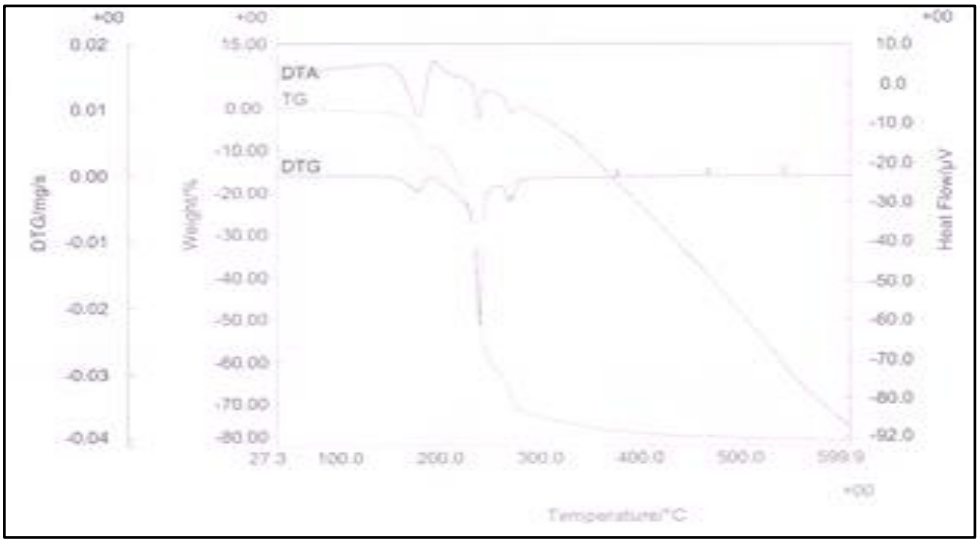

Figure 10: Simultaneous TG-DTA curve of [Cu (Ala.) (CCA).2H_2O] complex

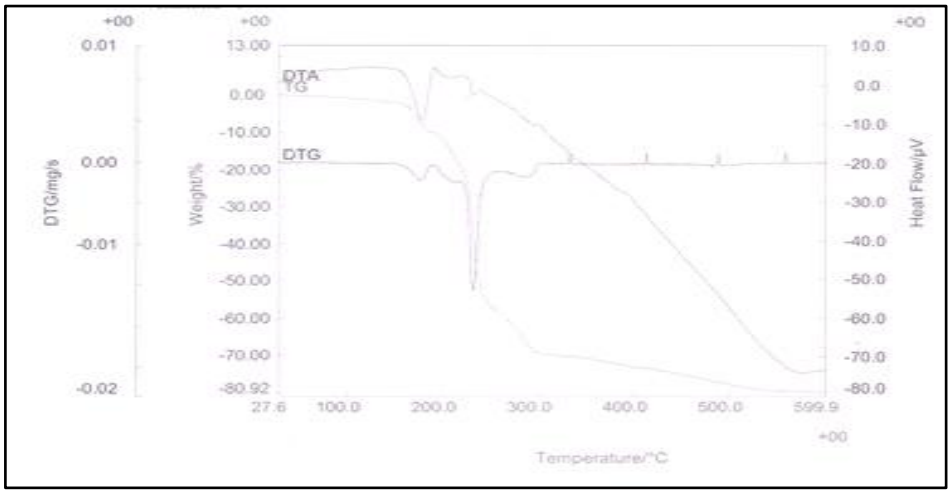

Figure 11: Simultaneous TG-DTA curve of [Cu (Gly.) (CCA).2H_2O] complex

The TG curves of the Cu-Ala-CCA complex indicates that as temperature increases the decomposition of complexes takes place due to fragmentation and show considerable loss in weight. The TG curve indicates that complex was stable up to temperature 175^{o}C. Also, the TG curve indicates that decomposition of Cu-Ala-CCA complex was takes place in three steps. In the first step, weight loss observed in the temperature range of 175^{o}C-200^{o}C may be due to loss of two water molecule. The TG illustrates further loss in weight in the temperature range of 200^{o}C -400^{o}C, which may be due to decomposition of comarin-3-carboxylic acid and alanine. The TG curve indicates that the decomposition of

the complex complete at 400^{o}C. After final decomposition of the complex, the product was CuO. The DTA curve of Cu-Ala-CCA was shown in the figure 11. The DTA cure indicates that, the first broad endothermic peak observed between in the temperature range of 175-200^{o}C for the loss water molecule. The DTA curve also illustrates that the second sharp endothermic peak at the temperature 250^{o}C, due to the loss of the coumarine -3-carboxylic acid moiety. The third small sharp endothermic peak observed in the DTA between temperatures 270^{o}C- to 290^{o}C due to the loss of the alanine moiety present in the complex. From simultaneous TG–DTA curve it was confirmed that the stoichiometry of copper, alanine and coumarine -3- carboxylic acid in Cu-Ala-CCA is 1:1:1.

Also, the simultaneous TG-DTA curve of mixed ligand complex of copper, glycine and coumarine-3-carboxylic acid (Cu-gly-CCA) was recorded in the nitrogen atmosphere at a heating rate 10^{o}C/min is shown in figure 11 respectively. The TG curves of the Cu-gly-CCA complex indicates that as temperature increases the decomposition of complexes takes place due to fragmentation and show considerable loss in weight. The TG curve indicates that complex was stable up to temperature 160^{o}C. Also, the TG curve of Cu-gly-CCA complex was indicates that, the decomposition takes place in three steps. In the first step, weight loss observed in the temperature range of 160^{o}C- 190^{o}C may be due to loss of two water molecule. The TG illustrates further loss in weight in the temperature range of 200^{o}C -400^{o}C, which may be due to decomposition of comarin-3-carboxylic acid and glycine. The TG curve indicates that the decomposition of the complex complete at 400^{o}C. After final decomposition of the complex, the product was CuO.The DTA curve of Cu-Gly-CCA was shown in the figure 11. The DTA cure indicates that, the first broad and large endothermic peak observed between in the temperature range of 160-190^{o}C for the loss two water molecule. The DTA curve also illustrates that the second sharp endothermic peak at the temperature 240^{o}C, due to the loss of the coumarin-3-carboxylic acid moiety. The third small sharp endothermic peak observed in the DTA between temperatures 280^{o}C- to 290^{o}C due to the loss of the glycine moiety present in the complex. From simultaneous TG – DTA curve indicates that, stoichiometry of copper, glycine and coumarin-3-carboxylic acid in Cu-Gly-CCA is 1:1:1.Similarly, the simultaneous TG-DTA curve of mixed ligand complex of copper, serine and coumarine-3-carboxylic acid (Cu-gly-CCA) as well as copper, cysteine and coumarine-3-carboxylic acid was recorded in the nitrogen atmosphere at a heating rate 10^{o}C/min. These curves are not shown here but these curves also indicate that the stoichiometry of the complexes is (1:1:1).

Nuclear Magnetic Resonance (NMR):

The well resolved ^{1}H NMR spectra of mixed ligand copper complexes in DMSO are shown in Figure 12 to 15. The spectral data are given in Table 4.

Table 4: NMR spectral data of the copper complexes

Complex	ppm	Group assignment
[Cu (Ala.) (CCA).2H_2O]	1ppm(S), 3.5ppm, 4.3 ppm 7.3 -7.8 ppm	-CH_3 -NH_2 -C-H - aromatic hydrogen
[Cu (Gly.) (CCA).2H_2O]	2.7 ppm(S), 3.3 ppm, 7.3 -7.8 ppm	-CH_2 -NH_2 - aromatic hydrogen
[Cu (Cys.) (CCA).2H_2O]	2.7 ppm(S), 3.3 ppm, 4.3 ppm	-CH_2 -NH_2 -C-H

	1-1.2 ppm 7.3 -7.8 ppm	-SH - aromatic hydrogen
[Cu (Ser.) (CCA).2H_2O]	2.7ppm(S), 3.3ppm, 4.3 ppm 7.3 -7.8 ppm	-CH_2 -NH_2 -C-H - aromatic hydrogen

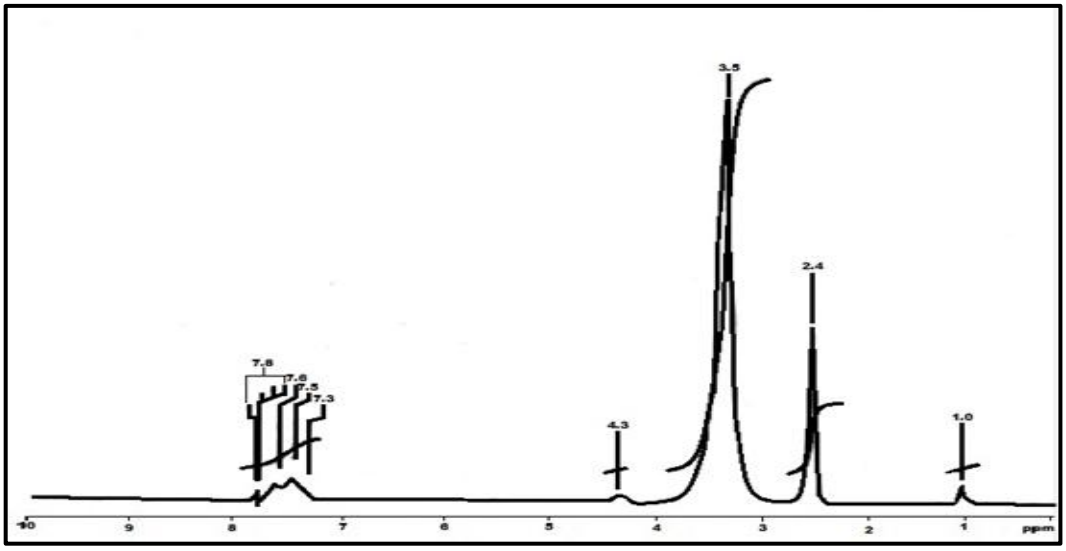

Figure 12: NMR spectra of [Cu (Ala.) (CCA).2H_2O] complex

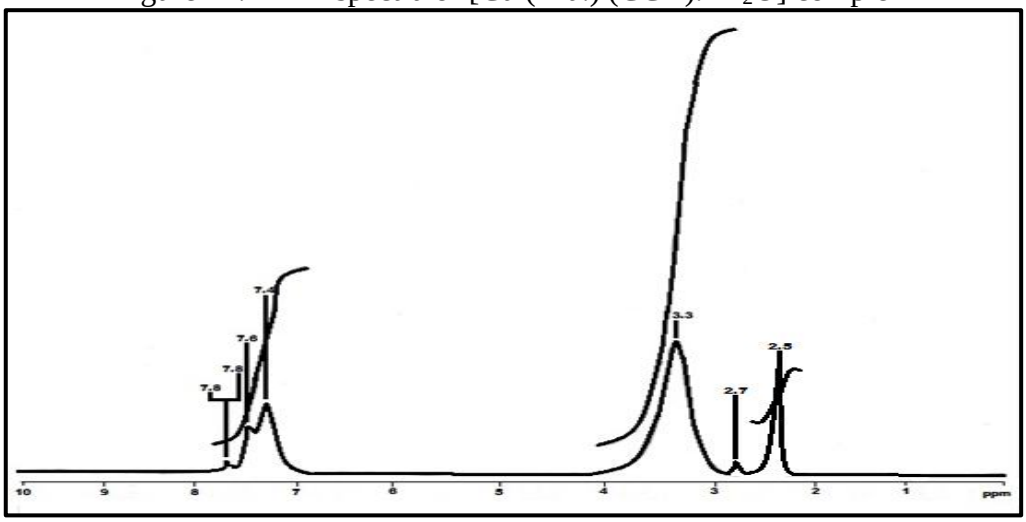

Figure 13: NMR spectra of [Cu (Gly)(CCA).2H_2O] complex

The ^{1}H-NMR spectra of -CH_2 in the amino acids ligands shows peaks as a singlet at 2.7 ppm. A downfield shift and broadening of peaks are observed in CH_2peak in the ^{1}H-NMR spectra of -CH_2 of amino acids in mixed ligand complexes. The shift in the CH_2peak in the spectra may be taken as an indication of coordination through the adjacent nitrogen and carboxylate oxygen.

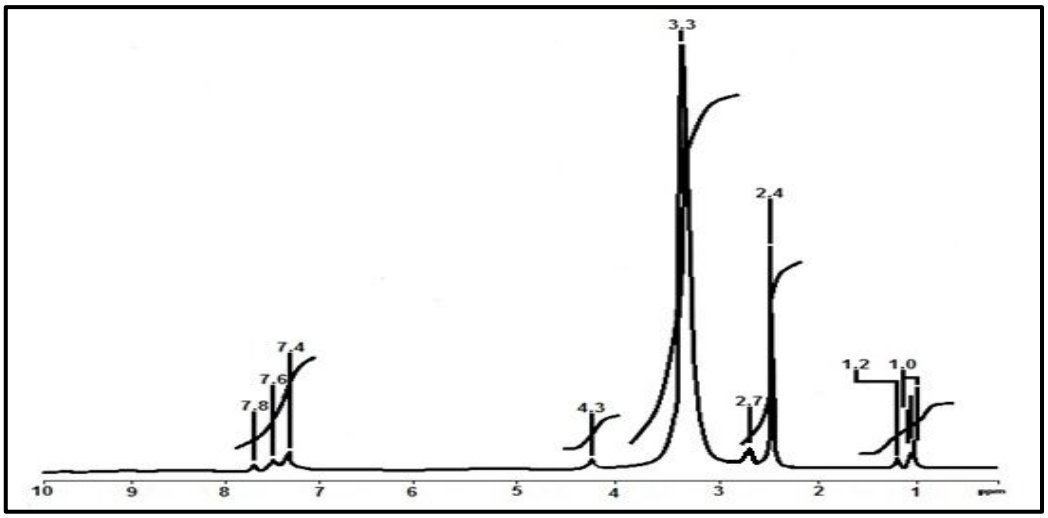

Figure 14: NMR spectra of [Cu (Cys.) (CCA).2H_2O] complex

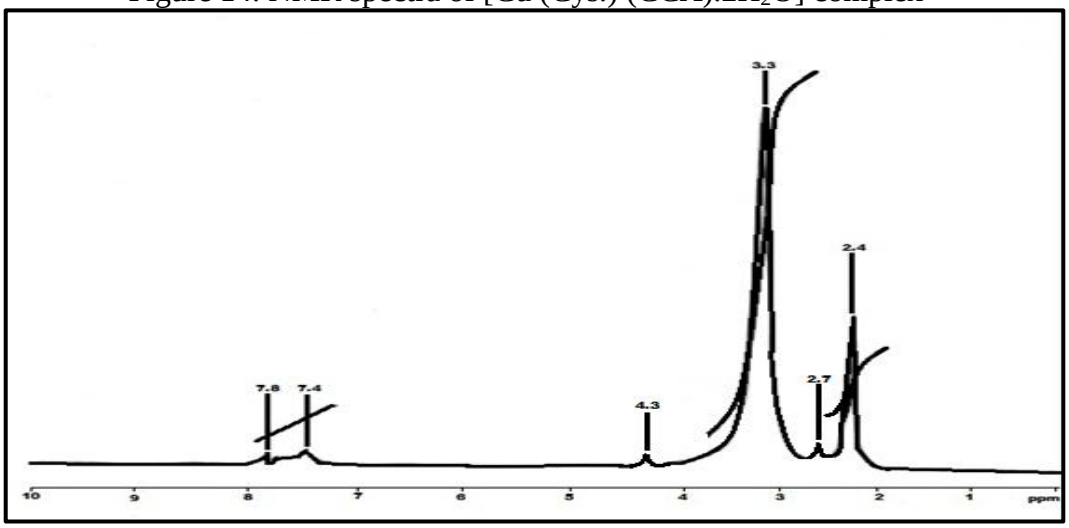

Figure 15: NMR spectra of [Cu (Ser.) (CCA).2H_2O] complex

The absence of the peak at above 9 ppm indicates that the deprotonation of the carboxylic group and hence the coordination of oxygen to metal. The peak observed in the NMR spectra of alanine, cysteine and serine complexes at 4.3 ppm as a singlet due to the C-H proton which was absent in the glycine complex. The peak at 3.3 ppm and 3.5 ppm in the spectra of all complexes indicates the H atoms of $–NH_2$ group of amino acids. The up field shifting of the peak from 5-6 ppm of free ligands to the 3.3-3.5 ppm in the complexes due to the nitrogen atom coordinates with metal ion.The peaks observed as a singlet at 1ppm in the NMR spectra of aniline complex, illustrates that the H of the free CH_3 group of aniline. The ^{1}H- NMR spectra of the cysteine complex shows that peak as a triplet at 1-1.2 ppm, indicates that, H atoms of S-H group of the cysteine moiety. The strong up field of the peak observed in the complex may be due to the decrease the electron density. The ^{1}H-NMR spectrum of all complexes shows five singles in the range of 7ppm to 8 ppm which is assigned to the protons of the CCA. The absence of the peak above 10 ppm in the spectra of the all complexes indicates that the carboxylate group coordinates with metal by deprotonation of carboxylic acid group. Based on these results the following structure is proposed for the mixed ligand complexes of Cu with amino acids and CCA.

ISSN 2063-5346 *Section A-Research paper*

X-Ray Diffraction:

XRD of the mixed ligand complexes are carried out between the ranges 20 -50, 2 Theta. X-ray powder diffraction patterns of the mixed ligand complexes Cu-amino acid-CCA are shown in the figure 16 to 19.

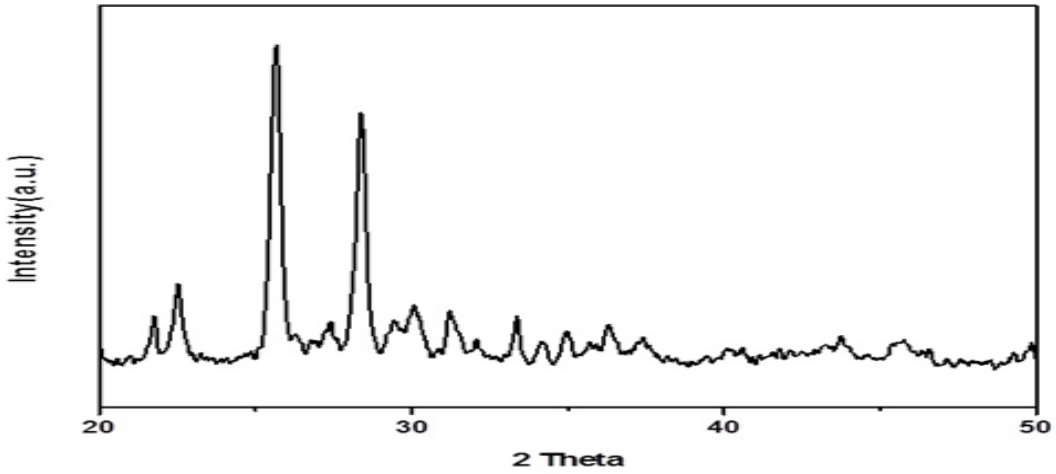

Figure 16: XRD pattern of [Cu(Ala.)(CCA).2H_2O]complex

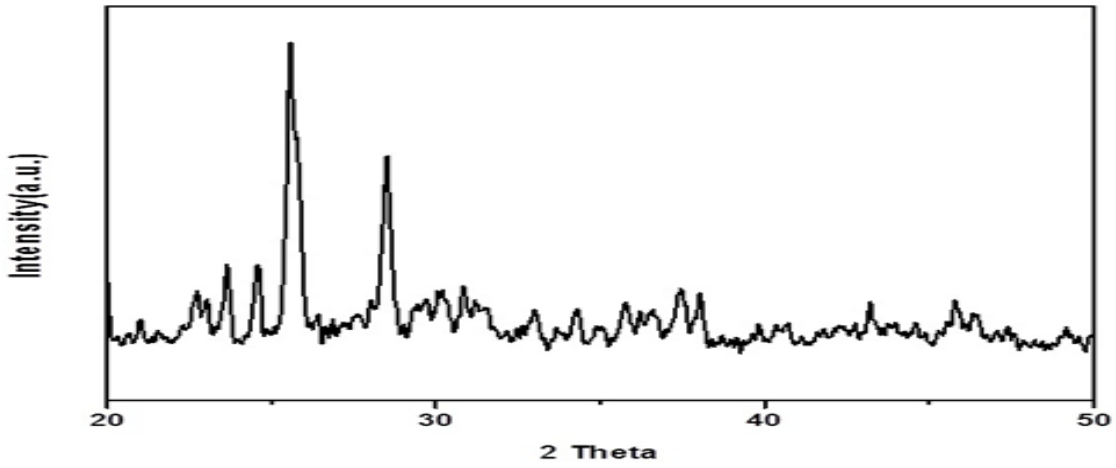

Figure 17: XRD pattern of [Cu (Gly.) (CCA).2H_2O] complex

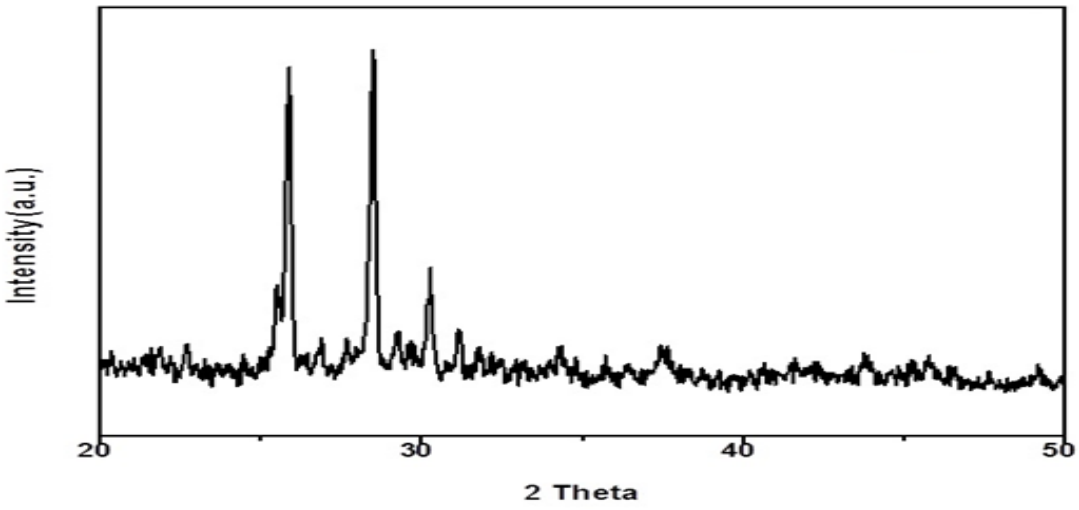

Figure 18: XRD pattern of [Cu(Cys.)(CCA).2H_2O] complex

ISSN 2063-5346 *Section A-Research paper*

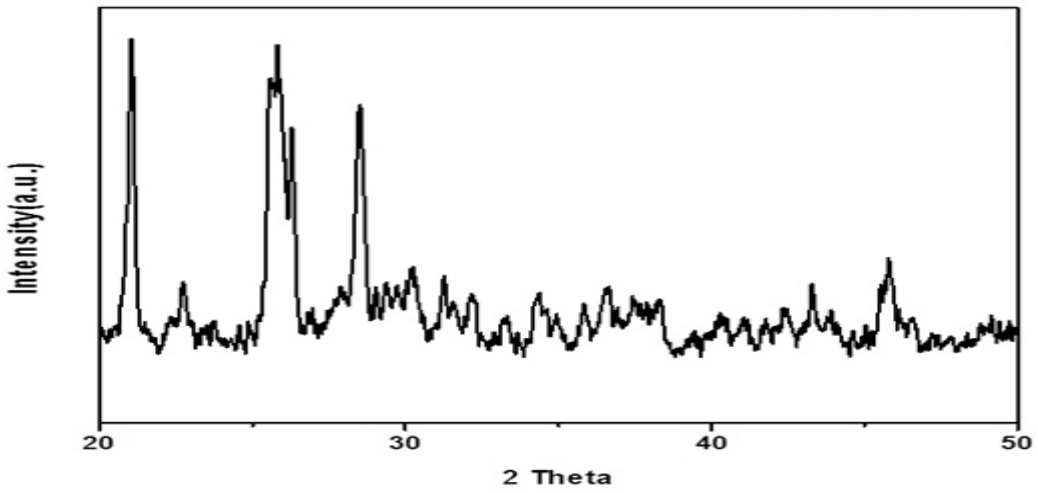

Figure 19: XRD pattern of [Cu (Ser.) (CCA).2H_2O] complex

XRD patterns of the mixed ligand complexes were carried in order to obtain an idea about the lattice dynamics of the complexes. The XRD pattern all complexes indicate that complexes have a definite structure which is not contaminated with starting materials. The XRD pattern of complexes illustrates that all complexes are crystalline one.

Nano Tracking Analysis:

The NTA data of all complexes are shown in figure 20 to 27.

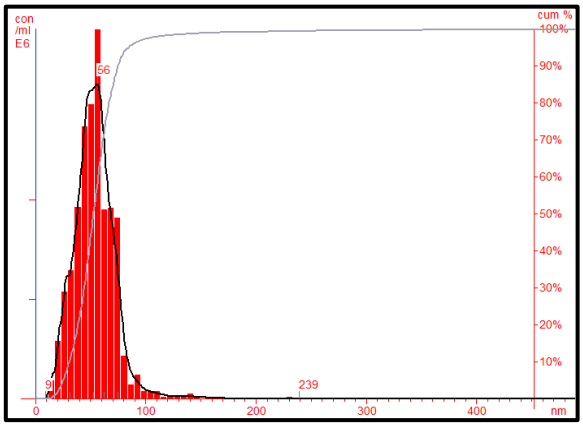

Figure 20: Particle Size/Conc. of [Cu (Ala.) (CCA).2H_2O] complex

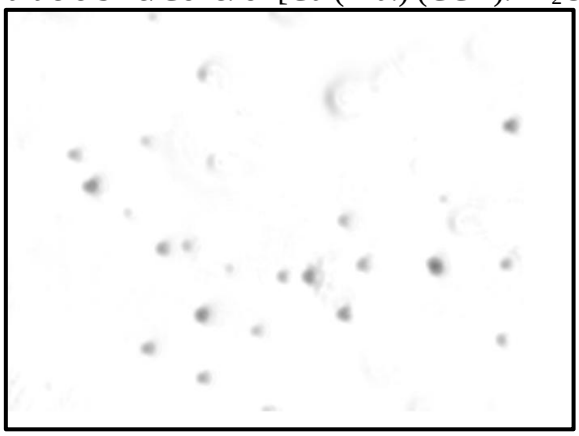

Figure 21: Sample Video Frame of [Cu (Ala.) (CCA).2H_2O] complex

ISSN 2063-5346 *Section A-Research paper*

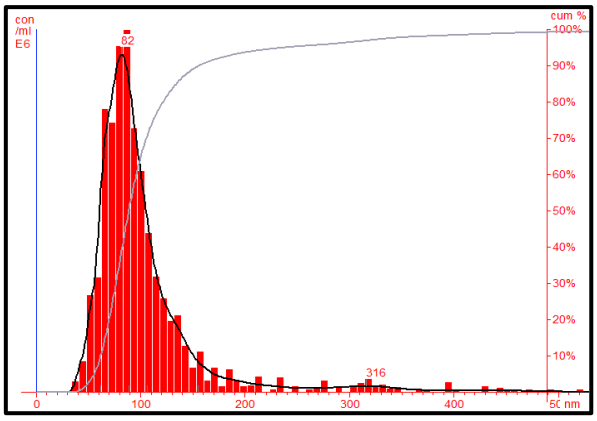

Figure 22: Particle Size/Conc. of [Cu (Gly.) (CCA).2H_2O] complex

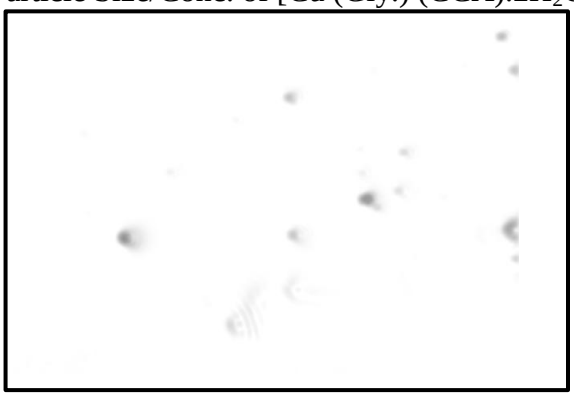

Figure 23: Sample Video Frame of [Cu (Gly.) (CCA).2H_2O] complex

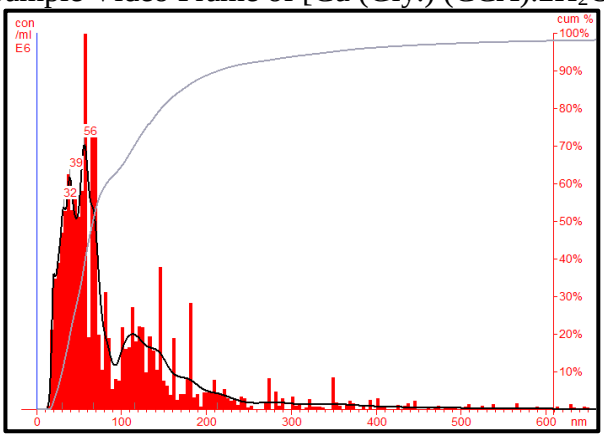

Figure 3.24: Particle Size / Conc. of [Cu (Cys.) (CCA).2H_2O] complex

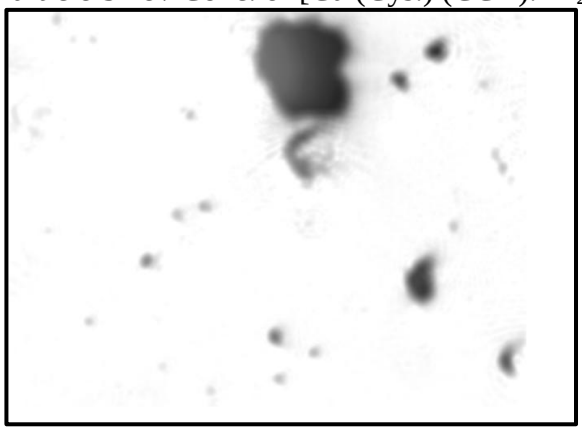

Figure 25: Sample Video Frame of [Cu (Cys.) (CCA).2H_2O] complex

ISSN 2063-5346 *Section A-Research paper*

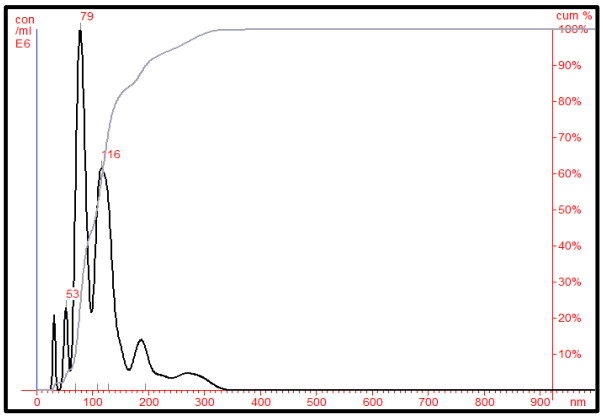

Figure 26: Particle Size / Conc. of [Cu (Ser.) (CCA).2H_2O] complex

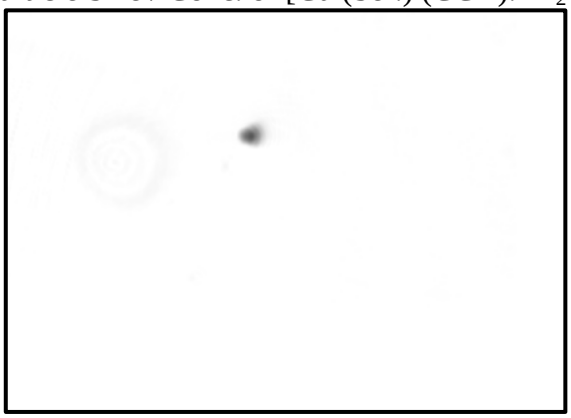

Figure 27: Sample Video Frame of [Cu (Ser.) (CCA).2H_2O] complex

The figure indicates that the particle size of all synthesized complexes in the range of 56-82 nm. The average particle size of alanine, glycine, cysteine and serine complexes were 56 nm, 82 nm, 56 and 79 nm. The size of the particles was depending on the P^H, time and temperature. The NPs of the all complexes shows higher cytotoxicity against MCF-7 cell line of breast cancer.

Solubility:

Solubility is an important parameter for characterization of chemical compound, the Mixed ligand copper complexes were characterized by solubility study. The solubility of all complexes was determined using different types of Solvents such as water, methanol, ethanol, and DMSO. It was observed that all synthesized complexes are soluble in only DMSO. The determined solubility was shown in table 5.

Table 5 Solubility of Complexes in different solvents

Complexes	**Water**	**Ethanol**	**Methanol**	**DMSO**
[Cu (Ala.) (CCA).2H_2O]	Insoluble	Insoluble	Insoluble	~11.4mg/10ml
[Cu (Gly.) (CCA).2H_2O]	Insoluble	Insoluble	Insoluble	~11.5 mg/10ml
[Cu (Cys.) (CCA).2H_2O]	Insoluble	Insoluble	Insoluble	~13 mg/10ml
[Cu (Ser.) (CCA).2H_2O]	Insoluble	Insoluble	Insoluble	~12.5mg/10ml

ISSN 2063-5346 *Section A-Research paper*

Conductivity:

The conductivities of the all complexes are measured for 100ppm solution in DMSO. The conductivity data reported for these complexes are given in Table 6. The Conductivity measurements in non-aqueous solutions have frequently been used in structural studies of metal complexes within the limits of their solubility are reported in the literature.[19-21] The conductivity measurement gives the degree of ionization of the complexes, the molar ions that a complex liberates in solution, the higher will be its molar conductivity and vice versa. Water molecules complete the coordination sphere of the studied complexes.

Table 6: Conductance of the copper complexes

Complex	Conductance(mhos)
[Cu (Ala.) (CCA).2H_2O]	10.50
[Cu (Gly.) (CCA).2H_2O]	9.20
[Cu (Cys.) (CCA).2H_2O]	8.10
[Cu (Ser.) (CCA).2H_2O]	9.50

The non-ionized complexes haven eligible value of molar conductance. It is clear from the conductivity data that the complexes present behave as weak electrolytes. All the complexes did not show electrolytic properties. This fact elucidated that the water molecules complete the coordination sphere of the studied complexes.

Anti-cancer activity of mixed ligand complex:

In the present study, we investigated the cytotoxic effects of the four newly synthesized mixed ligand copper complexes against the human breast cancer cell line MCF-7 using the standard MTT-dye reduction assay for cell viability. The cytotoxicity of the synthesized mixed ligand complexes is shown in Figure 27.

Table 7: Anti-cancer activity of the copper complexes

The data for the cytotoxicity of the synthesized complexes on MCF-7 cell line indicated

Complex	Human Breast Cancer Cell Line MCF-7			
	% Control Growth			
	Average ValuesDrug Concentrations (µg/ml)			
	10	**20**	**40**	**80**
[Cu (CCA)(CCA).2H_2O]	92.0	94.6	82.8	83.7
[Cu (Ala.) (CCA).2H_2O]	80.7	86.9	74.0	72.3
[Cu (Gly.) (CCA).2H_2O]	88.6	96.2	63.5	29.8
[Cu (Cys.) (CCA).2H_2O]	90.2	91.5	28.3	15.1
[Cu (Ser.) (CCA).2H_2O]	98.2	84.8	60.8	15.5
ADR	-62.5	-62.1	-67.8	-57.2

that amino acids complexes viz Cu (Ala)(CCA).2H_2O, Cu (Gly)(CCA).2H_2OCu (Cys)(CCA).2$H_2O$and Cu (Ser)(CCA).2H_2O shows more cytotoxicity than the Cu $(CCA)_2$. 2H2O shown in Table 7.The data for the cytotoxicity of the complexes on MCF-7 cell line indicated that Cu (Cys)(CCA).2H_2O and Cu (Ser)(CCA). 2H_2O proved to be the most active cytotoxic compound . From the result obtained Cu (Cys)(CCA).2H_2O exerted the most pronounced cytotoxic effects against the MCF-7 cell line with as low as, 40 ug/mL concentration. Whereas the Cu (Ser)(CCA). 2H_2O complex does not shows same cytotoxicity at same concentrations but cytotoxicity of serine complexes increases rapidly after the concentration 40ug/mL.

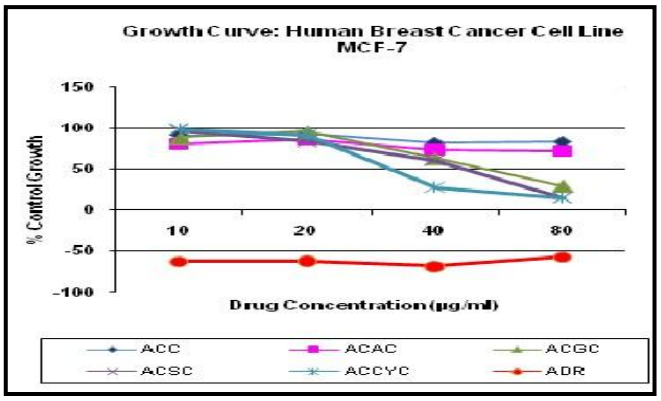

Figure 28: Anti-cancer activity of Cu mixed ligand complexes

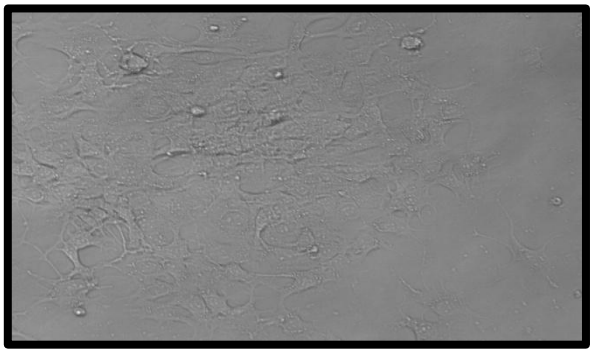

Figure 29.MCF-7 Control

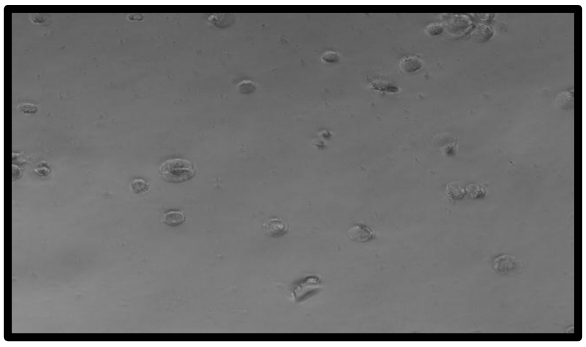

Figure 30. Positive control

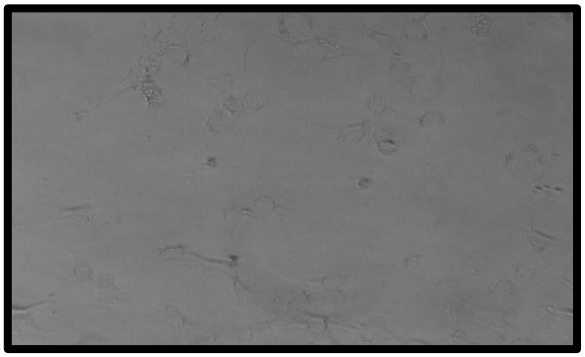

Figure 31 Anti-cancer activity of Cu-CCA Complex

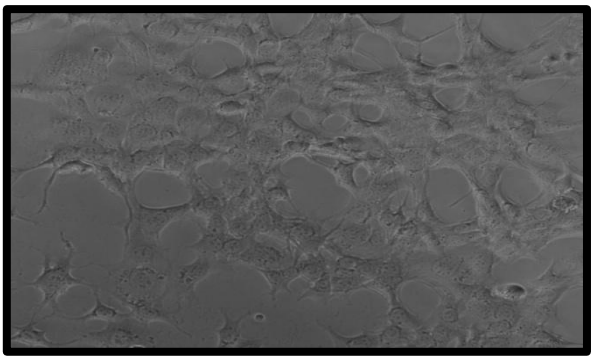

Figure 32 Anti-cancer activity of[Cu(Ala.)(CCA).2H_2O]Complex

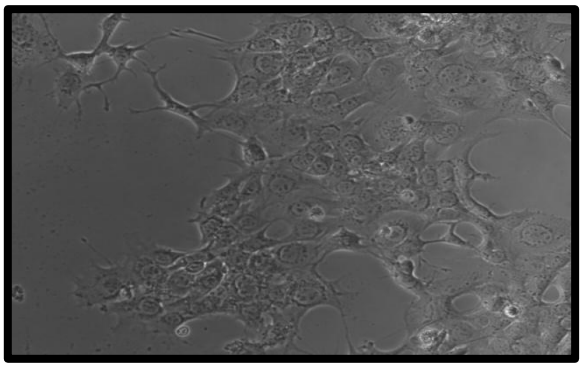

Figure.33 Anti-cancer activity of [Cu (Gly.) (CCA).2H_2O] Complex

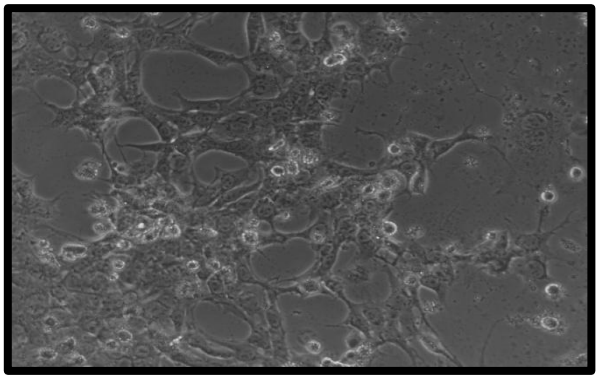

Figure 34 Anti-cancer activity of [Cu (Cys.) (CCA).2H_2O] Complex

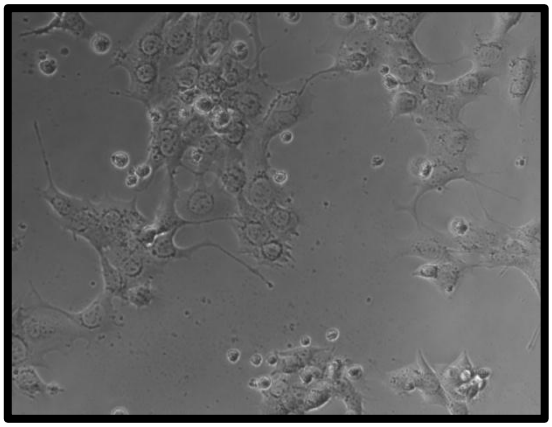

Figure 35 Anti-cancer activity of [Cu (Ser.) (CCA).2H_2O] Complex

The cytotoxicity of both these complexes are nearly same at concentration was 80 ug/mL. The cytotoxic effect of cysteine complexes slightly increases with increasing the

concentration 40 to 80 ug/mL whereas the cytotoxicity of the serine complex goes on increases with increasing the concentration from 40 to 80 ug/mL. This result indicates that the concentration of the serine complex more than 80 ug/mL may cytotoxicity of serine complex greater than the cysteine complex against MCF-7 cell line. From the data it clear that cytotoxicity of alanine complex is always lower for each concentration than the cytotoxicity of other complexes. The glycine complex showed the more cytotoxic activity than the alanine complex and less than the cysteine and serine complexes. The cytotoxic effect of glycine complex increases with increasing the concentration. The copper complexes under investigation exerted concentration-dependent cytotoxic activity against MCF-7 cell line. Several authors explained the binding mode of the copper complexes with DNA. The toxicity of the complexes depends on the concentration as well as the binding capacity of the complexes with DNA. Form the data it is clear that the cytotoxicity of serine [Cu (Ser)(CCA).$2H_2O$] and cysteine [Cu (Cys)(CCA).$2H_2O$] complex are more than the glycine and alanine complexes because the serine and cysteine complexes have higher binding capacity with DNA as compared to other complexes. The binding capacity of serine and cysteine complexes to DNA increases due to the free –OH and –SH group available in serine and cysteine ligands respectively. Whereas such free group are not available in the alanine and glycine complex to binding to DNA. Hence alanine and glycine complex show less cytotoxic effect against MCF-7 cell line of breast cancer. Finally, the overall results from the preliminary screening program revealed, that all the copper complexes with CCA and amino acids are potent cytotoxic agent against MCF-7 cell line as shown in figure 29 to 35. The only CCA complex of copper shows least cytotoxicity as compared to the all synthesized mixed ligands complexes.

CONCLUSION

According to a review of the literature, mixed ligands complexes of transition metal complexes are cytotoxic to cancer cells. Copper complexes have been shown to have cytotoxic effect against cancer cells by several writers. I. Kostova has discovered that transition metal complexes with coumarine derivatives have potent anticancer properties. They also discovered that copper complexes of coumarine -3- carboxylic acids had anticancer properties.Copper, coumarine-3-carboxylic acid, and amino acids alanine, glycine, serine, and cysteine were used to make mixed ligand complexes. Elemental analysis, simultaneous TG-DTA, XRD, IR, and 1H-NMR spectroscopy are used to characterise these synthesised mixed complexes. All of the complexes' solubility is tested in a variety of solvents, including methanol, ethanol, ethyl acetate, acetone, and DMSO. All of these mixed ligand complexes are shown to be soluble in DMSO.All of these mixed ligand complexes are shown to be soluble in DMSO. The particle size of all synthesised complexes was determined to be in the range of 56 nm to 83 nm utilising the NTA method. The formula of these complexes was [Cu (Ala)(CCA)] based on the elemental analysis and simultaneous TG-DTA, IR, and 1H-NMR spectra of the complexes. [Cu (Gly)(CCA)], 2H2O. 2H2O [Cu (Ser)(CCA)].2H2O and [Cu (Cys) (CCA)].2H2O and [Cu (Cys) (CCA)]. The XRD pattern of the compounds shows that they are all crystalline in character. Complexes are non-ionized in DMSO, according to conductivity measurements for all complexes in DMSO.The MCF-7 breast cancer cell line was used to test the pharmacological activity of the produced complexes.The produced mixed ligands complexes of copper are described using several ways to identify the molecular formula of the complexes, according to the findings of the research.Against the MCF-7 breast cancer cell line, the produced mixed ligands complexes had a greater cytotoxicity than the Coumarine -3-carboxylic acid copper complex.Against the MCF-7 cell line of breast cancer, mixed ligands complexes of coumarine -3-carboxylic acid, serine, and cysteine with copper are more cytotoxic than alanine and glycine complexes. Because of their

capacity to bind to DNA, the serine and cysteine complexes are more cytotoxic than the alanine and glycine complexes. Because these ligands have free –OH and –SH groups, the serine and glycine complexes have greater DNA binding.It is obvious from this work that mixed ligands complexes have stronger cytotoxic action against the MCF-7 breast cancer cell line if the ligands have free electronegative groups that create appropriate DNA binding.

The proposed research additionally revealed that mixed ligand copper has a favourable modulatory impact. In the end, it will result in improved therapeutic treatment of malignant cells. The current study optimized the synthesis of nano composite and mixed ligand complexes of copper with amino acids and coumarin-3-carboxylic acid for cancer treatments, and the produced complexes were tested against the MCF-7 human cancer cell line using the MTT assay. Thus, mixed ligand copper and nano Composite Formulation demonstrate a link to the proposed hypothesis, and the impact might be attributable to the metal complexes' improved antineoplastic action.

These complexes interact with DNA via intercalation or partial contact, which has anticancer properties. Because these complexes engage with DNA through intercalation or partly interaction, they have superior cytotoxicity, resistance to cancer cells, and less side effects than cisplatin. To improve their interaction with DNA, several metal complexes have been created utilising various modified ligands.

This research will broaden the research aspect with the goal of current research work to develop novel mixed ligand complexes with fewer side effects, better resistance, and the same or greater cytotoxicity to cisplatin for rational and scientific uses in the suffering community for the therapeutic management of this life-threatening disease.

ACKNOWLEDGEMENT

The authors acknowledge Tatyasaheb Kore College of Pharmacy and Shivaji University, Kolhapur (M.H), India, for their support and encouragement. This work did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

ETHICS

Ethics Committee Approval: No Need of approval.
Informed Consent: There was no investigation carried out using human volunteers.
Peer-review: Externally peer-reviewed.
Authorship Contributions Concept:All Authors equally contributed in the Design, Data Collection or Processing, Analysis or Interpretation, Literature Search, and writing works.
Conflict of Interest: No conflict of interest was declared by the authors.
Financial Disclosure: The authors declared that this study received no financial support.

REFERENCES:

1. Niakan B. Proposed Biology of the Spontaneous Remission of Cancer. International Journal of Cancer and Clinical Research. 2021;8(5), 365-367.
2. PoojaRokade, Aishwarya Patil, RutikaHarshad, Santosh Payghan. A Review on Carbon Nanotubes on Cancer Therapy, International Journal of Creative Research Thoughts, Volume 9, Issue 8 August 2021 | ISSN: 2320-2882, 469- 478.
3. Akshay S. Jirage, Vyankatesh S. Baravkar, Vaishali K. Kate, Santosh A. Payghan, John I. Disouza. Poly-β-Hydroxybutyrate: Intriguing Biopolymer in Biomedical Applications and Pharma Formulation Trends.International Journal of Pharmaceutical & Biological Archives 2013; 4(6): 1107 – 1118.
4. Nangare KA, Powar SD, Kate VK, Khavane KK, Payghan SA. Nanosuspension: Potential Applications of Nano Therapeutics in Ocular Delivery. Mod ApplBioequivAvailab. 2018; 3(2): 555608.

5. Sung H, Ferlay J, Siegel R, Laversanne M, Soerjomataram I, Jemal A et al. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. CA: A Cancer Journal for Clinicians. 2021;71(3):209-249.
6. S. Ramakrishnan and M. palaniandavar, Mixed-ligand copper (II) complexes of dipicolylamine and 1,10-phenanthrolines: The role of diamines in the interaction of the complexes with DNA. Journal of Chemical Sciences.2005; 117(2): 179–186.
7. Malathymoorthy and dr. rajavelrangappan, copper(ii) complexes as in vitro anticancer agents against human breast cancer mcf-7 cell line: a review. European journal of biomedical and pharmaceutical sciences. 2015;2 (3): 177-200.
8. C. Fernandes, G.L. Parillha, J.A. Lessa, L.J.M. Santiago, M.M. Kanashiro, F.S. Boniolo, A. Horn, Synthesis, crystal structure, nuclease and in vitro antitumor activities of a new mononuclear copper (ll) complex containing a tripodal N3O ligand, InorganicaChimica Acta.,2006; 359: 3167- 3176.
9. N. Farrell, Transition Metal Complexes as Drugs and Chemotherapeutic Agent,1989; 2777-2795.
10. Castellani C.B., Carugo D., Studies on fluorescent lanthamide complexes of lanthamides (III) with coumarinic-3-carboxylic acid. InorganicaChimica Acta.1989;159 (2): 157–161.
11. Satoshi Horikoshi and Nick Serpone, Microwaves in Nanoparticle Synthesis, First Edition ©, Wiley-VCH Verlag GmbH & Co. KGaA. Published. 2013; 1-5.
12. H.I wahara, H. Uchida, K. Ono and K. Ogaki, Proton Conduction in Sintered Oxides Based on BaCeO3. Journal of The Electrochemical Society.1988; 135: 529.
13. AlkaChoudhary, Renu Sharma, Meena Nagar, Synthesis, characterization and antimicrobial activity of mixed ligand complexes of Co (II) and Cu (II) with N, O/S donor ligands and amino acids, International Research Journal of Pharmacy and Pharmacology, 2011; 1(6): 172-187.
14. P. Kostova, I lia i. manolov, Maritza k. radulova, Stability of the complexes of some lanthanides with coumarin derivatives Cerium (III) complex with 4-methyl-7-hydroxycoumarin, ActaPharmaceutica. 2004; 54: 37–47.
15. Ammar J. Alabdali, Farah M. Ibrahim, Synthesis and Thermal Study of Co (II), Ni (II), Cu (II) Mixed Ligand Complexes Using Histidine as Tridentate Ligand, IOSR Journal of Applied Chemistry (IOSR-JAC), 2014; 6, (6): 60-63.
16. D. Kannan, M.N. Arumugham, Synthesis, Characterization, DNA-Binding Studies and antimicrobial activity of Copper (II) Complex with 1,10 Phenanthroline, L-Tyrosine and Thiourea as Ligands, International Journal of Research in Controlled Release. 2012;2(4): 10-17.
17. Gokhale N, Padhye S. Rathbone D, Billington D, Lowe P, Schwalbe C, Newton C. The crystal structure of first copper (II) complex of a pyridine-2- carboxamidrazone - a potential antitumor agent. Inorganic Chemistry Communications. 2001; 4: 26-29.
18. Georgieva, N. Trendafilova, W. Kiefer, V.K. Rastogi, I. Kostova, Vibrational and theoretical study of coumarin-3-carboxylic acid binding mode in Ce (III) and Nd (III) complexes, Vibrational Spectroscopy. 2007;44: 78–88.
19. H. H. Lin, R. K. Chiang, C. L. Kuo. C. W. Chang, Synthesis of BaCeO3 powders by a fast aqueous citrate–nitrate process. Journal of Non-Crystalline Solids. 2007; 353: 1188-1193.
20. C. Jun, L. Kate, G. S. Francis and S. L. Steven, Preparation Method and Cationopant Effects on the Particle Size and Properties of BaCeO3 Perovskites. Journal of the American Ceramic Society. 2005; 88 (10): 2729-2735.

21. K. Joo, J. Paitz, D. Klimm, P. Reiche, K. H. Auh and W. Schroder, Growth of Barium Cerate Crystals from BaCl2 Solution. Crystal Research and Technology. 2000; 35 (4): 493-496.
22. Nangare K. A., Kate V. K., Patwekar S. R. Payghan S. A Therapeutics applications of Nanosuspension in Topical/ Mucosal delivery drug Delivery Journal of Nanomedicine Research 7(1):00170. 10.15406/jnmr.2018.07.00170