ISSN: 2349 - 4891

International
Journal of Recent Research and Applied Studies
(Multidisciplinary Open Access Refereed e-Journal)

Anti diabetic activity, Anticancer activity and α-amylase enzyme inhibitory effect of Tetrakis (4-aminopyridine- $_kN^1$) di chloride copper (II) monohydrate, $[CuCl_2(C_5H_6N_2)_4].H_2O$

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Received 13th May 2020, Accepted 5th June 2020

Abstract

The functional group of the crystal Tetrakis (4-aminopyridine- $_kN^1$) di chloride copper (II) monohydrate, $[CuCl_2(C_5H_6N_2)_4].H_2O$ complex confirmed by FTIR spectrum. The crystal taken in the form of solution and subjected to biological activity test. Its particle size and Zeta potential calculated. It exhibit good zone of inhibition against bacteria Escherichia coli, Pseudomonas putida, Bacillus Badius, Pseudomonas aeruginosa and Staphylococcus aureus. Antibacterial activity of this complex showed 90% reduction in the Colony-forming Unit CFU and for this same concentration it displayed efficient radical scavenging activity compared to the standard antioxidant (Ascorbic acid). This complex show good anti diabetic property and Anti cancer activity. The molecular docking results show that this complex has greater binding affinity towards α-amylase enzyme when compared to HIV (human immunodeficiency virus) protease, COX (Cyclo-oxgenase) and EGFR (Epidermal growth factor Receptor) enzyme through $N_{(\text{amino radical of 4-aminopyridine})}$-H…$O_{(\text{carboxylic group of enzyme})}$ hydrogen bonding.

Keywords: Copper complex of 4-aminopyridine, α-amylase enzymes, antidiabetic activity, anticancer activity, Molecular docking.

Introduction

4-aminopyridine clinically approved specific blocker of voltage gated potassium channels for the treatment of patients with Multiple Sclerosis. It used in large scale human trials for compensating the loss of the myelin cover in damaged nerve, it slowly absorbed and eliminated from human and it identified as the novel compound for restoring conduction in injured spinal cord [1].Using the auto dock force field with a Lamarckian genetic algorithm, with atomic charges or the ligands derived from the electrostatic potential obtained at the B3LYP/cc-pVDZ level, a zone of α-subunit of the K^+ channel bearing common binding sites identified. At this zone 4-aminopyridine binds to the carboxylic oxygen of Thr 107 and Ala 111[2]. Aminopyridine act as mono dentate ligands by binding through the pyridine N, so that the coordination preferences of the metal ion and bridging modes may modify. The amino group present in aminopyridine enables the ligand to act as potential hydrogen bond donor or acceptor moiety [3]. For the development of metal complexes (like copper) it may posses anticancer activity when making complexes with organic motifs . For target specific- next generation anticancer therapeutic process copper based compounds plays vital role. The copper act as tracing element and it plays a key role in many physiological cellular process [4]. $Cu((C_5H_6N_2)_4).2Br.2(C_3H_7NO)$, a new mono nuclear copper complexes showed antibacterial and antioxidant activities[5]. The stability of the compound with its ligand leads to potential drug candidate towards bio molecules. Single crystal X-ray diffraction[6], charatcterization [7] and molecular docking[8] with sphingosine 1- phosphate lyase (S1PL) of Tetrakis (4-aminopyridine- $_kN^1$) di chloride copper (II) monohydrate, $[CuCl_2(C_5H_6N_2)_4].H_2O$were reported. In-silico docking study of a protein ligand interaction resulted in -6.11 kcal mol-1 and -6 kcal mol-1 free energy values for the GLN476 - ligand and TYR526 – ligand respectively. The digestive enzyme α-amylase forms hydrolyze glycosidic bonds with starch to glucose, Dexedrine, etc. This enzyme have applications in food industry, medical and clinical fields[9]. It have anti oxidant activity and anticancer activity[10]. This work focus on investigating the important biological activity of six coordinated copper complex of 4-aminopyridine along with chlorine molecules.

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Experimental process

The $[CuCl_2(C_5H_6N_2)_4].H_2O$ complex prepared according to the literature review H.K.Fun et al,.(2008) [6]. The functional group presence[7] (sample in liquid

form) compared with FTIR spectrum.

Materials and methods

1. Hydrodynamic particle size analysis

After the formation of nucleation inside the solvent, the blue colored solution of $[CuCl_2(C_5H_6N_2)_4].H_2O$ diluted and the mean hydrodynamic diameter and poly disparity index of $[CuCl_2(C_5H_6N_2)_4].H_2O$ particles present in the diluted solution determined by the dynamic light scattering (DLS) using Malvern Zetasizer nano ZS instrument at 37ºC.

2. Anti microbial activity

Colony forming unit : Antimicrobial assay carried out by dilution of mid-log liquid cultures into the appropriate assay buffer to a concentration of approximately 2 x 10^5 CFU/ml. Bacteria grown in MH (Mueller Hinton) broth to an $OD_{600\ nm}$ of 0.5. 2µl aliquot of the bacteria then taken and added 5 ml of fresh MH broth. The log_{10} reduction in CFU/ml determined by subtracting the log_{10} transformed counts of viable bacteria in$[CuCl_2(C_5H_6N_2)_4].H_2O$ containing assays from the log_{10} transformed counts of viable bacteria in control assays.

Well diffusion method: $[CuCl_2(C_5H_6N_2)_4].H_2O$ diluted and Serial dilutions such as 100, 80, 60, 40, 20 mg/ ml made ready. The material containing bacteria uniformly spread using a sterile cotton swab on a sterile Petri dish MH agar. 50 µL of diluted $[CuCl_2(C_5H_6N_2)_4].H_2O$ added to each well with 7 x 10^3m diameter holes cut in the agar gel. The systems incubated for 24 hr at 36ºC ± 1ºC, under aerobic conditions. After incubation, confluent bacterial growth observed. Inhibition of the bacterial growth measured in mm.

3. Anti oxidant activity

The anti oxidant activity of $[CuCl_2(C_5H_6N_2)_4].H_2O$ calculated using the formula

$$\%\ \text{inhibition} = 100\ \text{x}\ \frac{V_c - V_t}{V_c}$$

Where V_t = absorbance of test sample and V_c = absorbance of control.

The ABTS(2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) produced by mixing 0.35 mL of ABTS diammonium salt (7.4 mM/L) with 0.35 mL of potassium persulfate (2.6 mM/L). To determine the scavenging activity , 1.2 mL of ABTS reagent mixed with 0.3 mL of sample (95% ethanol), and the absorbance at 734 nm measured after six minutes as initial mixing, using 95% ethanol as the blank.

Ferric Reducing Antioxidant Power Assay: 300 mM acetate buffer, 10 mM TPTZ solution in 40 mM HCl, and 20 mM $FeCl_3$ $6H_2O$ solution used as stock solution. Result recorded for coloured product at 593 nm. The standard curve have linearity with ferrous sulphates.

1,1-diphenyl-2-picryl-hydrazyl Radical Scavenging Activity : Using the method of Blois (1958)[11], 0.1mM solution of DPPH(1,1-diphenyl-2-picryl-hydrazyl) in ethanol prepared and one ml solution of this solution was added 3 ml of $[CuCl_2(C_5H_6N_2)_4].H_2O$ solution in water at different concentrations (10-50 µg/ml). The free radical scavenging activity of $[CuCl_2(C_5H_6N_2)_4].H_2O$ determined at λ_{max} 517nm.

Hydroxyl Radical Scavenging Assay: Using modified method of Halliwell et al. (1987)[12], 1 ml of the final reaction solution consisted of aliquots (500 µL) of various concentrations of the $[CuCl_2(C_5H_6N_2)_4].H_2O$, 1 ml $FeCl_3$, 1 ml EDTA, 20 ml H_2O_2, 1 ml L-ascorbic acid, and 30 ml deoxyribose in potassium phosphate buffer (pH 7.4). The reaction mixture incubated at 37 °C for an hour, and further heated in a boiling water bath for 15 min after addition of 1 mL of 2.8% (w/v) trichloro acetic acid and 1 mL of 1% (w/w) 2- thiobarbituric acid. The color development measured at 532 nm against a blank containing phosphate buffer.

Hydrogen Peroxide Assay: $[CuCl_2(C_5H_6N_2)_4].H_2O$ dissolved in 3.4 ml of 0.1M phosphate buffer (pH 7.4) and mixed with 600 µ l of 43 m M solution of hydrogen peroxide. The absorbance value (at 230 nm) of the reaction mixture recorded at 10 min intervals between zero and 40 min. for each concentration, a separate blank sample used for background subtraction.

4. Anti diabetic activity

A total of 500 µ l of $[CuCl_2(C_5H_6N_2)_4].H_2O$ and standard drug (20- 100mg /mL) added to 500 µ l of 0.20 mM phosphate buffer (pH 6.9) containing α-amylase (0.5mg/ml) solution and incubated at 25°C for 10 min. After these, 500 µ l of a 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9) added to each tube. The reaction mixtures then incubated at 25°C for 10 min. The reaction stopped with 1.0 ml of 3, 5 dinitrosalicylic acid colour reagent. The reaction mixture then diluted after adding 10 ml distilled water and absorbance measured at 540 nm.

5. Anticancer activity

MDB-MB- 468 (Breast cancer) cells initially procured from National Centre for Cell Sciences (NCCS), Pune, India and maintained Dulbecco's modified Eagles medium (Gibco, Invitrogen).The cell line cultured in 25 cm^2 tissue culture flask with DMEM supplemented with 10% FBS, , L-glutamine, sodium bicarbonate and antibiotic solution containing: Penicillin (100U/ml), Streptomycin (100µg/ml), and Amphotericin B (2.5µg/ml). Cultured cell lines kept at 37ºC in a humidified 5% CO_2 incubator (NBS Eppendorf, Germany). The viability of cells evaluated by direct observation of cells by Inverted phase contrast microscope and followed by MTT assay method.

Cytotoxicity Assay by MTT Method: Fifteen mg of MTT (Sigma, M-5655) built up again in 3 ml PBS and

sterilized. After 24 hours of incubation period, the sample content in wells removed and 300µl of reconstituted MTT solution added to all test and cell control wells, the plate gently shaken well, then incubated at 37ºC in a humidified 5% CO_2 incubator for 4 hours. After the incubation period, the supernatant removed and 100µl of MTT Solution (DMSO) added and the wells mixed gently by pipetting up and down in order to increase the solubility of the crystals. The absorbance values measured by using micro-plate reader at a wavelength of 570 nm.

Cytotoxicity Evaluation: After 24 hours the growth medium removed, freshly prepared each sample in 5% DMEM five times serially diluted by two fold dilution (100µg, 50µg, 25µg, 12.5µg, 6.25µg in 100µl of 5% MEM) and 100µl, 50µl, 25µl, 12.5µl, 6.25µl of standard solution in 100µl each concentration of 100µl added in triplicates to the respective wells and incubated at 37ºC in a humidified 5% CO_2 incubator.

*Cytotoxicity Assay by Direct Microscopic observation:*Entire plate observed at an interval of each 24 hours; up to 72 hours in an inverted phase contrast tissue culture microscope (Olympus CKX41 with Optika Pro5 CCD camera) and microscopic observation recorded as images. Any detectable changes in the morphology of the cells, such as rounding or shrinking of cells, granulation and vacuolization in the cytoplasm of the cells considered as indicators of cytotoxicity.

6. Molecular docking

Computational bioinformatics tools have been employed in this study for the compound $[CuCl_2(C_5H_6N_2)_4].H_2O$. Biological databases like NCBI, PubChem and PDB (Protein Data Bank) and computational tools. The software used for this work for the compound $[CuCl_2(C_5H_6N_2)_4].H_2O$ are Jal viewer (version 2.8), PyRx with Autodock, PyMOL and Rasmol.

The protein sequence of the three enzymes was downloaded from protein data bank (*http://www.rcsb.org/pdb*). Protein Data Bank (PDB) is a structural archive for biological macromolecules which encompasses all the structural information of macromolecules as determined by X-ray crystallography, NMR studies, etc.

Validated chemical depiction information, pee-clustered structures and cross-referenced structures by identity and similarity groupsgot from PubChem Compound Database.

To screen libraries of compound against potential drug targets Virtual screening software used. To run virtual screening at any platform PyRx used and this helps in every step of result analysis.

Molecular modelling program Pymol effective for the construction, three dimensionalvisualization of macromolecules, including proteins and protein-ligand complexes

Result and discussion

1. Hydrodynamic Particle Size Analysis

The Zeta potential ZP (ζ) is related to the surface charge, a property that all materials possess, or acquire, when suspended in a fluid. ZP is an indicator of the stability of Nanoparticle suspensions. The positive zeta potential of $[CuCl_2(C_5H_6N_2)_4].H_2O$ infers that this complex provide only short term stability which leads to strong electrostatic potential it is shown in figure 1.

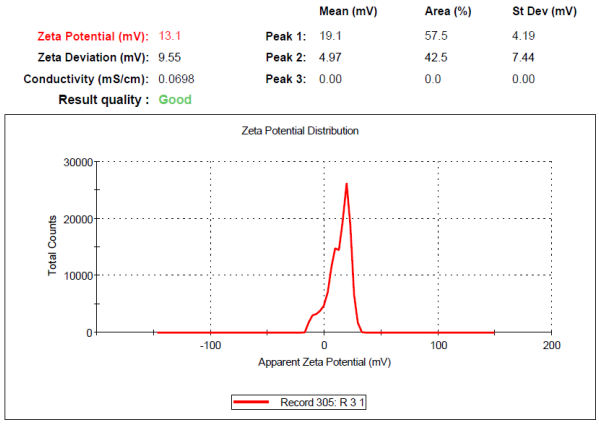

Figure 1. Particle size analysis for $[CuCl_2(C_5H_6N_2)_4].H_2O$ compound

2. Anti microbial activity

The plate count method involves serially diluting a culture of bacteria to count colony forming units (CFU) on a agar plate. Colonies of E.coli and Bacillus were counted at different concentrations. When the concentrations keeps on increasing from 1.25, 2.5, 5, 50 to 500 µG/ML the compound reduce the cell viability. Complete inhibition of bacteria at 500µG/ML was observed for both Bacillus and E.coli. The antimicrobial activity overall is more reactive in E.coli than in Bacillus is shown in figure 2.

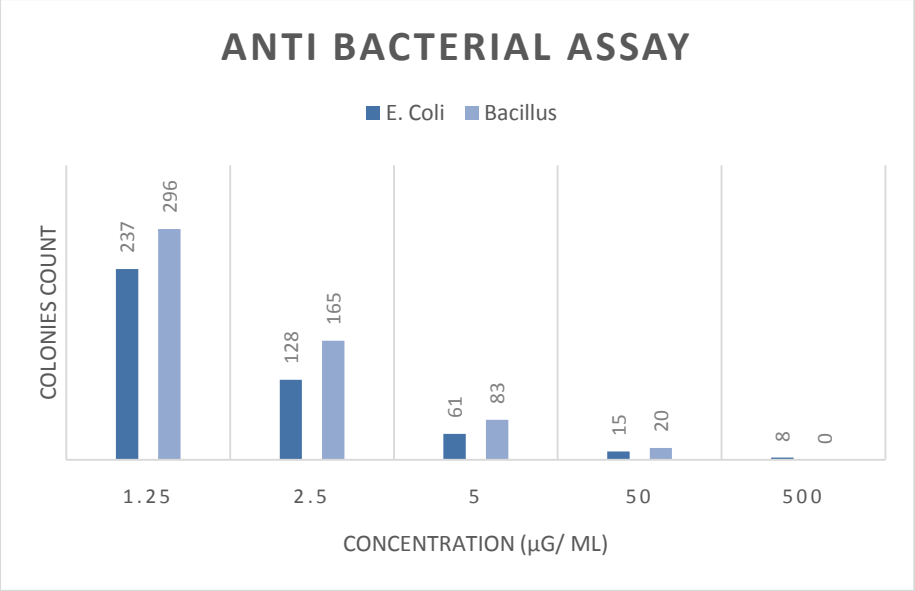

Figure 2. Anti -microbial activity of $[CuCl_2(C_5H_6N_2)_4].H_2O$ compound

Using well diffusion assay, the anti bacterial potential $[CuCl_2(C_5H_6N_2)_4].H_2O$ evaluated according to their zone of inhibition against various organism. The high degree of inhibition was found in Pseudomonas sp,with diameter of 1± 9mm.Followed by this P.putida showed maximum antimicrobial activity with diameter 1.2±6. B.badius (0.7-2.8mm) showed restrained and minimum activity shown in table 1.

Table 1. *Well Diffusion Assay*

Organism	**Zone of inhibition (mm)**	
	Solvent Control	$[CuCl_2(C_5H_6N_2)_4].H_2O$
E. coli	0.8	3
P. putida	1.2	6
B. badius	0.7	2.8
Pseudomonas sp.	1	9
Staphylococcus sp.	0.5	3.2

3. Anti oxidant activity

The radical scavenging activity (DPPH inhibition, FRAP, ABTS, hydroxyl and hydrogen peroxide) of $[CuCl_2(C_5H_6N_2)_4].H_2O$ have similar activity to the standard antioxidant (Ascorbic acid) at the concentration 100 mg/ml and it is shown in figure 3. All assays, however, had no genotype time interaction, indicating that all techniques gave a comparable ranking of antioxidant activity among clones within each time of determination. Therefore, the DPPH and FRAP assays could be used to determine antioxidant activity in guava as both showed high reproducibility. Synthesised compound showed 10-60 % antioxidant activity at 100 mg/ml for ABT assay, DPPH model systems also depicts the similar result. Synthesised compound showed 5- 45 % antioxidant activity at 100 mg/ml for HRA assay, HPS and FRAP assay also depicts the similar result. This is the first report on the antioxidant properties of the $[CuCl_2(C_5H_6N_2)_4].H_2O$ Owing to this property, the studies can be further extended to exploit them for their possible application for the preservation of food products as well as their use as health supplements.

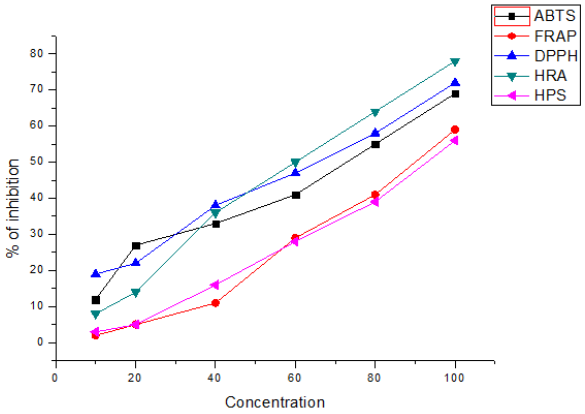

Figure 3. Radical scavenging activity of $[CuCl_2(C_5H_6N_2)_4].H_2O$ compound

4. *Anti diabetic activity*

α- amylase is a prominent enzyme found in pancreatic juice and saliva which breaks down large insoluble starch molecules into absorbable molecules. Inhibitors of α- amylase delay the breaking down of carbohydrate in the small intestine and diminish the postprandial blood glucose excursion.$[CuCl_2(C_5H_6N_2)_4].H_2O$ concentration dependent increase in percentage inhibitory activity against α- amylase enzyme is shown in figure 4.

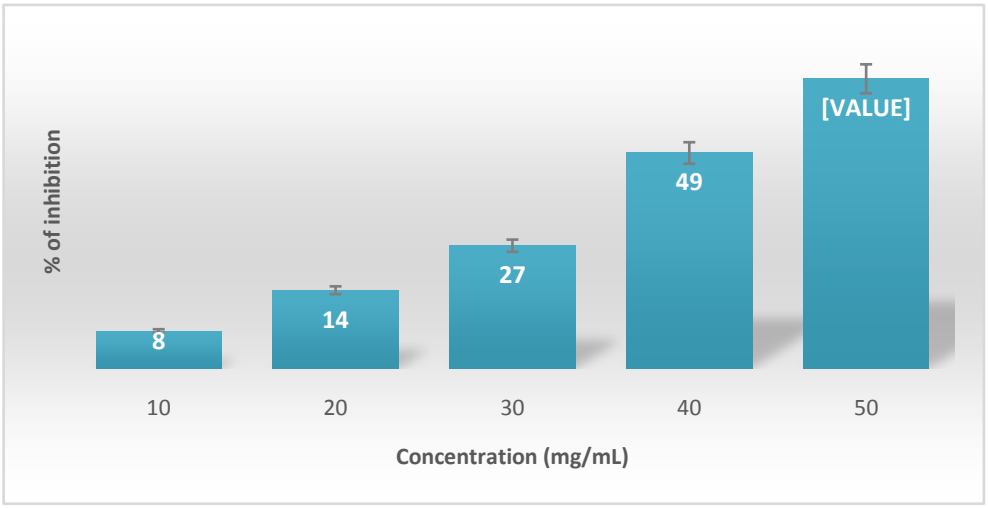

Figure 4. Anti-Diabetic Activity of $[CuCl_2(C_5H_6N_2)_4].H_2O$ compound

The inhibition of α- amylase by $[CuCl_2(C_5H_6N_2)_4].H_2O$ showed no significant difference at lower concentration from 0-10 mg/ml. At a concentration 10 mg/ml of $[CuCl_2(C_5H_6N_2)_4].H_2O$the percentage of inhibition was 8 ± 0.12 and the inhibitory potentials keeps on increasing as the concentration goes higher. At a concentration of 50 mg/ml it was 78 ± 3.8.

5. Anti cancer activity

The result of MTT assay revealed that $[CuCl_2(C_5H_6N_2)_4].H_2O$ decrease the percentage viability of MDB-MB-468 cells. In vitro cytoxity test using MDB-MB-468 cell lines was performed to screen potentially toxic compounds that affect basic cellular functions and morphology of the cells. The title compound showed in vitro growth inhibition effect on the cancer cell line such selective effect were concentration as well as incubation time period dependent. With respect to concentrations (6.25µg, 12.5µg, 25µg, 5µg, 100µg) of $CuCl_2(C_5H_6N_2)_4.H_2O$ were evaluated in two fold dilution.Figure 5 image shows the shrinking of cells, granulation and vacuolization in the cytoplasm of the cells at the different concentration and at 100µg there is complete inhibition of cancer cells. The results states that the title compound significantly inhibited the cell lines and was the most potent with LC_{50} value 32.67µg/ml. Therefore $[CuCl_2(C_5H_6N_2)_4].H_2O$ is an excellent source of antioxidant which helps in decrease the free radicals in the body and prevent cancer cells.

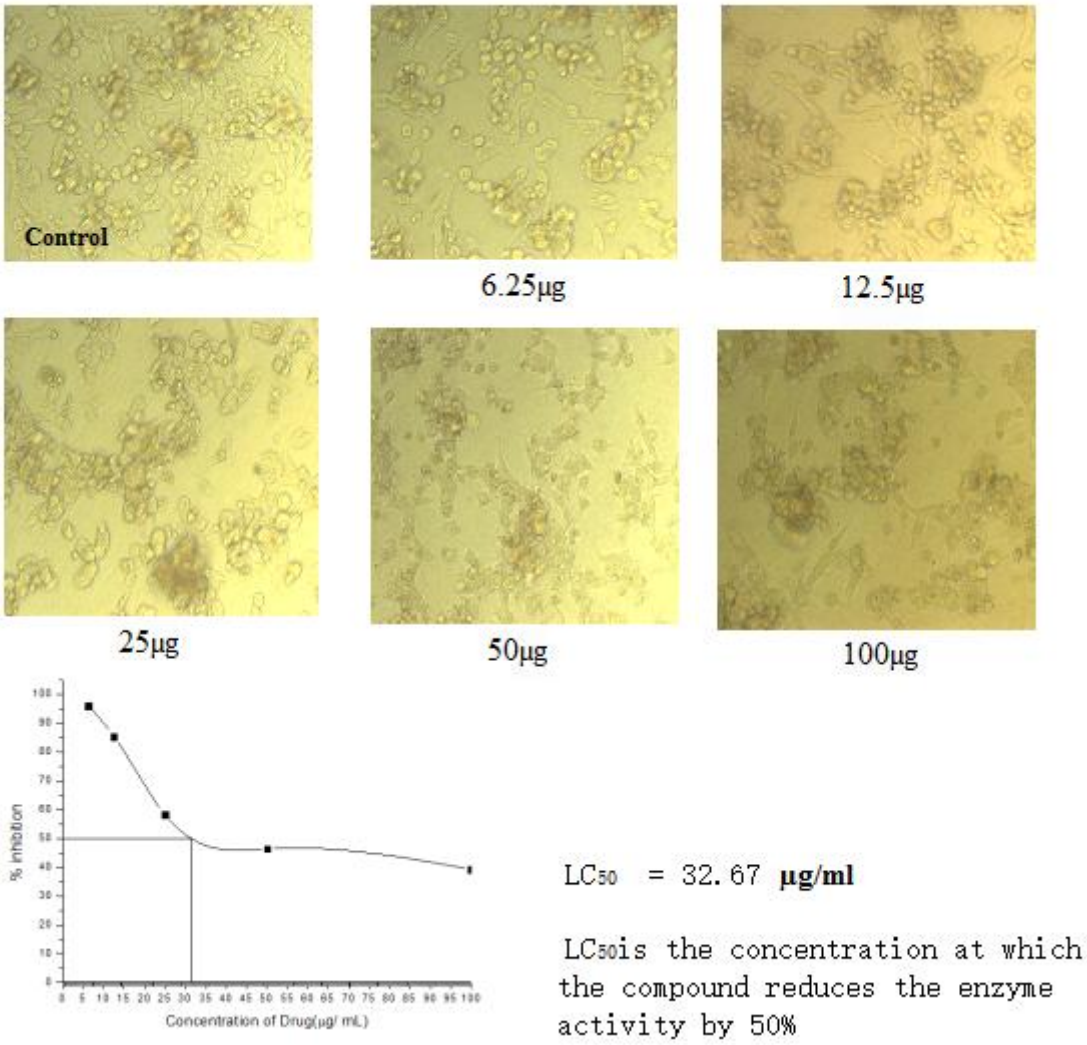

Figure 5. Image of anticancer effect at different concentration.

6. Molecular docking

Using auto dock software the enzyme molecules targeted by $[CuCl_2(C_5H_6N_2)_4].H_2O$ bind at the active sites in that enzyme, which can inhibit the activity of enzyme and is shown in figure 6. Based on the binding energy the ability of $[CuCl_2(C_5H_6N_2)_4].H_2O$ analyzed. Binding energy for the various targets tabulated in Table 2.

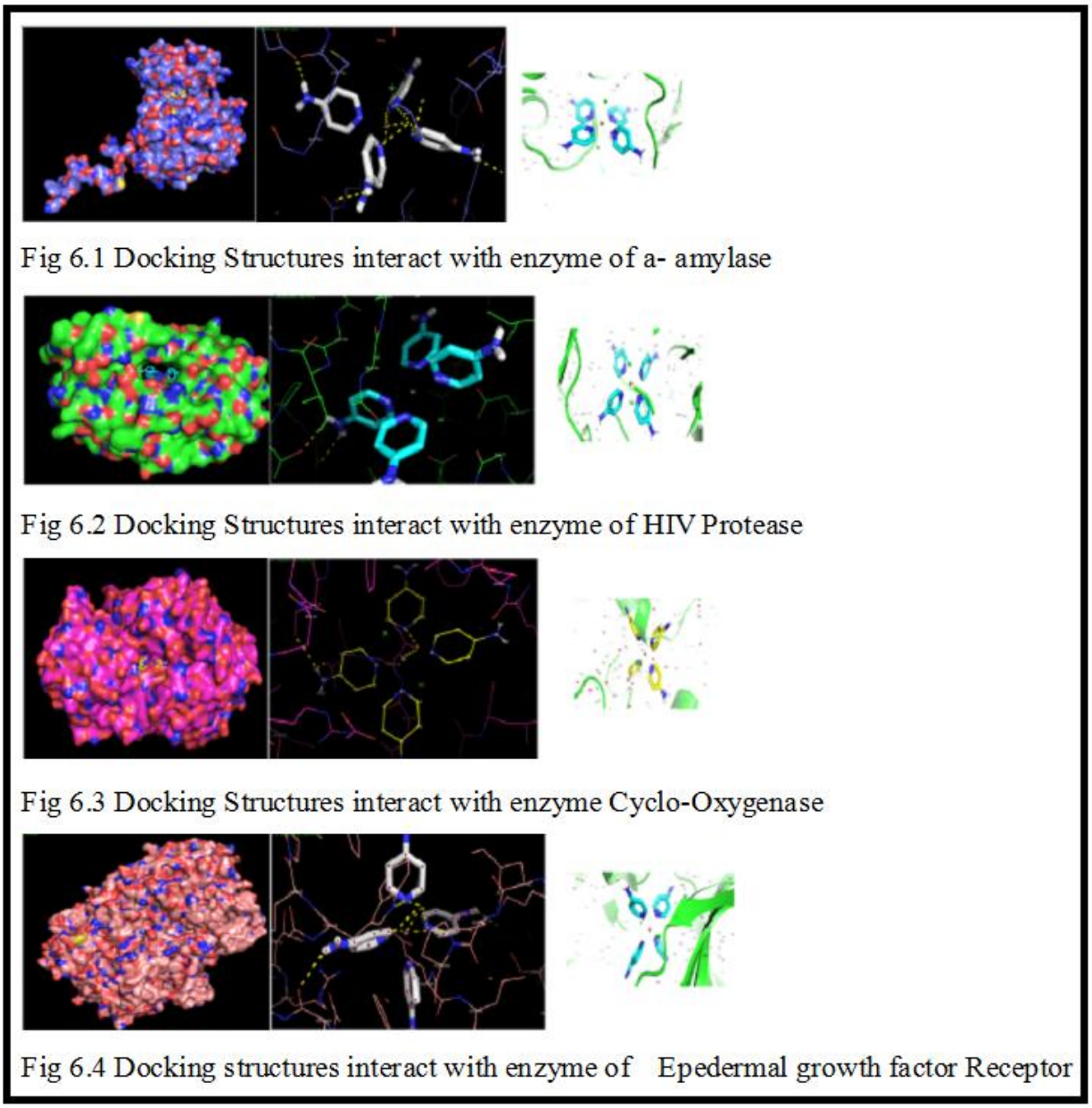

Figure 6. Molecular Docking studies for [CuCl2(C5H6N2)4].H2O compound. Blue colored atoms indicate the Nitrogen atom and red colored atom indicate the Oxygen atom.

Table 2.*Binding energy for* $[CuCl_2(C_5H_6N_2)_4].H_2O$ *for various targets*

Enzymes	**Interacted amino acid**	**Hydrogen bonding**	**Binding Energy kcal/mol**
α - amylase	Glutamine-318 Lysine-2	N-H…O	-6.2
HIV Protease	Aspatic acid-25 Glysine-49	N-H…O	-5.4

Cyclo-oxgenase	Aspringine-11 Glutamine-365	N-H…O	-5.1
Epedermal growth factor Receptor	Glysine-772 Lysine-773	N-H…O	-3.2

Among these enzymes the Tetrakis (4-aminopyridine- $_{k}N^{1}$) di chloride copper (II) monohydrate interact through N-H…O hydrogen bonding with amino acids present in each enzymes.

Conclusion

Anti microbial, anti oxidant, anti diabetic, anti cancer activity of Tetrakis (4-aminopyridine- $_{k}N^{1}$) di chloride copper (II) monohydrate complex were studied. Because of the excellent antioxidant behaviour of $[CuCl_2(C_5H_6N_2)_4].H_2O$, it reduce the free radicals in the body and prevent cancer cells. The binding energy calculated using molecular docking software displays better binding energy for α-amylase compared to other enzyme and also to the early reported results.

Acknowledgment

The authors wish to thank the management of St.Joseph's College (Autonomous), Tiruchirappalli 620002, Tamil Nadu, India, for their encouragement and also thanks to Biospark Biotechnological Research Center (BBRC), Tiruchirappalli, Tamil Nadu, India, for biological activity study.

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