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RESEARCH ARTICLE

# Cytotoxicity and Anticancer Activity of [Cu(tet-am)]$(CH_3COO)_2.4H_2O$ Complex Against Ehrlich Ascites Carcinoma (EAC) Cells

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## Abstract

Cancer is a fatal disease characterized by aberrant cell proliferation, invasion, and spread to many body parts. Many chemotherapeutics, including metal complex cisplatin and carboplatin, have been utilized in cancer treatment throughout history. However, a lack of selectivity for cancer cells and related adverse effects with present treatment choices have fueled global attempts to find new anticancer drugs. In this study, a metallo-azamacrocyclic complex [Cu(tet-am)] $(CH_3COO)_2.4H_2O$ has been synthesized, and its antineoplastic activity against EAC cells was measured *in vivo*. To identify the potent chemotherapeutic drug with minimal or no side effects, cytotoxicity, tumor cell growth inhibition, haematological and biochemical parameters were assessed. The complex showed significant antitumor efficacy against EAC cells. It inhibited cell growth by 38.1% and 70.78% at doses of 2.0mg/kg and 4.00 mg/kg i.p., respectively, compared to control EAC-bearing mice ($p<0.0001$). Furthermore, EAC-bearing control mice revealed significant deterioration of RBC, WBC, and hemoglobin percentage; while, in complex-treated mice, these parameters were recovered to normal levels. Toxicological studies showed that there were changes in hematological (RBC, WBC, Hb gm/dl) and biochemical (serum glucose, cholesterol, creatinine, SGOT, SGPT) parameters during 10 consecutive days of treatment. However, the parameters were found to be gradually returned to normal levels after the treatment period, indicating that the complex had no adverse toxic effects on the host. The complex exhibits promising anticancer properties with minimal host toxicity. Thus, it has the potential to be developed into an effective chemotherapeutic drug; nevertheless, more preclinical and clinical research using animal and human models is required.

**Keywords:** Anticancer activity, Toxicity assays, Haematological parameters, Metallo-Aza Macrocyclic Complex.



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## Introduction

Cancer remains one of the leading causes of morbidity and mortality worldwide, posing a significant global health challenge. According to the World Health Organization (WHO), cancer accounted for nearly 10 million deaths in 2020 alone, making it the second leading cause of death globally (Gritsch et al. 2022). The increasing incidence of cancer is driven by various factors, including aging populations, lifestyle changes, and environmental exposures (Bray et al. 2018). Chemotherapy, which employs cytotoxic drugs to kill or inhibit cancer cells, remains a fundamental treatment due to its broad applicability across many cancer types despite advances in targeted therapies and immunotherapy (Holohan et al. 2013).

Metal-based drugs have gained increasing interest as potential chemotherapeutic agents because of their unique mechanisms of action and ability to interact with biological molecules differently from organic compounds (Johnstone et al. 2014). Both natural and synthetic compounds can target specific organs, tissues, and biomolecules within biological systems. Metal complexes, particularly Schiff bases, are known for their biological activity. In addition, metal ions, especially copper are essential for many enzymes, including cytochrome oxidase, superoxide dismutase, ceruloplasmin, and dopamine-β-hydroxylase. Copper (II) complexes have many pharmacological applications, such as antibacterial, antifungal, antidiabetic, antiarthritic, and enzymatic agents (Kuwabara and Sigman 1987, Mazumder et al. 1995, Nocentini and Barzi 1996). Moreover, they are of great interest in cancer therapy because they are less toxic to normal cells than malignant cells (Mazumder et al. 1995). Copper's innate reactivity and flexibility can affect cancer cell homeostasis, resulting in cytotoxicity and death (Engleka and Maciag 1992, Gao and Zhang 2023), keeping the normal cells unaffected. Moreover, copper, a vital trace element in the human body, adds another layer of intricacy to the metallodrug's anticancer properties (Ji et

al. 2023). Copper Schiff base complexes utilize the body's natural absorption and distribution processes for copper, allowing them to penetrate into tumor tissues more efficiently (Beckford et al. 2012, Pinheiro et al. 2023). This intrinsic characteristic, when paired with the customized selectivity of Schiff base ligands, shows potential for use as anticancer drugs (Mahendiran et al. 2017).

Recent studies have shown that Tetra azamacrocyclic Schiff base complexes showed better activity as compared to macrocyclic Schiff base ligand against cancer cell lines including cervical, epithelioma, human breast and cell line of normal human dermal fibroblast (Paulpandiyan et al. 2021). Additionally, metal retention or depositiom in tissues can increase the toxicity of metal complexes, which in turn can be detrimental to the host (Sarkar et al. 1995, Hultberg et al. 1998). Therefore, the presence of Cu (II) ions *in vivo* may be hazardous. A copper toxicity study showed that unbound copper in the blood can cause hemolytic anemia (Linder and Hazegh-Azam 1996), which is the main reason for copper-induced oxidative stress on erythrocytes (Rolfe and Twedt 1995). Furthermore, long-term toxicity can lead to liver cirrhosis, hemolysis, and damage to organs (Chang et al. 1992). However, the mode of action, type of ligand, chemical structure, configuration, and metal type would control their biological activity and toxicity (Köpf-Maier 1994). Considering the therapeutic potential and toxicity concerns of copper-based metallodrugs, it is imperative to investigate both their anticancer efficacy and biosafety profile. Therefore, this study aims to evaluate the cytotoxicity, *in vitro* anticancer activity, and *in vivo* toxicity of a newly synthesized copper (II) Schiff base complex, $Cu(tet\text{-}am)_2{\cdot}4H_2O$. The investigation is designed to determine its selectivity toward cancer cells, potential mechanisms of action, and any adverse systemic effects in animal models, providing a comprehensive assessment of its viability as an anticancer candidate.

## Materials and Methods

### Chemicals and reagents

Throughout the experiment, only reagent-grade chemicals and reagents were used. WBC and RBC fluid were purchased from D Lab Chemicals (Dhaka, Bangladesh). Biochemical assay kits, such as glucose, HDL, LDL, TG, creatinine, SGOT, and SGPT, assays kits were acquired from Human Diagnostic Worldwide (Wiesbaden, Germany).

### Source of the compound

The copper(II) Schiff base complex, $Cu(tet\text{-}am)_2{\cdot}4H_2O$, used in this study was previously synthesized and characterized in our laboratory. The synthesis involved multiple steps, including the preparation and resolution of isomeric ligands, and the final formation of the tet-am ligand and its copper complex. Detailed procedures, along with characterization data such as IR spectroscopy, electronic spectra, magnetochemical, and physical properties, have been reported in our earlier publications (Yasmin et al. 2013, Yasmin et al. 2017, Yasmin et al. 2021).

### Animals and cancer cells

In this study, adult male Swiss albino mice weighing 24-30 g were used. These mice were born and grown up in our laboratory following the regulations and criteria of the Institution of Biological Sciences at the University of Rajshahi in Bangladesh. Approval for the use of experimental animal (mouse) was obtained from the University ethic committee (Institutional Animal, Medical Ethics, Bio-Safety and Bio-Security Committee for Experimentations on Animal, Human, Microbes, and Living Natural Sources (No. 174/320(69)/IAMEBBC/IBSc), Institute of Biological Sciences, University of Rajshahi, Bangladesh. Ehrlich Ascites Carcinoma (EAC) cells were kindly provided by the Indian Institute for Chemical Biology (IICB) in Kolkata, India. These cells were maintained through regular intraperitoneal inoculation at a rate of $10^5$ cells per mouse every two weeks, in a controlled laboratory environment.

### Brine shrimp Nauplii lethality assay

Lethality of brine shrimp bioassay indicates cytotoxicity as well as a broad range of pharmacological activity of bioactive compounds (Hossain et al. 2024). For this bioassay, 0.5, 1, 2, 8, 10, and 20 µl of the test $[(Cu(tet\text{-}am)]\ (CH_3COO)_2.4H_2O$ complex (10 µg/µl) were taken in different vials, and ten brine shrimp nauplii in artificial seawater were added to each vial. Sea water is prepared by dissolving sodium chloride (38 g) in one liter of sterilized distilled water and then filtering off to get a clear solution. The pH of the seawater was maintained between 8 and 9 by using $NaHCO_3$ solution. Finally, each volume was adjusted to 5 mL by adding prepared seawater. All tests were carried out at room temperature (30°C) and under continuous lighting and were kept for 24 hours. The vials were then observed, the numbers of surviving nauplii in each vial were counted, and the results were noted. From this, the percentage of lethality of brine shrimp nauplii was calculated for each concentration of the samples. The nauplii mortality percentage was calculated for each concentration, and the $LC_{50}$ values were determined using Probit analysis (Hossain et al. 2024).

### *In vivo* cell growth inhibition

*In vivo*, tumor cell growth inhibition was performed as described by Siddika et al. (2021). In this study, Swiss albino mice weighing 30±6 grams were placed into four experimental groups (n=6). To evaluate the therapeutic potential of the compound, mice were injected intraperitoneally with $1\times10^6$ exponentially growing EAC cells. Treatment was initiated 24 hours post tumor cell inoculation. The compound was administered daily for a total of 5 consecutive days. The test complex was dissolved in distilled water and mice were injected intraperitoneally. Groups 2 and 3 received the test sample at doses of 2.0 mg/kg i.p. and 4.0mg/kg body weight i.p., respectively, per mouse per day. Whereas Group 4 served as a positive control where each mouse received a treatment of 0.3 mg/kg/day of bleomycin per day. Group 1 was deemed the control (untreated). On the sixth day, the mice were euthanized, and peritoneal fluid was collected using 0.9% saline. Viable cells were detected with trypan blue and counted using a hemocytometer under an inverted microscope (XDS-1R, Optika, Bergamo, Italy) on day six of initial inoculation. The total number of viable cells in every treated group animal was compared with that of the control (EAC-bearing only) group.

The cell growth inhibition was calculated using the following formula:

$$\%\ cell\ growth\ inhibition = (1 - \frac{Tw}{Cw}) \times 100$$

Where,
Cw = Mean of the number of tumor cells of the control group of mice.
Tw = Mean of the number of tumor cells of the treated group of mice.

### Monitoring of blood parameters in EAC-bearing mice

Following the intraperitoneal injection of the complex, haematological profiles in the experimental mice were evaluated for abnormalities. The effects of the complex on haematological parameters such as WBC, RBC, Hb content, etc., were measured using published protocols with cell dilution fluids (Mukherjee 2017). The mice were divided into four groups: 1st group was normal mice (n = 6), 2nd group's mice (n = 6) treated with EAC regarded as control, 3rd and 4th contained EAC bearing mice (n = 6) received complex at 4.00 mg/kg/day i.p. and 2.00 mg/kg/day i.p. for 10 consecutive days, respectively. And mice were sacrificed on day 11 with, and blood was taken from each group of mice using standard methods. The blood parameters were analyzed as previously described (Auwal et al. 2025).

### Host toxicity

### Blood parameters

Mice were divided into two groups: group 1, normal control mice (n = 6), and group 2, normal mice treated with test complex at 4.00 mg/kg/day (i.p.) (n = 18). The mice were treated with the test complex for 10 consecutive days and were sacrificed on days 5 (6 mice), 10 (6 mice), and 25 (6 mice), and blood was taken from each group of mice using previously published methods (Auwal et al. 2025).

### Biochemical parameters

After the end of the treatment, the mice in all groups were sedated and slaughtered using surgical blade No. 22, and the blood was collected in plastic centrifuge tubes. These were then left to solidify at 40°C for four hours. Following clotting, the blood samples were centrifuged at 4000 rpm for 15 minutes on a WIFUNG centrifuge LABO-50 M. The clear straw color serum was then collected in vials with a Pasteur pipette and stored at -20°C. All parameters were determined using the previous procedures (Jesmin et al. 2008).

### Statistical analysis

Each experiment was carried out in triplicate and the results of the study have been used to express the mean ± SEM (standard error of mean). Data has been analyzed by one-way ANOVA followed by Duncan's multiple range test using GraphPad Prism 8 software. A $p<0.05$ value was considered as significant.

## Results

### The structure of the Copper (II) complex [Cu(tet-am)]$(CH_3COO)_2.4H_2O$

The complex was synthesized in the laboratory of the Department of Chemistry, University of Rajshahi. The purity of the complex was checked by T.L.C. and characterized by Infra-red (IR) spectrometric data, UV-Visible spectrometric data, and physical data. Its melting point was 220-225°C. This complex had a fully equatorially oriented structure according to Fig.1. A structurally detailed explanation of the copper (II) complex [Cu(tet-am)]$(CH_3COO)_2.4H_2O$ was given in an already published article (Yasmin et al. 2021).

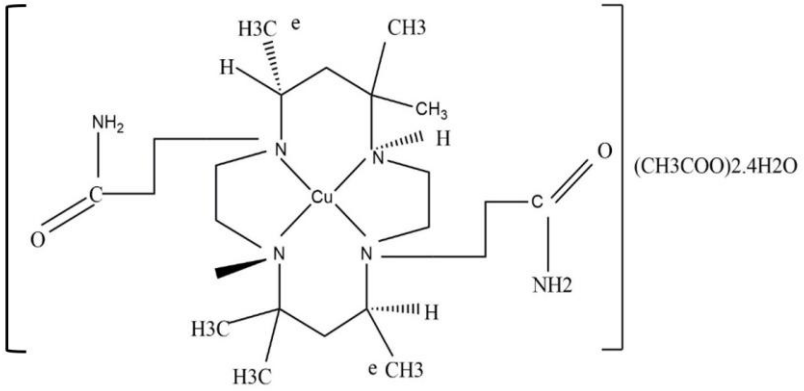


**Fig. 1:** Structure of the Copper (II) complex [Cu(tet-am)]$(CH_3COO)_2.4H_2O$.

### Cytotoxic effects of the test complex

The [Cu(tet-am)]$(CH_3COO)_2.4H_2O$ complex exhibited moderate cytotoxicity against brine shrimp nauplii with an $IC_{50}$ of 119.063 µg/ml after 24 hours of exposure (Fig. 2).

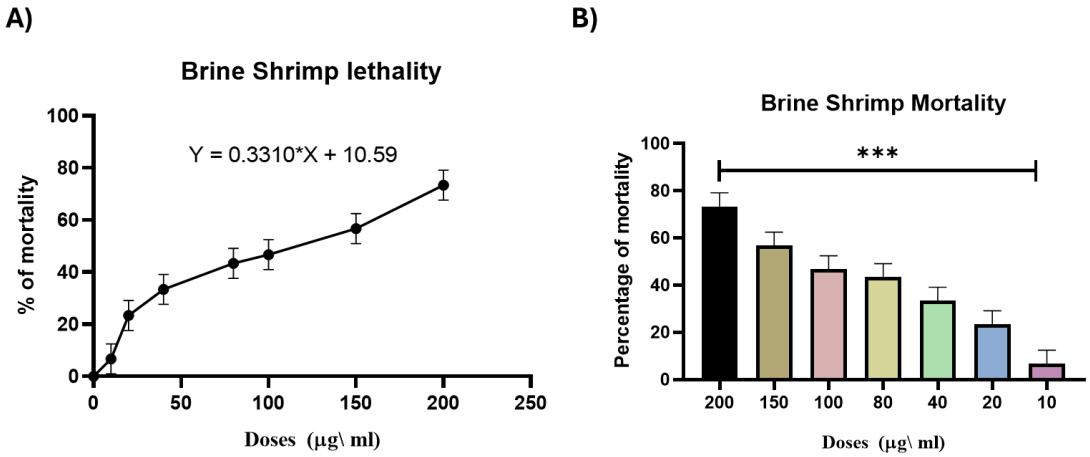


**Fig. 2:** Brine shrimp lethality bioassay.

**(A)** Cytotoxicity of [Cu(tet-am)] $(CH_3COO)_2.4H_2O$ against brine shrimp nauplii in a dose dependent manner. The results are presented as mean ± SEM. significant values are indicated by ***$p<0.001$ when compared to the control group. **(B)** Percent mortality of brine shrimp treated with test compound after 24 hours exposure.

### *EAC cell growth inhibition*

After treatment with the [Cu(tet-am)] $(CH_3COO)_2.4H_2O$ complex, the growth of EAC cells was effectively inhibited. Fig. 3A showed the number of viable EAC cells in untreated and treated cells. It was noted that the number of viable EAC cells significantly reduced in treated mice in comparison to that of untreated control EAC-bearing animals. The reduction in cell growth was 38.1% at dose of 2 mg/kg/day i.p whereas it showed 70.78% at 4 mg/kg/day i.p when compared to that of untreated control EAC bearing mice (Fig. 3B).

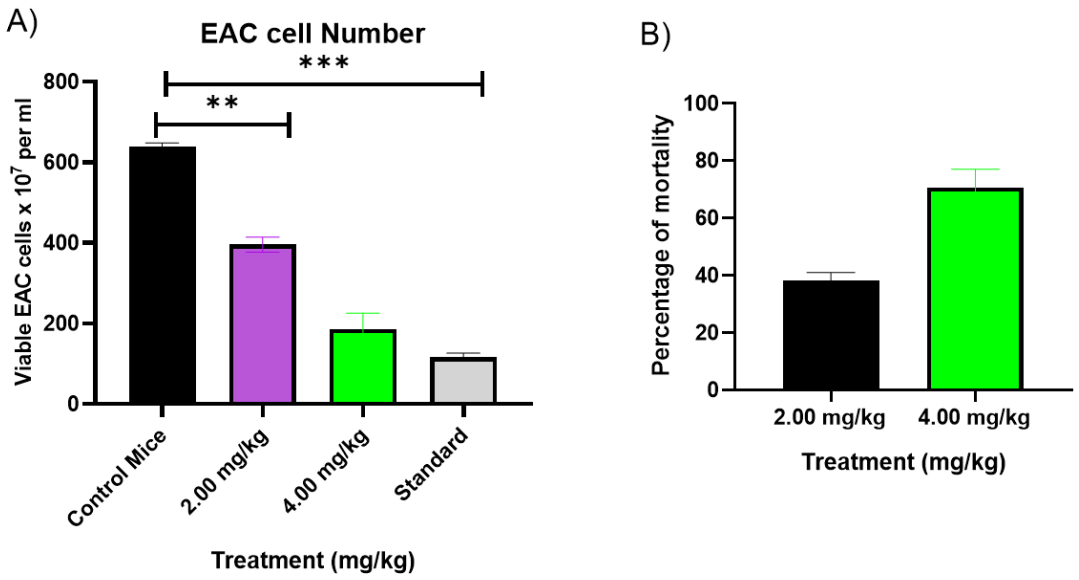


**Fig. 3:** Effects of test complex on EAC cell growth inhibition.

**A)** Number of viable EAC cells in control EAC-bearing and treated EAC-bearing mice. **B)** % of growth inhibition of EAC cells. Results are used as mean ± SEM, and significant values are indicated by **p<0.01 and ***p<0.001 when compared to the control group.

### Effects of the complex on haematological parameters in EAC cell-bearing mice

RBC, WBC, and Hb (gm/dl) showed moderate changes in control and treatment (2.00 mg/kg) groups, however, the depleted blood parameters of EAC-bearing mice returned towards normal levels after treatment with the test complex at high dose (4.00 mg/kg) (Fig. 4). This indicates that the test complex has no long-term harmful effects on the host.

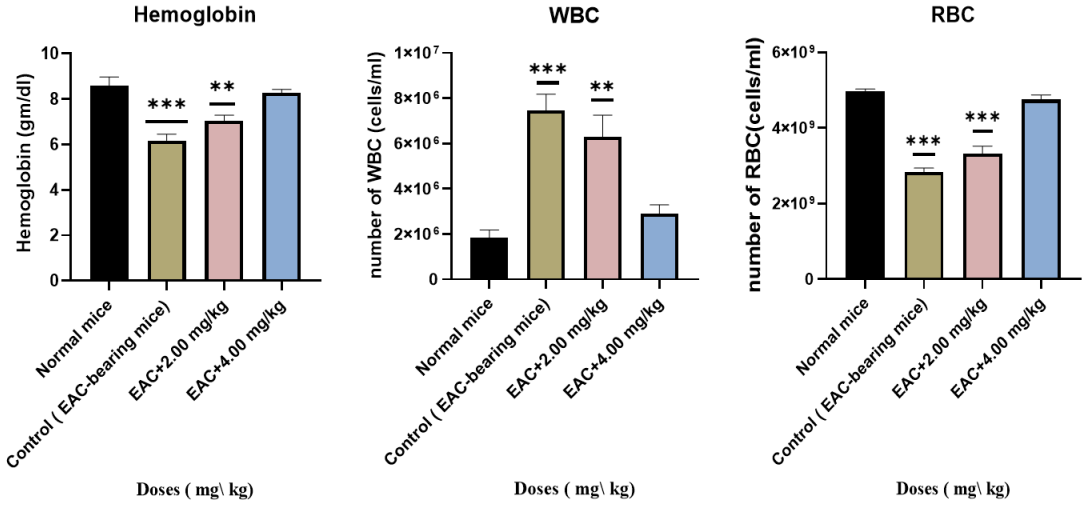


**Fig. 4:** Effect of test compound on blood parameters of EAC-bearing mice (Treated and Untreated) and normal mice.

Hemoglobin (Hb), WBC and RBC in both treated and normal mice were analyzed in experimental animals. In the untreated EAC-bearing control group, significant alterations in blood parameters were observed. However, treatment helped restore the blood parameters in the treated mice to values comparable to those of the normal group. Values are expressed as mean ± SEM, n = 6/group. Significant values are indicated by **p<0.01 and ***p<0.001 when compared to the normal mice by Dunnett's test.

## Toxicity study *in vivo*

### Hematological parameter

Table 1 presents the hematological parameters of normal Swiss albino mice (not inoculated with EAC cells) treated with the test complex at a dose of 4.00 mg/kg via intraperitoneal injection for 10 consecutive days, assessed on days 5, 11, and 25. It has been observed that RBC, WBC, and Hb gm/dl showed moderate changes during the treatment period but gradually returned to near-normal levels by day 25, after the initial dose. ($p<0.05$). This suggests that the test compound does not exhibit any long-term toxic effects.

**Table 1: Effect of test complex on blood parameters of normal mice on day 5, 10 and 25.**

| Name of experiment | | RBC (Cells/ml) | WBC (Cells/ml) | Hemoglobin (gm/dl) |
|---|---|---|---|---|
| Normal mice | | $(4.96\pm6.55)\times10^{9}$ | $(18.33\pm3.51)\times10^{3}$ | 8.57±0.40 |
| Treatment (4.00 mg/kg) | Day 5 | $(6.66\pm5.29)\times10^{9*}$ | $(12\pm2.64)\times10^{3*}$ | 8.5±0.5 |
| | Day 10 | $(6.42\pm13.11)\times10^{9*}$ | $(13.67\pm1.15)\times10^{3*}$ | 7.9±0.36 |
| | Day 25 | $(4.81\pm6.55)\times10^{9}$ | $(17.67\pm2.08)\times10^{3}$ | 9±0.5 |

Values are expressed as mean ± SEM, n = 6/group. *$p<0.05$ compared to the normal mice by Dunnett's test.

### Biochemical analysis

When biochemical parameters were evaluated (Fig. 5A and B), no significant difference was observed between treated (4.0 mg/kg i.p.) and normal control mice groups. During the 10-day treatment period, significant changes in biochemical parameters were observed on day 6 and 10 however, on day 25, the biochemical levels of these mice were remarkably back to normal levels. It was observed that there was a significant change between the HDL value of treated mice and normal mice (Fig. 5B).

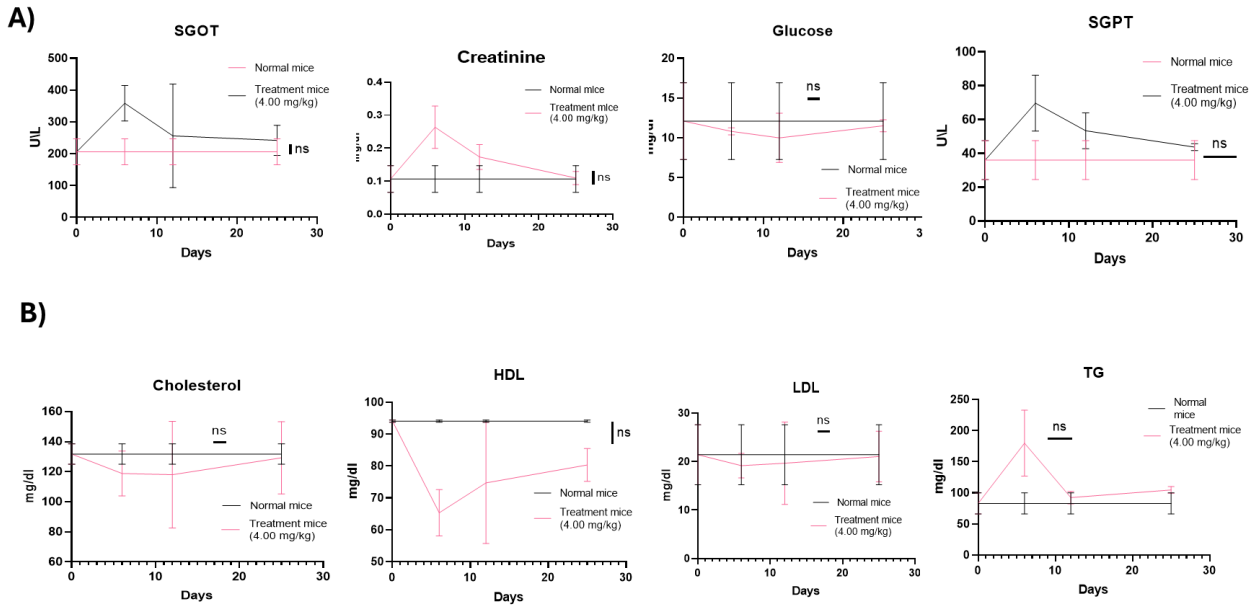


**Fig. 5:** Effect of the test complex on biochemical parameters of treated and normal mice.

**(A)** Serum Glucose, Creatinine, SGOT, and SGPT and **(B)** Cholesterol, HDL, LDL and TG in both treated and normal mice were analyzed in experimental animals. It was noted that the tested parameters were slightly changed during the treatment period, however, they have been restored towards the normal level after 25 days. The results indicated that the compound has no long-term effects on the biochemical parameters of mice. Values are expressed as mean ± S.E.M., n = 6/group. $p>0.05$ compared to the normal mice by Dunnett's test.

## Discussion

The cytotoxic potential of the complex $[Cu(tet\text{-}am)](CH_3COO)_2.4H_2O$ was evaluated against brine shrimp nauplii, illustrating significant cytotoxicity in a dose-dependent manner (Fig. 2), with an $LC_{50}$ of 119.063 µg/ml after 24 hours of treatment. The result suggests its potential pharmacological applications of the test complex. Anticancer activity of the complex was assessed against EAC cells in Swiss albino mice *in vivo*. Cell growth inhibition and restoration of haematological parameters have been measured to determine the efficiency of the complex in EAC cell-bearing mice. All effective chemo drugs have shown promising activity against examined parameters (Anand et al. 2022). According to our study, the growth of EAC cells treated with the test complex was significantly reduced. The highest growth inhibition noticed about 70.78% at the dose of 4.0 mg/kg/day i.p. While the dose decreased to 2.0 mg/kg/day i.p., about 38.1% inhibited EAC cell growth compared to control EAC-bearing mice ($p<0.01$). Similar copper complexes showed more than 80 % growth inhibition against EAC cells at 50 mg/kg (Sathisha et al. 2008). Thus, further increments of the dose of the tested complex could enhance the growth inhibition of cancer cells. The anticancer activity of $Cu(tet\text{-}am)_2{\cdot}4H_2O$ may be attributed to copper's redox cycling between Cu(I) and Cu(II), which generates reactive oxygen species (ROS) via Fenton-like reactions (Santini et al. 2008). This oxidative stress can lead to DNA damage, lipid peroxidation, and activation of apoptotic pathways. Cancer cells are particularly vulnerable due to their elevated basal ROS, higher copper uptake, and weaker antioxidant defenses, allowing selective toxicity over normal cells. These redox-based mechanisms likely contributed to the observed tumor growth inhibition and merit further molecular investigation.

The evaluation of haematological parameters differed among normal mice, EAC cell-bearing mice and EAC cell-bearing mice treated with the test complex. The haematological parameters, including RBC, WBC, and Hb, were deteriorating in mice with cancer indicating cancer induced anemia and systemic inflammation. Following treatment with the complex, the total RBC and hemoglobin levels in treated EAC-bearing mice increased while WBC counts decreased towards normal levels. Typically, anemia develops in EAC-bearing mice due to decreased RBC and hemoglobin percentage. RBC and hemoglobin levels increased following the complex treatment, whereas infection was reduced as the WBC returned towards normal. When animals with EAC cells were treated with the test complex, their blood parameters eventually returned to normal. As a result of the treatment with the test complex, key characteristics of an effective chemotherapeutic agent, such as significant cell growth inhibition and restoration of haematological parameters, were observed. Restoration of these parameters in treated mice underscores the complex's potential as a chemotherapeutic agent with minimal hematological toxicity. Thus, the chemical is believed to have anticancer properties and could be a candidate for further development.

Afterwards, we investigated the complex's overall host toxicity using Swiss albino mice. In the toxicity experiment, we compared the haematological parameters of normal control group mice to normal mice treated that had been receiving treatment for 10 days continuously. After treatment, we checked their haematological parameters on days 5, 10, and 25. The findings of this study revealed that RBC remained normal during and after 25 days of initial treatment, and RBC count did not change significantly. Although, WBC levels fluctuated substantially during therapy for the first five days, but were normalized by day 25. Similarly, the percentage of hemoglobin remained constant over the treatment regimen when compared to that of normal mice. Chemotherapy drugs often cause anaemia, leucopenia, but mice treated with this complex did not develop anaemia during treatment and also had no significant effect on leucocytes. These findings suggest the absence of long-term hematological toxicity.

For further analysis of the biochemical markers relating to liver functions, such as SGOT and SGPT, and renal functions, such as serum creatinine, and serum glucose, serum cholesterol in mice were assayed. Serum levels of these parameters were found to be altered along with the pathological changes of these organs. In the event of hepatic necrosis, cirrhosis, and obstructive jaundice, the serum level of SGOT and SGPT might rise to 200 IU/L. If a drug possesses any effect on the liver and kidney, several pathological changes may occur, and ultimately, the serum level of these parameters will be altered. From literature study it has been shown that lower cholesterol level (Wang et al. 2013) is present in cancer and higher levels of SGOT and SGPT resulted in serum of damaged liver (Chauhan et al. 2016) which support our study. Additionally, we analyzed the biochemical parameters, including blood glucose, cholesterol, HDL, LDL, Triglyceride, creatinine, SGOT, and SGPT, of treated

mice, who received treatment for 10 days at a dosage of 4.00 mg/kg body weight i.p., in comparison to normal mice. The biochemical measurements indicated that the serum glucose, cholesterol, and LDL levels were nearly equivalent to normal mice during and after the treatment. From the experiment on creatinine, HDL, SGOT, and SGPT moderately changed in 5 days of treatment, but regained normal levels during and after treatment in comparison to the normal mice which was a good sign for no damage or disease. These outcomes suggest the compound does not induce persistent hepatotoxicity or nephrotoxicity in host.

## Conclusion

Collectively, these findings suggest that the produced [Cu(tet-am)]$(CH_3COO)_2.4H_2O$ complex is a promising anticancer therapeutic candidate. Furthermore, it can be considered a drug for cancer and infectious disease treatment. Its anticancer efficacy, characterized by significant tumor growth inhibition and normalization of haematological parameters, and minimal systemic toxicity, supporting its potential for therapeutic development. Nonetheless, the present study primarily reports phenotypic outcomes without delving into the underlying molecular mechanisms. Future investigations should focus on elucidating mechanistic pathways-particularly those involving oxidative stress, DNA damage, mitochondrial dysfunction, and apoptotic signaling cascades-that may account for the observed cytotoxicity and antitumor activity. Techniques such as caspase activation assays, DNA fragmentation analysis, and mitochondrial membrane potential studies could provide valuable mechanistic insights. Moreover, further optimization of the compound's bioactivity, comprehensive safety assessments, and evaluation of synergistic interactions with established chemotherapeutics will be crucial. Preclinical validation using human cancer cell lines and mechanistic assays will serve as a necessary foundation for translational advancement and potential clinical application.

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**Conflict of interest:** The authors hereby declare that there is no conflict of interest regarding the publication of this work.

**Author's contribution**: MA collected samples and data, conducted experiments, and wrote the original draft of the manuscript. AA, MMH, and TK analyzed and processed the data. SY, PGR, and MHR synthesize and characterize the compound. MAH, FI, and JAK designed the experiment, supervised the study, and corrected the manuscript. All authors have read and approved the final manuscript.

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**Data availability**: All data generated in the study are reported in the manuscript, and unprocessed data will be available to the corresponding author upon request.

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