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Original Article

# Hepatoprotective Assessment of Copper Calx against Anti-Tubercular Drug-induced Hepatotoxicity in Rats

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## ABSTRACT

**Background:** Anti-Tubercular Drugs (ATDs), while effective in treating tuberculosis, are associated with hepatotoxicity, leading to liver damage and complications. Calx of Copper, a traditional Ayurvedic preparation, has shown potential hepatoprotective properties. **Objectives:** To investigate the potential hepatoprotective role of Calx of Copper in mitigating ATD-induced hepatotoxicity and to examine its impact on liver function markers and histopathological changes in rats. **Materials and Methods:** Thirty male Wistar rats were randomly divided into five groups ($n$=6 per group): control, ATD, Calx of Copper, ATD+Calx of Copper, and silymarin (used as a standard hepatoprotective agent). Hepatotoxicity was induced in the ATD, ATD+Calx of Copper, and silymarin groups by administering a combination of isoniazid, rifampicin, and pyrazinamide for 25 days. Calx of Copper and silymarin were orally administered at doses of 6.17 mg/kg and 12.33 mg/kg, and 300 mg/kg b.w, respectively, in their respective groups. Liver function markers, including serum transaminase and alanine Aminotransferase (ALT), were measured at the end of the study. A histopathological examination of liver tissues was also performed. **Results:** ATD-induced hepatotoxicity was evident through elevated serum SGPT, SGOT, ALT, and ALP levels and histopathological alterations in liver tissue. Co-administration of Calx of Copper significantly reduced SGPT, SGOT, ALT, and ALP ($p$<0.05) and improved liver histopathological changes compared to the ATD group. The hepatoprotective effect of Calx of Copper was comparable to that of silymarin. **Conclusion:** Copper calx demonstrated significant hepatoprotective activity against ATD-induced hepatotoxicity in rats, as evidenced by normalizing liver function markers and histopathological improvements. These findings suggest that Calx of Copper may be a promising adjuvant therapy for mitigating liver damage associated with anti-tubercular drug treatment.

**Keywords:** Anti-Tubercular, hepatotoxicity, Copper Calx, Antioxidant, Rat.

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## INTRODUCTION

Tuberculosis (TB) constitutes a persistent infectious ailment predominantly impacting pulmonary tissue. But can infect other body parts.[1] *Mycobacterium tuberculosis* is the etiological agent of tuberculosis and a significant contributor to global morbidity and mortality. The World Health Organization reported that approximately 1.4 million fatalities were attributed to TB in 2019, with an estimated 10 million individuals contracting the illness within the same period.[2] The situation is further aggravated by the increasing prevalence of drug-resistant TB strains, which are more challenging to treat. The contemporary primary therapeutic approach for tuberculosis employs a multi-drug regimen encompassing rifampicin, isoniazid, ethambutol, and pyrazinamide. These drugs are administered for at least six months to eradicate the bacteria effectively. However, TB treatment's significant challenge is the hepatotoxicity associated with anti-tubercular drugs. Hepatotoxicity refers to the potential of these drugs to cause liver damage, which can lead to severe complications or even death.[3,4] Anti-tubercular drug-induced hepatotoxicity has been reported in various studies, with isoniazid being the primary culprit. This dose-dependent hepatotoxicity can manifest as hepatitis, hepatic necrosis, or even acute liver failure in severe cases. The incidence of drug-induced liver injury varies across populations and depends on factors such as age, gender, genetic predisposition, and concomitant drug use.[5] Given the significance of this issue, there is a pressing need for effective hepatoprotective agents that can mitigate the adverse effects of anti-tubercular drugs on the liver. Several natural products, including herbal extracts and minerals, have

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been investigated for their hepatoprotective potential. One mineral-based hepatoprotective agent is copper calx, an ayurvedic medicine known for its anti-inflammatory, antioxidant, and immunomodulatory properties.[6]

Copper calx, known as *Tamra bhasma* in Ayurveda, is an ancient Indian therapeutic agent prepared from purified copper through calcination.[7] Copper calx is utilized in traditional medicine primarily for treating various conditions, including anemia, liver disorders, and inflammatory diseases. Recent studies have shown that copper calx possesses antioxidant, anti-inflammatory, and immunomodulatory activities, crucial in mitigating liver damage caused by various factors.[8] The liver is an essential organ, performing multiple metabolic and detoxifying functions. Oxidative stress and inflammation are critical factors in developing a drug-induced hepatic injury. An asymmetry in the generation of Reactive Oxygen Species (ROS) and the organism's antioxidant defense mechanisms can result in oxidative stress, leading to cellular harm and hepatotoxicity.

Inflammation also significantly contributes to liver damage, as it can generate pro-inflammatory cytokines and chemokines, which attract immune cells and exacerbate tissue injury.[9] Copper calx has exhibited potential in mitigating oxidative stress and inflammation owing to its antioxidative and anti-inflammatory characteristics. It can neutralize free radicals, subsequently reducing ROS concentrations in the body. Furthermore, copper calx modulates the immune response by inhibiting the synthesis of pro-inflammatory cytokines while enhancing the secretion of anti-inflammatory cytokines.[10] These attributes imply that copper calx could be a promising hepatoprotective agent against hepatotoxicity induced by anti-tubercular drugs.

## MATERIALS AND METHODS

### Chemicals and Drugs

Silymarin was sourced from (West-Coast Pharmaceutical Works Ltd., India), Calx of Copper was obtained from (Pentacare Ayurpharma, Bengaluru, India), and Isoniazid was procured from (the Drug Testing Laboratory in Bengaluru, India). At the same time, Rifampicin was acquired from Lupin Ltd., India. Pyrazinamide was secured from Yarrow Chem Products in Mumbai, India. All other chemical reagents employed in this study were of analytical grade.

### Animals

Male Wistar rats with an average weight of 180 ± 20 g were obtained from a Licensed breeder (Vaarunya Biolabs Pvt. Ltd.), Bangalore. Before and during the experimental initiation, the rats were housed in individual polypropylene cages under controlled environmental conditions, including a temperature of 23°C ± 2°C, 55±5% relative humidity, and a 12 hr light/dark cycle, and the acclimation period lasted for one week. Throughout the study, the rats were provided with a standard pellet diet and water *ad libitum*. All experimental procedures involving the animals adhered to the guidelines set forth by the Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA). Furthermore, the research protocol received approval from the Ethical Institutional Animal Ethical Committee with Reference no-(05/HP/2021).

### Experimental Design

Male Wistar rats were utilized and distributed into five distinct groups, each comprising six rats ($n$=6). Group I, a control group, received vehicle 1% Carboxy Methyl Cellulose (CMC). Group II, positive control, administered a combination of anti-TB drugs (Isoniazid 30.85 mg/kg, Rifampicin61.7 mg/kg, and Pyrazinamide 132.65 mg/kg, p.o.) for 25 days to produce hepatotoxicity.[11] Group III and IV were administered with Calx of Copper (6.17 mg/kg and 12.33 mg/kg, b.w.), respectively,followed by anti-TB drugs administration orally (INH 30.85 mg/kg, RIF 61.7 mg/kg, and PYZ132.65 mg/kg) for 25 days. Group V, a standard group, received silymarin, a standard drug (300 mg/kg b.w.), followed by administering anti-TB medications orally (INH 30.85 mg/kg, RIF 61.7 mg/kg, and PYZ 132.65 mg/kg).

After completing the 25-day experimental phase, the body weight of each rat was measured before euthanasia. Following an overnight fasting period, the subjects were anesthetized and euthanized 24 hr post-administration of the final drug dose. Blood samples were collected via the retro- orbital plexus and subsequently through the cardiac puncture, allowing for clot formation before serum separation was conducted using centrifugation at 2,500 x g for a 15 min duration at 4°C. The hepatic tissue was cleansed in saline solution twice, gently dried, and weighed to determine the relative liver weight as a percentage of the animal's total body weight. A formalin solution for histopathological assessment preserved a portion of the hepatic tissue. The remaining tissue specimens were maintained at -20°C for 12 hr before further analysis.

### Evaluation of Hepatic Function Test

Biochemical markers, including Serum Glutamic-Oxaloacetic Transaminase (SGOT), Glutamic Pyruvic Transaminase (SGPT), Alkaline Phosphatase (ALP), total bilirubin, total protein, Albumin (ALB), urea and creatinine were analyzed using established kit adhering to the protocols provided by the respective kits.

### Evaluation of Antioxidant Parameters

Liver tissues from rats were prepared as a 10% homogenate in phosphate buffer (pH 7.4) using a Potter-Elvehjem glass homogenizer. The resulting homogenate was subjected to centrifugation at 12,000 rpm for 20 min at 4°C to obtain The Post-Mitochondrial Supernatant (PMS), which was then employed for Malondialdehyde (MDA), Catalase (CAT), Reduced Glutathione (GSH), and Superoxide Dismutase (SOD) activities

in the liver PMS were determined according to the methods delineated by Aebi, Kakkar *et al*., and Upadhyay.[12-14]

### Histopathological Evaluation

For histological analysis, liver tissues were preserved in 10% neutral buffered formalin. Subsequently, the samples underwent dehydration through a graded alcohol series (50%–100%) and were embedded in paraffin. Finally, sections approximately 5 µm thick were prepared and stained using conventional hematoxylin and eosin staining techniques for microscopic examination. This qualitative assessment aimed to identify and quantify histopathological alterations in the collected liver specimens.

### Statistical analyses

Values are presented as Mean ± SEM, with $n$=6 in each group. GraphPad Prism was used for the statistical analysis, which involved one-way ANOVA followed by Tukey's *post hoc* tests.

## RESULTS

### Influence of Calx of Copper on body weight, and liver weight

The findings are depicted in Figure 1, which shows the initial and final body weights of rats subjected to Isoniazid, Rifampicin, and Pyrazinamide treatment. A substantial decrease in body weight was observed, from a control value of 220±1.8g to 170±4.7g ($p$<0.001). In contrast, administering Calx of Copper at concentrations of 6.17 mg/kg and 12.33 mg/kg, b.w led to a notable increase in the weight of rats treated with Isoniazid, Rifampicin, and Pyrazinamide, reaching 187±6.9g ($p$<0.05) and 210±4.2g ($p$<0.001), respectively. Additionally, 300mg/kg silymarin administration significantly increased body weight, reaching 215±1.7g ($p$<0.001) compared to the control group I. Figure 2 displays the liver weights of rats treated with Isoniazid, Rifampicin, and Pyrazinamide, demonstrating a significant increase ($p$<0.001) compared to control group I. The administration of Calx of Copper at 6.17 mg/kg and 12.33 mg/kg, b.w led to a significant reduction in liver weight, with values ranging from $p$<0.05 to $p$<0.001.

### Impact of Calx of Copper on liver marker levels

The impact of the Calx of Copper on the combination of INH, RIF, and PYZ-induced hepatotoxicity in rats is demonstrated in Figures 3-5. The co-administration of INH 30.85 mg/kg, RIF 61.7 mg/kg, and PYZ 132.65 mg/kg, p.o notably increased the levels of SGPT, SGOT, ALP, Urea, and Serum total Bilirubin ($p$<0.001), while concurrently decreasing total protein ($p$<0.001) in comparison to the control group I. Pre-treatment with the Calx of Copper at 6.17 mg/kg and 12.33 mg/kg, b.w, given 2 hr before INH, RIF, and PYZ exposure, significantly mitigated the upregulation of transaminases, ALP, urea, and total bilirubin closer to normal levels. The hepatoprotective effect was more pronounced at a dose of 6.17 mg/kg and 12.33 mg/kg, b.w, resulting in a considerable increase ($p$<0.01) in total protein levels in the serum as opposed to the hepatotoxic control group II. Furthermore, the use of silymarin markedly ameliorated ($p$<0.001) the perturbations in liver marker levels compared to the hepatotoxic group II.

### Effect of Calx of Copper on anti-oxidant parameters

The hepatotoxic control group (Group II) exhibited a marked ($p$<0.001) decline in GSH levels in comparison to the normal control, suggesting a reduction in GSH values that safeguard hepatocytes from the harmful consequences of INH, RIF, and PYZ (Figure 6). Nevertheless, GSH levels significantly increased ($p$<0.001) following 25 days of Calx of Copper treatment (6.17 mg/kg and 12.33 mg/kg, b.w) relative to the hepatotoxic control (Group II).

The enzyme activities of MDA and SOD in homogenates from normal, hepatotoxic control, and Calx of Copper-treated groups are depicted in Figures 7 and 8. The hepatotoxic control group (Group II) revealed a significant decrease SOD activities

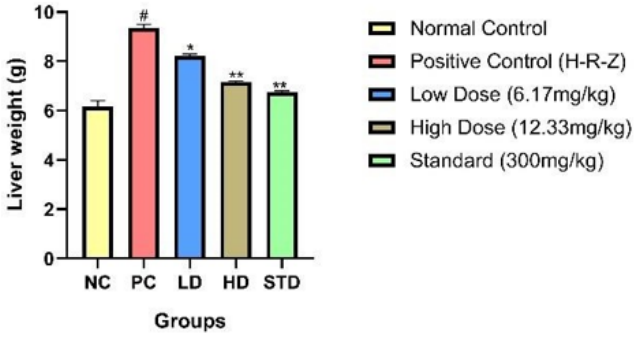


**Figure 1:** Effect of Calx of Copper on body weight of (INH, RIF, and PYZ) induced hepatotoxicity in experimental groups of rats. Values are expressed as mean ± SEM of 6 rats in each group. *P* values: #<0.001 compared with respective normal control group I *P* values: *<0.05 and **<0.001 compared with group II (INH, RIF, and PYZ).

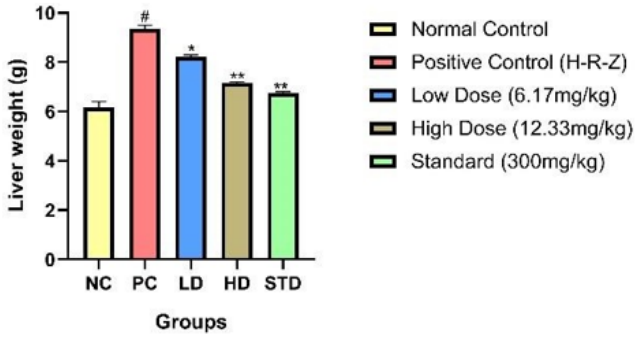


**Figure 2:** Effect of Calx of Copper on liver weight and relative liver weight of (INH, RIF, and PYZ) induced hepatotoxicity in experimental groups of rats. Values are expressed as mean ± SEM of 6 rats in each group. *P* values: #<0.001 compared with respective normal control group I *P* values: *<0.05 and **<0.001 compared with group II INH, RIF, and PYZ).

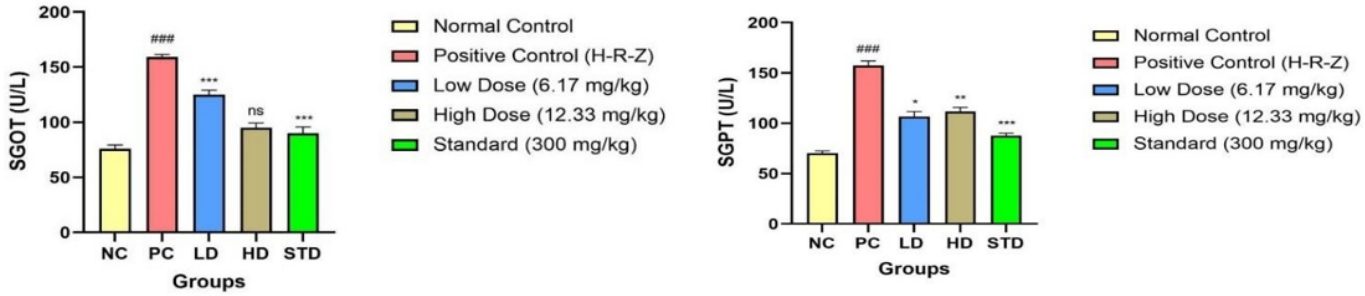


**Figure 3:** Effect of Calx of Copper treatment on SGOT and SGPT levels against the hepatotoxic effect of INH, RIF, and PYZ in Wistar rats. ###$p$<0.001 versus group I (normal control), ***$p$<0.001 versus group II (hepatotoxic control), *$p$<0.01 - **$p$<0.001 versus group II (hepatotoxic control), nsp>0.05 versus group I (normal control).

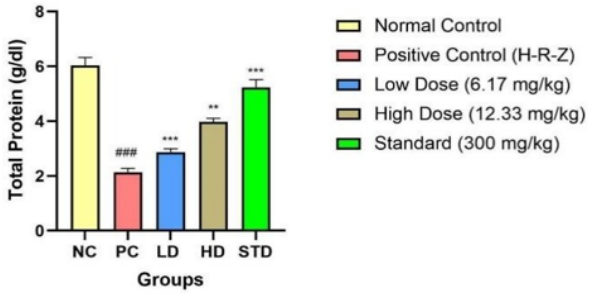


**Figure 4:** Effect of Calx of Copper treatment on total protein level against the hepatotoxic effect of INH, RIF, and PYZ in Wistar rats. ###$p$<0.001 versus group I (normal control), ***$p$<0.001 versus group II (hepatotoxic control), **$p$<0.001 versus group II (hepatotoxic control).

compared to the normal control ($p$<0.001), while there is an increase in MDA activities compared to the normal control ($p$<0.001). Treatment with Calx of Copper (6.17 mg/kg and 12.33 mg/kg, b.w) resulted in a substantial enhancement in the activities of these antioxidant enzymes relative to the hepatotoxic control ($p$< 0.01–$p$<0.001). No significant difference was detected in the MDA and SOD activities in the standard group (silymarin, 300 mg/kg) compared to the normal control.

### Histopathological observations

Figure 9A-D demonstrates the microscopic analysis of a normal liver section revealed intact parenchymal cells and well-organized mucosal glands. These glands comprised cells containing vesicular nuclei containing nucleoli and abundant eosinophilic cytoplasm. The basement membrane was found to be thick and intact. However, in the group of rats exposed to a hepatotoxic substance, the liver cells exhibited degeneration and necrosis. Pycnotic nuclei, granular cytoplasm, and an increase in intercellular spaces with inflammatory collections were observed, indicating the loss of cellular boundaries.

On the other hand, when rats were treated with Calx of Copper at two different doses, marked changes were observed at the periphery of the cells, granular cytoplasm, and a decrease in intercellular spaces compared to the hepatotoxic control group. The liver sections displayed minimal degenerative changes of hepatocytes and minimal swelling. The treatment with Calx of Copper also demonstrated a significant reduction in tissue damage and minimal evidence of inflammation. A reference drug, silymarin (300 mg/kg body weight), was administered to a separate group (Group V), and the liver tissue displayed normal architecture without any abnormality or degenerative changes in hepatocytes.

## DISCUSSION

This investigation evaluated the hepatoprotective properties of *Tamra bhasma* (Calx of Copper) in mitigating liver toxicity induced by antitubercular medications (isoniazid, rifampicin, and pyrazinamide) in a rat model. Combining these drugs in antitubercular chemotherapy may lead to drug-induced hepatotoxicity, a severe adverse effect. Hepatocellular steatosis and centrilobular necrosis, possibly accompanied by cholestasis, may occur when these three drugs are used together. Both animal and human studies have proposed that toxic isoniazid metabolites covalently bind to cellular macromolecules.[15] Hepatotoxicity arises from converting monoacetyl hydrazine to a poisonous metabolite via the cytochrome P450 pathway. Rifampicin stimulates cytochrome P450 enzyme activity, increasing the production of toxic acetyl hydrazine metabolites. Furthermore, rifampicin can enhance the metabolism of isoniazid to isonicotinic acid and hydrazine, exhibiting hepatotoxic properties. By accelerating the oxidative elimination rate, rifampicin shortens the plasma half-life of acetyl hydrazine and its conversion to active metabolites, contributing to the higher incidence of liver necrosis caused by the combined use of isoniazid, rifampicin, and pyrazinamide.[16]

Hepatotoxicity from antitubercular drugs can be attributed to various mechanisms, including oxidative stress-induced liver injury. The combination of isoniazid, rifampicin, and pyrazinamide has been demonstrated to impede biliary secretion and elevate liver cell lipid peroxidation, possibly due to the involvement of cytochrome P450. Additionally, increased serum levels of hepatic enzymes, such as Serum Glutamic Pyruvic Transaminase (SGPT), Serum Glutamic Oxaloacetic Transaminase (SGOT),

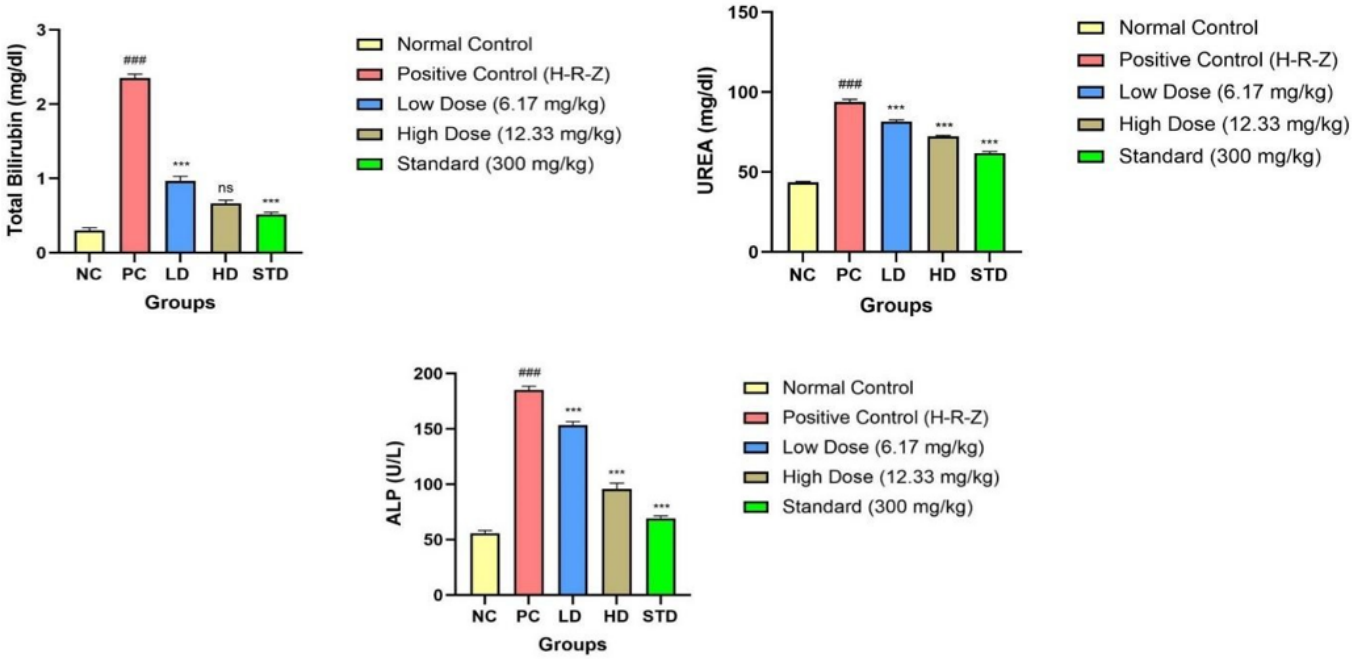


**Figure 5:** Effect of Calx of Copper treatment on total bilirubin, urea, and ALP levels against the hepatotoxic effect of INH, RIF, and PYZ in Wistar rats. ###$p$<0.001 versus group I (normal control), ***$p$<0.001 versus group II (hepatotoxic control), nsp>0.05 versus group I (normal control).

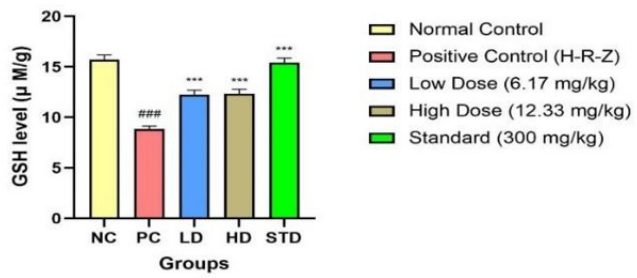


**Figure 6:** Effect of Calx of Copper treatment on GSH contents against the hepatotoxic effect of INH, RIF, and PYZ in Wistar rats. ###$p$<0.001 versus group I (normal control), ***$p$<0.001 versus group II (hepatotoxic control).

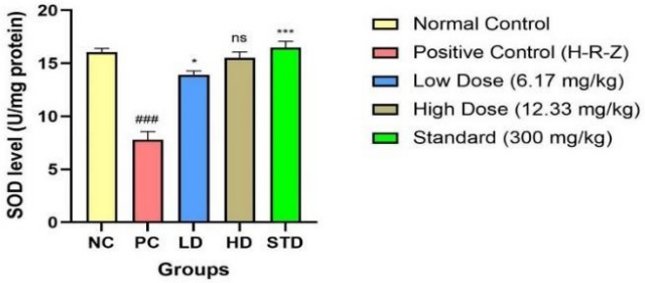


**Figure 7:** Effect of Calx of Copper treatment on SOD contents against the hepatotoxic effect of INH, RIF, and PYZ in Wistar rats. ###$p$<0.001 versus group I (normal control), *$p$<0.01 versus group II (hepatotoxic control), nsp>0.05 versus group I (normal control), ***$p$<0.001 versus group II (hepatotoxic control).

and Alkaline Phosphatase (ALP), indicate liver damage as these enzymes leak from the injured liver tissue. However, this study

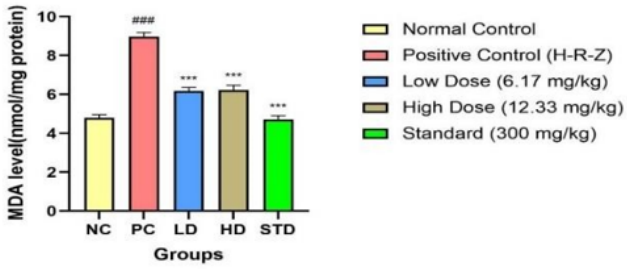


**Figure 8:** Effect of Calx of Copper treatment on MDA contents against the hepatotoxic effect of INH, RIF, and PYZ in Wistar rats. ###$p$<0.001 versus group I (normal control), ***$p$<0.001 versus group II (hepatotoxic control).

found that co-treatment with Calx of Copper at doses of 6.17 mg/kg and 12.33 mg/kg, b.w, resulted in near-normal levels of serum marker enzymes, suggesting protection against liver damage. This protective effect might be due to the reduced tissue damage from Calx of Copper administration. Moreover, rats treated with isoniazid, rifampicin, and pyrazinamide exhibited significantly elevated Serum Bilirubin (SBL) levels and decreased Total Protein (TP), indicative of liver damage. However, treatment with silymarin or Calx of Copper restored all studied parameters to normal levels. Combining isoniazid, rifampicin, and pyrazinamide can cause liver damage through various mechanisms, including oxidative stress-induced injury. Nonetheless, treatment with Calx of Copper can protect against liver damage and restore liver function.

The body possesses a defense mechanism to guard against the detrimental effects of free radicals, which can damage cells. This

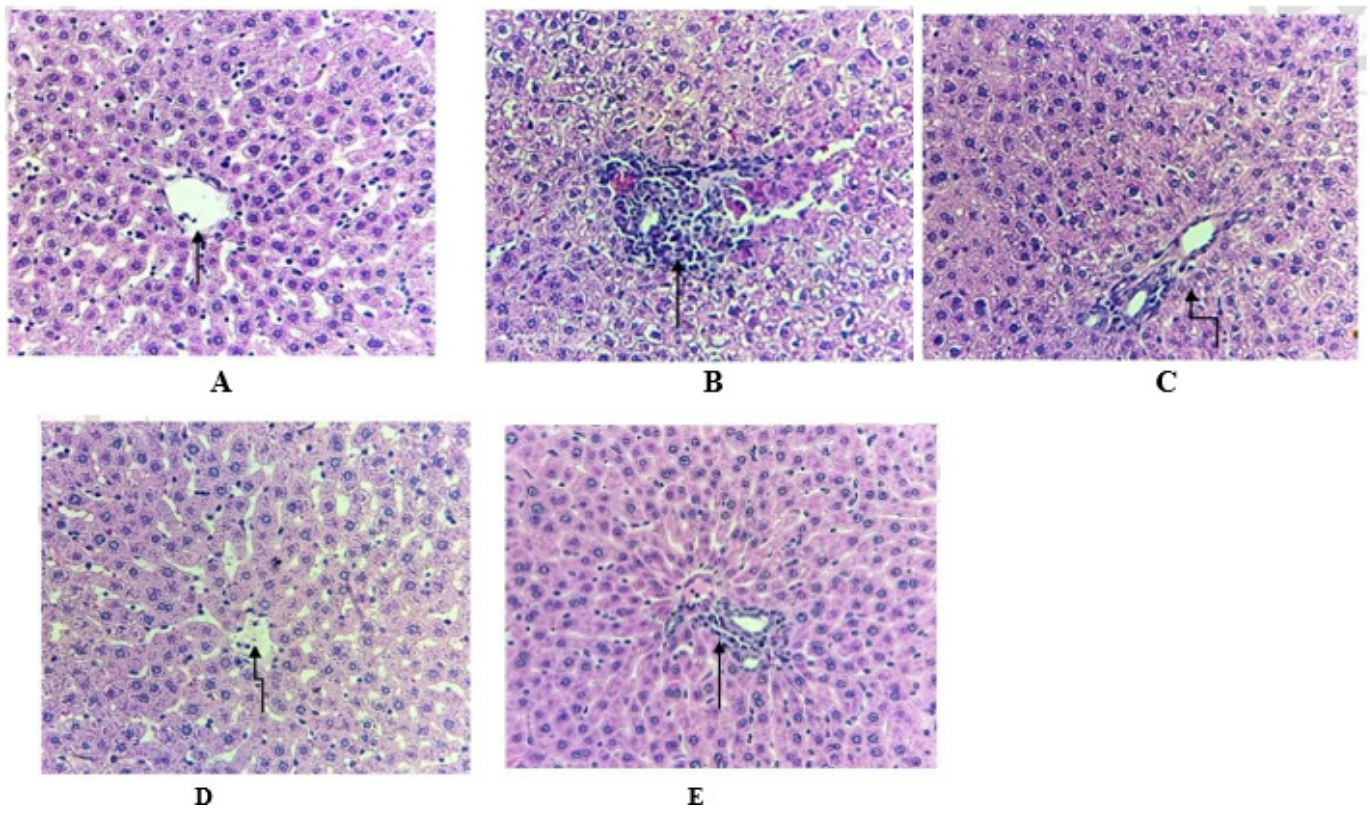


**Figure 9:** Effect of Calx of Copper against antitubercular drugs (INH, RIF, and PYZ) induced histopathological alteration in experimental groups of rats (H&E staining @ 40X). (A) Normal control, (B) positive control anti- tubercular drugs (INH, RIF, and PYZ) induced rat liver damage, (C and D) Histological Examination of Hepatic Tissue in Rats Administered with Copper Calx at Doses of 6.17 mg/kg and 12.33 mg/kg, (E) Histological Analysis of Hepatic Tissue in Silymarin-Treated Rats.

defense system is maintained by antioxidant enzymes, such as Superoxide Dismutase (SOD) and catalase. Prior research has revealed that antitubercular drugs can inhibit the activity of the body's antioxidant system in rats. In this investigation, rats treated with antitubercular drugs displayed reduced SOD and GSH activity in the liver. However, administration of Calx of Copper at doses of 6.17 mg/kg and 12.33 mg/kg, b.w, significantly increased these enzymes' activity, indicating the scavenging ability of reactive oxygen species Calx of Copper. Oxidative stress-induced cellular death can occur due to diminished antioxidant defenses or increased generation of free radicals.[17] Furthermore, a decrease in Glutathione (GSH) levels can lead to heightened lipid peroxidation, and excessive lipid peroxidation may subsequently cause increased glutathione consumption.[18] Nevertheless, the administration of Copper Calx led to a considerable elevation in liver GSH concentrations, signifying the antioxidant properties of Copper Calx. Furthermore, the hepatoprotective influence of Copper Calx was corroborated by histopathological evaluations, demonstrating a comprehensive protective effect conferred by Copper Calx. The current investigation implies that Copper Calx may offer potential benefits in mitigating the oxidative stress triggered by anti-tuberculosis medications, analgesics, and other agents capable of inducing liver damage.[19]

## CONCLUSION

The Calx of Copper demonstrated a dose-responsive hepatoprotective effect in rats with Isoniazid (INH), Rifampicin (RIF), and Pyrazinamide (PYZ) induced liver damage. The histopathological analysis provided additional support for these protective properties. Further research into Calx of Copper's potential to mitigate antitubercular drug-induced hepatotoxicity may contribute to developing clinically viable therapeutic approaches.

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## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest

## ABBREVIATIONS

**INH:** Isoniazid; **RIF:** Rifampicin; **PYZ:** Pyrazinamide; **TB:** Tuberculosis; **ROS:** Reactive oxygen species; **CMC:** Carboxy methyl cellulose; **SGPT:** Alanine transaminase; **SGOT:** Aspartate transaminase; **ALP:** Alkaline phosphatase; **MDA:** Malondialdehyde; **CAT:** Catalase; **GSH:** Reduced glutathione.

## SUMMARY

The present work was an attempt to check the hepatoprotective activity of Calx of Copper (*Tamra bhasma*), which was obtained as a gift sample from Pentacare Ayurpharma, Bangalore, India. Hepatoprotective activity was investigated in anti-tubercular drugs (H-R-Z)-induced experimental hepatotoxicity model in Wistar rats. Significant hepatic injury was produced by (H-R-Z administration: 30.85-61.7-132.65 mg/kg *p.o*), which was identified and validated by observation of uplifted serum levels of several liver and renal biomarkers and also by a reduction in total protein levels. Most importantly, the alterations in the anti-oxidant status by an increase in the MDA levels and reduction of SOD and GSH activity also confirmed hepatotoxicity. Pre-treatment of the two doses of Calx of Copper 6.17 mg/kg and 12.33 mg/kg by oral route caused a reduction in the levels of several liver and renal biomarkers and increased total protein level, also reversed the oxidative damage by improving the antioxidant status. Further, the results of this work were well established by a histopathological examination of liver tissues which revealed that the test drug significantly decreased the hepatic damage by improving the cytoplasmic vacuolations and chronic inflammation in hepatocytes.

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