

**Prophylactic Effect of Ascorbic Acid On Certain Biochemical and Histopathological Indices In Rats Treated With Repeated Dose of Cyclophosphamide**

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ABSTRACT

The limited use of cyclophosphamide in cancer treatment resulted from its role in multiple organ toxicities. The present study aimed to investigate the prophylactic capacity of ascorbic acid against hepatotoxicity induced by repeated dose of cyclophosphamide in male rats. Twenty-four male Wistar rats were randomly selected into 4 groups of 6 rats each. Group 1 served as the control and received intraperitoneal injection of normal saline BP 0.9% w/v. Group 2 received freshly prepared cyclophosphamide 4 mg/kg intraperitoneally once daily throughout the period of administration. Group 3 received an oral dose of 100 mg/kg ascorbic acid and then cyclophosphamide while Group 4 received ascorbic acid only. After 21 days of administration, the animals were sacrificed and biochemical estimation as well as histological assessment of the liver were carried out. The result showed significant ($P < 0.05$) decrease in protein concentration, significant ($P < 0.05$) elevation in serum alanine transaminase (ALT), alkaline phosphatase (ALP), gamma glutamyl transpeptidase (GGT) and disrupted liver histological assembly in rats treated with repeated dose of cyclophosphamide as compared to control. Ascorbic acid showed prophylactic capacity against liver damage in cyclophosphamide treated rats. The total protein concentration was normal, the serum liver enzyme activities were not significantly affected and there was protection of the hepatic histoarchitecture when compared with cyclophosphamide treated group. Therefore, the supplementation of ascorbic acid with the use of chemotherapeutic agent such as cyclophosphamide during cancer treatment may prevent related symptoms.

Keywords: Prophylactic, ascorbic acid, biochemical indices, cyclophosphamide.

Introduction

Since ages past, man has been bewildered by diverse ailments. Among such diversity is the severity of cancer. Multiple studies have demonstrated crude method of disease mitigation. However, advancement in medicine has helped develop novel drugs which can aid disease mollification and management.

Cyclophosphamide is a widely used DNA alkylating agent that has been employed in the treatment of metastatic breast cancer, malignant lymphomas, retinoblastomas, leukaemia, carcinomas and neuroblastoma of the ovary, lung and endometrium. It has also been used as an immunosuppressive agent during organ transplant as well as the treatment of some chronic autoimmune disorders such as multiple sclerosis, systemic lupus erythematosus and vasculitides.^{1,2} Despite its anticancer and immunosuppressive capacity, the use of cyclophosphamide is now limited, due to its ability to damage normal cells.

Cyclophosphamide is an inactive pro-drug that is activated in the liver by cytochrome P450 enzyme system.^{3,4} The metabolic activation of cyclophosphamide initially produces 4-hydroxycyclophosphamide which is an unstable compound that can spontaneously degenerate to

phosphoramidate mustard and acrolein.⁵ The phosphoramidate mustard is a bifunctional DNA alkylating agent which is responsible for the therapeutic effect of cyclophosphamide.⁶ It produces an aziridinylium ion intermediate in order to alkylate nucleophiles thereby inhibiting DNA replication and retarding tumour growth.^{4,7}

In recent time, the use of cyclophosphamide and other DNA alkylating agent has been limited; due to their ability to damage normal tissues.⁴ Studies have shown that the acrolein formed by spontaneous β -elimination from 4-hydroxycyclophosphamide is responsible for the cytotoxic effect of cyclophosphamide. Acrolein is a highly reactive aldehyde and may deplete antioxidant glutathione via conjugation.⁸⁻¹⁰ Alarcon (1976) reported that the conjugate produced by acrolein and glutathione is eliminated in urine as 3-hydroxypropylmercapturic acid.¹¹

Many authors have correlated the use of cyclophosphamide to hepatotoxicity.^{12,13} In another study; the hepatotoxic effect of cyclophosphamide was linked to oxidative stress.¹⁴⁻¹⁶ One major consequence of cyclophosphamide-induced hepatotoxicity is the leaking out of liver enzymes into the systemic circulation.¹⁷ However, the increase in serum enzyme activities is a reflection of hepatic necrosis and altered functional membrane. Similar study has shown that cyclophosphamide can induce disruption in hepatic histoarchitecture.^{18,19}

The possibility of ascorbic acid prophylaxis in cancer patients threatened with chemotherapy-related adverseness has been an area of great interest. One study from literature indicated that doses of ascorbic acid can prevent a number of chemotherapy-related symptoms.²⁰ Compelling evidences that ascorbic acid may improve the quality of life of cancer patients receiving chemotherapy have been accumulating.^{21,22} The chemoprotective effect of ascorbic acid has been reported.²³ In another study, ascorbic acid prevented the disturbed hepatic architecture caused by cyclophosphamide administration in rat.¹⁸

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Therefore, this study aimed to evaluate the daily efficacy of oral dose of ascorbic acid as a prophylactic measure against cyclophosphamide-induced hepatotoxicity.

Materials and Methods

Reagents and Materials

Pure cyclophosphamide (white crystalline powder, CTX-GLS, 200 mg) was purchased from GLS Pharmaceutical Limited, India. Ascorbic acid (crystalline powder, CAS No 50-81-7, containing 99.0-100.5% of pure ascorbic acid) was purchased from FoodChem, China. Commercially prepared biochemical reagents (of standard analytical grade) were purchased from Randox Diagnostics, United Kingdom and were refrigerated for later use.

The stock solution of cyclophosphamide was prepared by scaling down the manufacturers recommended dose. The solution was prepared fresh and administered daily. The daily stock solution was prepared by dissolving 3.84 mg of cyclophosphamide in 1.2 mL of sterile water. The stock solution of ascorbic acid was prepared by dissolving 80g of ascorbic acid into 1000ml of distilled water to obtain a concentration of 80 g/1000 mL.

The homogenizing buffer was prepared by dissolving 7.88 g of Tris-HCl and 11.2 g of KCl in 1000 ml of distilled water and adjusted to a pH of 9.4. The washing buffer was prepared by dissolving 11.5 g of KCl in 1000 ml of distilled water and the pH was adjusted to 7.4. However, the homogenising and washing buffer were refrigerated at 4°C for later use.

Animals

Twenty-four (24) male Wistar rats (average weight of 160 g) were purchased from the Department of Biomedical Sciences, Ladoko Akintola University of Technology (LAUTECH), Oshogbo. All animals were housed in clean and well-aerated metal cages. They were acclimatized for seven days with moderate feeding (twice daily) at a temperature (23.2 – 25.3°C), relative humidity (42 - 65%) and 12 hours light/12 hours dark sleep/wake cycle.²⁴

Ethical Approval

This research was ethically considered and approved by the Ethical Review Committee of the Ladoko Akintola University of Technology, Ogbomosho, Nigeria. The experiments were conducted in concord with the ethical regulations of the Organization for Economic Co-operation and Development (OECD) for the use of laboratory animals.²⁵

Experimental groups

The animals were randomly distributed into four groups of 6 rats per group after 7 days of acclimatization. Rats in each group were moderately fed throughout the period of administration. In addition, the animals in group 1 (control group) received intra-peritoneal injection²⁶ of 0.2 mL normal saline (BP 0.9% w/v) for 21 days. Animals in group 2 received intra-peritoneal injection²⁷ of freshly prepared cyclophosphamide (4 mg/kg body weight) daily for 21 days. Animals in group three received an oral dose²⁸ of ascorbic acid (100 mg/kg body weight) and then intra-peritoneal injection of cyclophosphamide for 21 days. Animals in group 4 received an oral dose of ascorbic acid only, for 21 days.

Blood Sample Collection for Biochemical Analysis

About 24 hours after 21 days of administration, the test animals were sacrificed by cervical dislocation²⁹ and carefully opened, blood was collected into a bottle via cardiac puncture using sterile syringe and needles. The blood collected were spun at 4000 rpm for 5 minutes using a bucket centrifuge. The serum obtained were stored in a separate bottle at 4°C for later use.

Liver Excision for Histopathological Assessment

The Liver was completely excised from the test animals. About 1 g each of the liver (from an animal in each group) that had been thoroughly washed in sufficient volume of the washing buffer were completely immersed separately in 10% formalin contained in universal bottle.

Liver Homogenate preparation for protein Analysis

Liver obtained was thoroughly washed in sufficient volume of cold washing buffer. About 1 g of the intact liver and homogenising buffer

were vigorously agitated using a homogeniser. The homogenised sample were centrifuged at 4000 rpm for 5 minutes using a bucket centrifuge (model 800DCE). The supernatant obtained were stored in a separate bottle at 4°C for later use.

Biochemical Assessment of Liver Function

The liver function was investigated by measuring total plasma protein concentration, total protein concentration of the liver tissue and determination of the liver enzyme activities (Alanine transaminase, alkaline phosphatase and γ -glutamyl transpeptidase). Quantitative assessment of total protein concentration was determined by Brunet (Colorimetric) method.^{30,31} Alanine transaminase (ALT) was assessed by kinetic determination of its activity. The method of determination was based on the recommendation of the International Federation of Clinical Chemistry (IFCC). Alkaline Phosphatase (ALP) activity was measured by the optimised standard method recommended by the Deutsche Gesellschaft für Klinische Chemie.³² γ -glutamyl transpeptidase (GGT) activity was determined as the quantitative measure of 5-amino-2-nitrobenzoate at 405 nm following GGT-catalysed reaction between L- γ -glutamyl-3-carboxy-4-nitroanilide and glycylglycine.

Histopathological Analysis

The histopathological processing of the liver organ as well as slide elucidation were carried out in the Department of Morbid Anatomy and Histopathology of the Lagos University Teaching Hospital, Lagos, Nigeria. The formalin-fixed liver was dehydrated in ascending grades of alcohol and cleared before paraffin embedding. A microtome was used to cut out thin sections from the embedded tissue after which the slides were prepared and stained appropriately. The histological features and a micrograph were obtained by qualitative examination of the stained sections under a compound light microscope.^{33,34}

Statistical Analysis

Data collected were expressed as mean \pm SD, subjected to one-way analysis of variance (ANOVA) using the statistical SPSS 20 (SPSS Inc. Chicago, IL, USA). Least significant differences (LSD) were used as a point of significance among treatment groups. $P < 0.05$ was considered statistically significant in statistical comparison.

Results and Discussion

Cyclophosphamide, a potent DNA alkylating agent is now limitedly used in the treatment of cancer, due to its ability to damage normal cell.^{1,35} As an inactive prodrug; it becomes metabolically activated by the hepatic cytochrome P450 enzyme system.^{36,37} Acrolein, one of the active metabolites of cyclophosphamide is responsible for its toxicity.³⁸⁻⁴⁰

Multiple studies have shown that the toxic metabolite of cyclophosphamide can induce hepatotoxicity. Moreover, cyclophosphamide-induced hepatotoxicity is known, and it has been correlated with weight loss, decreased total protein concentration, oxidative stress, leaking out of liver enzymes^{14,15,17} and alteration in hepatic histological architecture.^{18,19} The present study showed that repeated administration of cyclophosphamide at a low dose regimen culminate to hepatic damage. There were decreased total protein concentration in serum and liver (figures 1 and 2) and marked ($P < 0.05$) elevation of liver enzymes (alanine transaminase, alkaline phosphatase, gamma glutamyl transpeptidase) in the serum (figures 2, 3 and 4). The result is in agreement with the report of other investigators.^{18,41} The decrease in total protein concentration may be due to DNA damage and the inhibition of protein synthesis caused by cyclophosphamide-induced free radical generation.¹⁴ The altered level of liver enzyme activities in the serum of Group 2 animals may be a reflection of hepatocellular damage as well as compromised functional membrane.⁴² Ascorbic acid supplementation provided prophylactic measure against cyclophosphamide-induced hepatotoxicity. In this study, rats treated with oral dose of ascorbic acid before intraperitoneal administration of cyclophosphamide (Group 3) showed prophylaxis against total protein loss and prevented the abrupt increase in serum enzyme activity. This significant decrease in serum enzyme activity as compared to rats challenged with repeated dose of cyclophosphamide agrees with a report that provided experimental evidence on the protective role of ascorbic acid against cyclophosphamide-induced hepatotoxicity.¹⁸ The antioxidant prowess and free radical scavenging potential of ascorbic

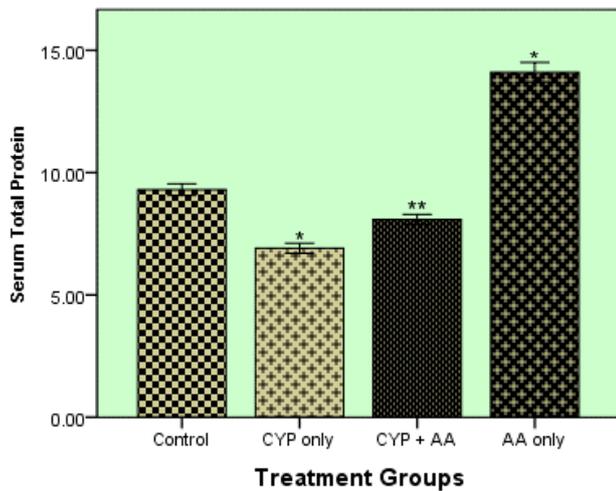


Figure 1: Serum total protein concentration (g/dL). Values are expressed as mean \pm SD, n = 6. * Value differ significantly (P<0.05) from group 1 (control). ** Value differ significantly (P<0.05) from cyclophosphamide treated group 2

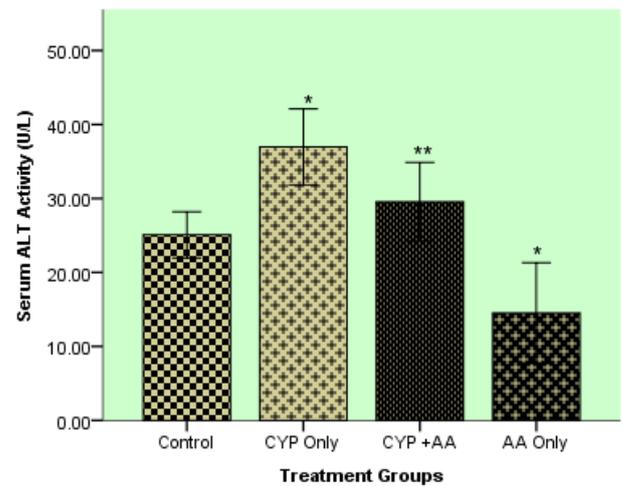


Figure 4: Serum Alanine Transaminase (ALT) activity (U/L). Values are expressed as mean \pm SD, n = 6. * Value differ significantly (P<0.05) from group 1 (control). ** Value differ significantly (P<0.05) from cyclophosphamide treated group 2.

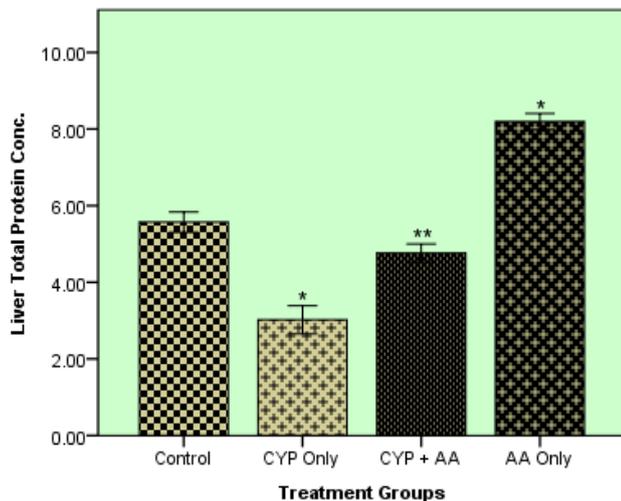


Figure 2: Liver total protein concentration (g/dL). Values are expressed as mean \pm SD, n = 6. * Value differ significantly (P<0.05) from group 1 (control). ** Value differ significantly (P<0.05) from cyclophosphamide treated group 2.

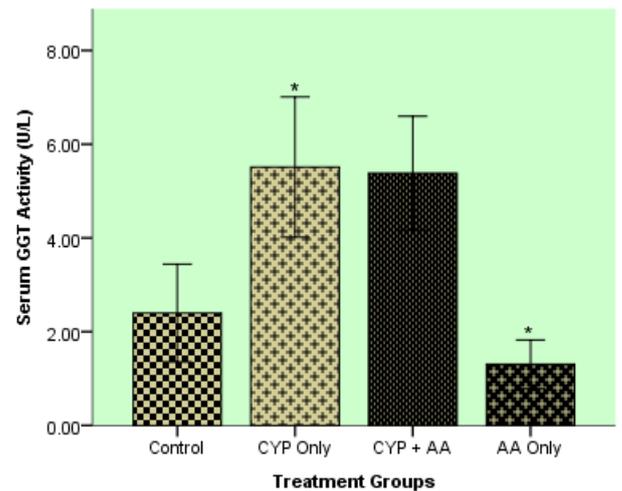


Figure 5: Serum Gamma Glutamyl Transpeptidase (GGT) activity (U/L). *Value differ significantly (P<0.05) from group 1 (control)

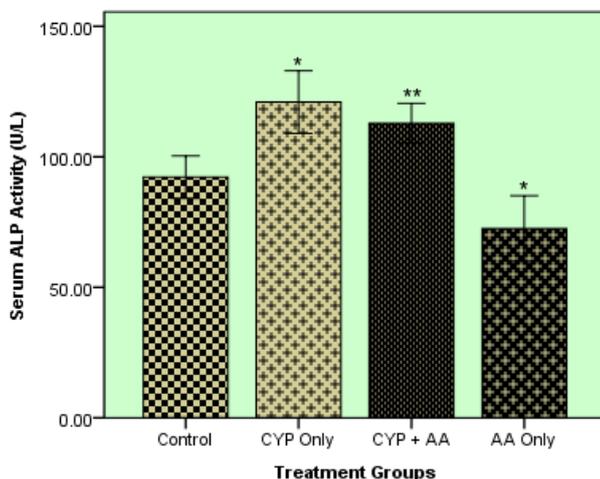


Figure 3: Serum Alkaline Phosphatase (ALP) activity (U/L). Values are expressed as mean \pm SD, n = 6. * Value differ significantly (P<0.05) from group 1 (control). ** Value differ significantly (P<0.05) from cyclophosphamide treated group 2.

acid may be the underlying prophylactic measure provided against cyclophosphamide-induced ROS generation in the hepatocyte; thereby preventing DNA strand breaks, inhibiting lipid peroxidation and markedly increasing protein biosynthesis.⁴³ The present findings is consistent with an earlier report.⁴⁴

Recent studies have characterised biomarkers used in clinical and preclinical screening for Drug-Induced Liver Injury (DILI).⁴⁵ Alanine transaminase which is primarily localised in the liver has been reportedly shown to be one of the liver-abundant enzyme that leaks out into the extracellular space and or the systemic circulation during hepatocellular injury, alanine transaminase remains the most universally accepted biomarkers of hepatocellular necrosis.⁴⁵ In our studies, repeated intraperitoneal administration of cyclophosphamide (Group 2) led to hepatocellular necrosis and culminating into marked (P<0.05) increase in serum level of Alanine transaminase as compared to the control (figure 4). This reflects hepatic damage. The present data is consistent with a recent report.⁴⁶ In another study, increase in serum alkaline phosphatase (ALP) may be due to biliary obstruction.³¹ A report in 2006 provided a supporting evidence to one of our findings that correlated significant (P<0.05) increase in serum ALP to cyclophosphamide-induced hepatotoxicity (figure 3). The increase in ALP may contribute to cholestatic liver damage.¹⁷ this study also showed marked (P<0.05) elevation in serum gamma glutamyl transpeptidase (GGT) level following repeated administration of cyclophosphamide as compared to control (figure 5). This may be due

to cholestasis and conventional hepatobiliary injury.⁴⁵ Moreover, in this studies, the cyclophosphamide-elevated serum liver enzymes (excluding GGT) were normal in groups that received ascorbic acid supplementation before intraperitoneal injection of cyclophosphamide (Group 3). Ascorbic acid prophylactic capacity may be due to its profound ability to bind the cytotoxic metabolite of cyclophosphamide thereby rendering it inactive.⁴⁷ The limited potency of ascorbic acid to maintain normal GGT activities in the serum may be due to the contribution made by the destruction of other extrahepatic organs which can also synthesize GGT, since it is an isoenzyme.

The liver is a pivotal organ and the central city of metabolism. It consists the hepatocyte and other microanatomical features.⁴⁸ Multiple studies have shown that repeated administration of cyclophosphamide can disrupt hepatic architecture.⁴⁹ We found in our studies that repeated dose of cyclophosphamide in Group 2 (plate 2) caused significant distortion in hepatic architecture as compared to control (plate 1); there were interstitial vascular congestion and hepatic centrilobular vacuolization. Our present study is in accordance with the report of Ademola *et al.* (2016) who demonstrated that the histological assessment of hepatic tissue in rat treated with cyclophosphamide showed haemorrhage, interstitial congestion and periportal inflammation.¹⁹

Also, it was observed that ascorbic acid supplementation to rat before treatment with cyclophosphamide in Group 3 (plate 3) showed marked protection of the hepatocellular structure as well as sustained hepatic histopathological architecture. The present data agree with one report that correlated ascorbic acid supplementation with significant decrease in histopathological degeneration.⁵⁰

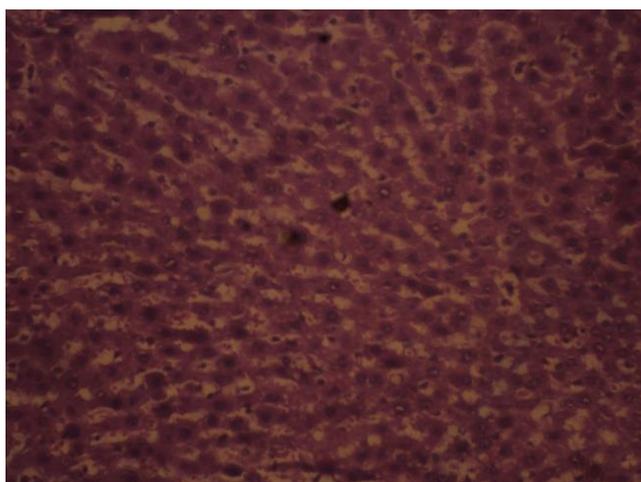


Plate 1: Histology of the liver (control) showing normal hepatic architecture (H & E 1000x).

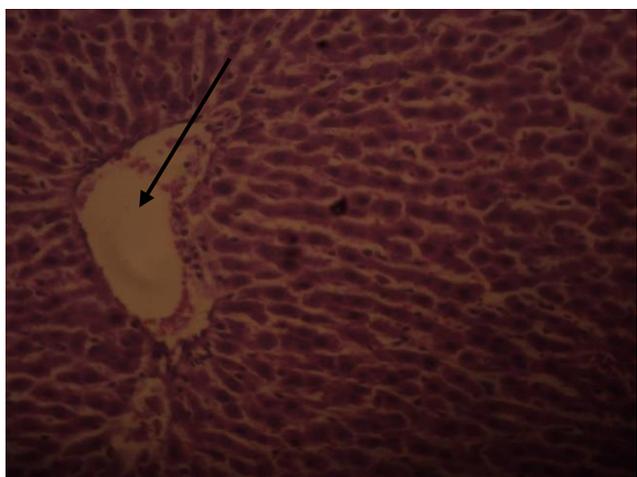


Plate 2: Histology of the liver (CYP only) showing significant distortion of the hepatic architecture and interstitial vascular congestion as well as hepatic centrilobular vacuolization (H & E 1000x).

Conclusion

The results of this study showed that repeated intraperitoneal administration of cyclophosphamide is associated with hepatic injury, cholestasis and altered histology of the hepatic architecture. However, ascorbic acid supplementation provided prophylactic capacity against cyclophosphamide-induced hepatotoxicity and concurrent protection of the distorted liver histological architecture. Consequently, we recommend ascorbic acid supplementation with the use of cyclophosphamide and other DNA alkylating agent during the treatment of malignant neoplasm.

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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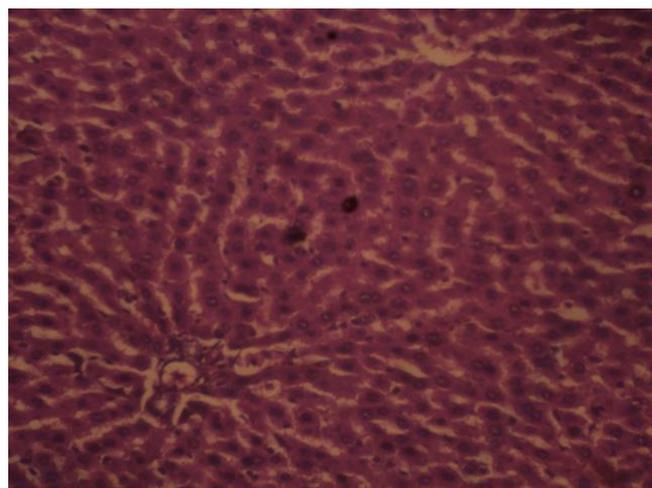


Plate 3: Histology of the liver (CYP + AA) showing significant protection of the hepatic architecture (H & E 1000x).

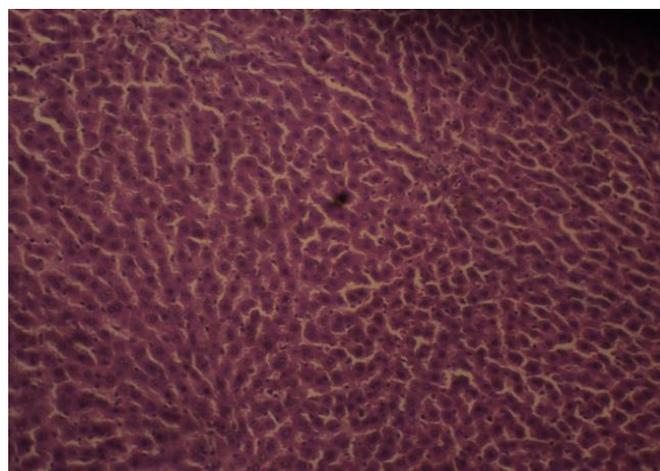


Plate 4: Histology of the liver (AA only) showing normal hepatic tissue architecture just like the control group (H & E 1000x).

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