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Hepatocurative Effects of Alpha Lipoic Acid in 5-Fluorouracil-Induced Toxicity Involves Activation of TNF Alpha and NF-*κβ* Signaling Pathway

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ABSTRACT

Alpha Lipoic Acid is an antioxidant capable of inhibiting xenobiotic-induced liver toxicity as reported in adriamycin-induced hepatotoxicity in rats. The present research was designed to study the hepatocurative effects of Alpha Lipoic acid (ALA) in 5-fluorouracil (5-FU)-induced hepatotoxicity of Wistar rats. Intraperitoneal injection of 50 mgkg^{-1} of 5-FU was associated with significant ($p \leq 0.05$) increase in the Phenobarbital-induced sleeping time, NF-κB, TNF-α, AST, ALP, ALT, triglyceride, cholesterol and LDL levels in the toxicity control when compared to the untreated control rats. However, intraperitoneal injection of 50 mgkg^{-1} of 5-FU was associated with significant ($p \leq 0.05$) decrease in albumin, total protein and HDL levels in the toxicity control when compared to the negative control rats. Daily oral post-treatment with 400 mgkg^{-1} ALA after 5-FU injection non-significantly ($p > 0.05$) attenuated increase in the HDL level when compared to the negative control group. Intraperitoneal injection of 50 mgkg^{-1} of 5-FU was associated with remarkable necrosis when compared to the normal liver architecture. Daily oral post-treatment with 400 mgkg^{-1} ALA after 5-FU injection significantly ($p \leq 0.05$) attenuated these trends. ALA has hepatocurative potential in 5-FU-induced hepatotoxicity of Wistar rats.

Keywords: Alpha Lipoic Acid, 5-Fluorouracil, Hepatocurative, Nuclear transcription factor kappa baeta, Tumour Necrosis factor alpha, Wistar Rats .

Introduction

5-fluorouracil (5-FU) is a fluoropyrimidine antimetabolite agent, used to treat cancer of the colon, breast, gastrointestinal, head, neck, and pancreas.[1] 5-Fluorouracil (5-FU) is one of the most widely used antineoplastic drugs, mainly because of its efficacy against various malignancies. 5-FU is extensively metabolized in the liver and the production of toxic intermediate may trigger liver injury. Fluorouracil is reported to exhibit severe toxicity and adverse effects which have restricted its chemotherapeutic use, limiting its potential as an effective anti-cancer agent. 5-FU severe side effects are based on its systemic toxicity, including hepatotoxicity.[2]

Hepatotoxicity refers to liver dysfunction or liver damage that is associated with an overload of drugs or xenobiotics. Drug-induced hepatotoxicity can be caused by anticancer, antidepressant, anti-inflammatory and analgesic agents. The liver is constantly involved in biotransformation and this could lead to hepatotoxicity.

The pathophysiological mechanisms underlying this toxicity is yet to be elucidated but increased oxidative stress, apoptosis and inflammation have all been associated with 5-FU toxicity. Production of free radicals leads to oxidative stress and this is the basis of drug-induced hepatotoxicity.[3] Like other chemotherapeutic agents, 5-FU creates over reactive oxygen species (ROS) and suppresses the antioxidant defense mechanism. 5-FU generates ROS and RNS. When ROS is excessive, the homeostasis is disturbed leading to oxidative stress.[4]

Oxidative stress produces cytokines, thus increasing inflammation and apoptosis. The oxidative stress alters structures of lipids, proteins and DNA contents. It also modulates pathways that control normal biological functions, e.g. NF-KB pathway.[5]

The generation of reactive oxygen species (ROS) incurs membrane lipid peroxidation and oxidative cellular damage. Several studies have suggested that ROS and proinflammatory cytokines are engaged in the activation of mitogen-activated protein kinases (MAPKs) including p38 MAPK, Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK). Stimulation of p38 MAPK and JNK contributes to the upregulation of cytokines and renal apoptotic cell death in several renal pathologies. In addition, stimulation of ERK pathway drives the activation of transcription factors such as nuclear factor kappa B (NF- κB) which controls the expression of diverse proinflammatory cytokines and proteins. Recently, there has been a revival of interest in the search for potential protective agents against chemotherapy-associated adverse effects. A number of natural agents have been reported to possess therapeutic properties against 5-FU toxicity.[6] Previous studies have revealed the protective effects of natural antioxidants such as propolis and chrysin in 5-FU-induced renal damage. Therefore, much attention has been paid to the potential role of antioxidants in protecting against chemotherapy-induced hepatotoxicity.[7] Hence, agents that possess antioxidant features with minimal adverse effects may represent a potential cost-effective intervention against 5-FU toxicity.[8] Alpha-lipoic acid

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(ALA) is a naturally occurring dithiol compound synthesized from octanoic acid in the mitochondrion and acts as a coenzyme for the mitochondrial respiratory enzymes.[9] It is able to protect cells and tissues from ROS and free radicals due to its antioxidant properties. Diseases mediated by oxidative stress are treated effectively with alpha lipoic acid according to several reports. It is also able to reduce apoptosis in the liver because of its ability to reduce oxidative stress. ALA and DHLA derivatives also have anti-inflammatory activities.[11] Even though a number of studies on the hepatoprotective effects of ALA has been carried out, none of them reported its hepatocurative effects. The aim of the present study is to investigate the curative roles of ALA against 5-FU-induced oxidative stress and hepatic injury in rats.

Materials and Methods

Animals

Wistar rats of either sexes weighing 225 ± 5 g were obtained from the Faculty Animal House, Department of Pharmacology and Therapeutics, ABU Zaria. The animals were housed in the Animal house, Department of Pharmacology and Therapeutics, A.B.U, Zaria under room temperature. The animals were housed in the Department of Pharmacology and Therapeutics, Ahmadu Bello University, Zaria. They were given access to pelletized growers marsh and water *ad libitum*. All experimental protocols were as approved by the University Animal ethics committee. The rats were acclimatized for two weeks in the home cages and environment before commencement of the experiment. All experimental protocols were in accordance with the Ahmadu Bello University research policy (Revised, 2010 and accepted internationally, NIH 1985), Revised) and of regulations governing the care and use of experimental animals with ethical approval number ABUCAUC/2020/013.

Chemicals

NF-kB and TNF alpha ELISA kits were purchased from Wuhan Fine Biotech Co.,Ltd,China.NFκB p65 (Total) Capture Antibody,NFκB p65 (Total) detector Antibody, NFκB p65 Lyophilized Recombinant Protein, TNF alpha Microplate (6 x 8 well strips),TNF alpha Standard (Lyophilized), 10X Standard Diluent Buffer,Biotinylated anti-TNF alpha,Biotinylated Antibody Diluent, Streptavidin-HRP,HRP Diluent. Alpha lipoic acid was obtained from Sigma Chemical Company, USA. All other chemicals were of analytical grade and obtained from local commercial sources.

Experimental design

Wistar rats were randomly divided into three different groups (n = 6 each). The first group received normal saline (1 mL/kg) for 14 days and used as a negative control. The second group received a daily oral dose of normal saline (1 mL/kg) for 14 days and 5-fluorouracil (50 mg/kg body weight) for only the first four days. This served as the toxicity model group. The third group received 5-fluorouracil (50 mg/kg body weight, orally) for only the first four days and 400 mg/kg alpha lipoic acid for 14 days. This served as the hepatocurative group. All the experimental animals were sacrificed on the 15th day under chloroform anesthesia.

Effect of alpha lipoic acid on NF-κβ expression by ELISA

The animals were sacrificed under mild ether anesthesia and blood was collected in clean centrifuge tubes 24 hours after the final experimental procedures. The serum was obtained by centrifugation and used for the estimation of various biochemical parameters. Serum levels of NF-κ β were quantified by using an enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's specifications.[12]

Effect of alpha lipoic acid on TNF-α expression by ELISA

Serum levels of TNF-α were quantified using an enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's specifications.[13]

Effect of alpha lipoic acid on phenobarbital induced sleeping time

The experimental design previously described by Al-Howriny *et al.(*2004)[14] was adopted with modifications. Phenobarbital, also known as phenobarbitone or phenobarb, is a medication recommended by the World Health Organization for the treatment of certain types of epilepsy in developing countries. Phenobarbital is occasionally used to treat sleep disorder, anxiety, and drug withdrawal and to help with surgery. This test is based on the fact that induced hepatotoxicity causes enhancement of sleeping time due to hepatic injury as barbiturate metabolism is delayed and excretion is slowed. Rats were grouped and treated as discussed previously. 24 hours after the last treatment, all rats received phenobarbital 150 mg/kg intraperitoneally (i.p). The time interval between the onset and the regaining of the right reflex was measured as sleeping time. The duration of "sleep" of the first group was taken as the normal sleeping time.

Effect of alpha lipoic acid on antioxidant indices in 5-FU treated Wistar rats

The dissected tissues were washed with 50 mM sodium phosphate-buffered saline (100 mM Na_2HPO_4/NaH_2PO_4, pH 7.4) in an ice-containing medium, with 0.1 mM EDTA to remove any RBCs and clots. Then tissues were homogenized in 5–10 mL cold buffer per gram tissue and were centrifuged at 5000 rpm for 30 min. The resulting supernatant was transferred into an Eppendorf tube and was preserved at -80°C into aliquots for the spectrophotometric estimation of tissue LPO biomarker (MDA), SOD, catalase (CAT), GSH, glutathione peroxidase (GSH-Px).

Effect of alpha lipoic acid on biochemical parameters in 5-FU treated Wistar rats

Serum biochemical parameters were measured spectrophotometrically according to manufacturers' instructions.

Effect of alpha lipoic acid on histopathology in 5-FU treated wistar rats

After the animals were euthanized, vital organs including liver and kidneys were removed from the rats and fixed in 10% formalin for at least 48 h. They were then processed routinely, and the tissues were embedded in paraffin wax. Histological sections were cut at 5 – 6 µm and stained with routine haematoxylin and eosin (H & E). Detailed microscopic examinations were carried out by a consultant histopathologist. Photomicrographs of the organs were taken at various magnifications (× 100, × 250, and × 400).

Statistical analysis

The differences between the obtained values (mean ± SEM, n = 6) were analyzed with One-Way Analysis of Variance followed by the Tukey–Kramer multiple comparison using Graph pad prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA) The differences were considered statistically significant when $p \leq 0.05$.

Results and Discussion

Although, a number of studies have been carried out on the protective effect of natural agents against chemotherapeutic drugs, this present study, to the best of our knowledge, would be the first to investigate the potential hepatocurative effect of ALA against 5-fluorouracil-induced liver damage. 5-FU is used in the treatment of a wide range of cancers. Some of these cancers include that of the pancreas, neck, head, gastrointestinal tract, breast and that of the colon. It is however found to be hepatotoxic and nephrotoxic.[15]

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In the present study, injection of 5-FU in rats resulted in deterioration of hepatic function as indicated by elevation in ALT, AST and by a significant decrease in total proteins and albumin. Intraperitoneal injection of 50 $mgkg^{-1}$ of 5-FU was associated with significant ($p \leq 0.05$) increase in the AST, ALP and ALT levels in the toxicity model control when compared to the untreated control rats (Table 1). However, daily oral post-treatment with 400 mgkg-1 ALA (5-FU injection significantly ($p < 0.05$) attenuated increase in the AST,ALP and ALT levels when compared to the toxicity model group. However, a single intraperitoneal injection of 50 $mgkg^{-1}$ of 5-FU was associated with significant ($p \leq 0.05$) decrease in the albumin and total protein levels in the toxicity model control when compared to the untreated control rats. However, daily oral post-treatment with 400 $mgkg^{-1}$ ALA after 5-FU injection significantly ($p < 0.05$) attenuated decrease in the albumin and total protein levels when compared to the toxicity model group.

Liver function tests are very important in the diagnoses of diseases. An increase in AST and ALT levels mean there is a possible leakage from injured tissues as a result of a hepatocellular necrosis.[16] Elevated ALP levels mean there is cholestasis and hepatobiliary injury which leads to overproduction and release in the blood.[17] In the present study there were elevated ALP, AST and ALT levels in the toxicity control model (1 mL/kg Normal Saline + 50 mg/kg 5-FU) as compared to the negative control (1 mL/kg Normal Saline). This is an ample demonstration of the extent to which 5-FU is toxic to the liver. This is in agreement with the findings of Zhou *et al*. (1995).[18] Decrease in albumin levels meaning there was hepatic dysfunction leading to a reduction in protein synthesis. It could also be due to increased protein loss via the kidney or gut.[19] It could also be due to increased protein loss via the kidney or gut.[20] This trends were reversed by post-treatment with ALA.

Intraperitoneal injection of 50 $mgkg^{-1}$ of 5-FU was associated with significant ($p \leq 0.05$) increase in the triglyceride, cholesterol and LDL levels in the toxicity model control when compared to the untreated control rats (Table 2). However, daily oral post-treatment with 400 $mgkg^{-1}$ ALA after 5-FU injection significantly ($p < 0.05$) attenuated increase in the triglyceride, cholesterol and LDL levels when compared to the toxicity model group. However, a single intraperitoneal injection of 50 $mgkg^{-1}$ of 5-FU was associated with significant ($p \leq 0.05$) decrease in the HDL levels in the toxicity model control when compared to the untreated control rats. However, daily oral post-treatment with 400 $mgkg^{-1}$ ALA after 5-FU injection significantly ($p < 0.05$) attenuated decrease in the HDL levels when compared to the toxicity model group (Figure 2). Increased serum concentrations of triglycerides, total cholesterol and LDL, and the decreased level of HDL were restored to normal values with alpha lipoic acid post-treatment. This may be explained on the basis that alpha lipoic acid has a strong ability to chelate multivalent metal ions, especially zinc, calcium and iron. Indeed, its ability to chelate minerals has been reported to have some protective effects, such as decreasing iron mediated free radical formation and lowering serum cholesterol, triglycerides and lipid peroxides in experimental animals.[21] The significant decrease in the concentration of the albumin could be attributed on one hand to an under nutrition and on the other hand to a reduction in the protein synthesis in the liver resulting in a decreased plasma total protein which confirmed the direct damaging effect of 5-FU on liver cells.[22] Similarly, the stimulatory effect of 5-FU on liver enzymes was likely due to the production of ROS, which enhanced Lipid Peroxidation (LPO) and the production of toxic aldehydes such as MDA which is consistent with our findings (unpublished data). The exhaustion of antioxidant defense enzymes ultimately led to hepatocellular injury and necrosis and the release of intracellular enzymes including ALT, AST, ALP as well as cholesterol and triglycerides. Increases in these biomarkers are evidence of active liver dysfunction.[23]

The increased ROS production due to depletion of GSH (consistent with our findings in unpublished data) might lead to oxidative damage and a critical role in the development of hepatic damage. ALA is effective in preventing the development of hepatic damage.[24,25] ALA is found to be a potential therapeutic agent in the treatment and prevention of different pathologies that are related to an imbalance of the oxidoreductive cellular status, which occurs in the case of hepatic disorder status.[26] In addition, several researchers have recently reported the protective effects of ALA on the liver which is induced by oxidative agents.[27] An elevation in MDA level usually occurs with a decrease in endogenous antioxidants (SOD, CAT, GPx, and GSH) in the presence of oxidative stress.[28] which agrees with findings of our study (unpublished data). 5-FU significantly increased MDA levels and significantly decreased GSH, CAT, GPx and SOD levels in the toxicity control when compared to the negative control. This shows that free radicals are generated in the metabolism of 5-FU which leads to hepatic damage. Post-treatment with 400 $mgkg^{-1}$ ALA reversed these trends. This once again is indicative of the protective role of alpha lipoic acid in the presence of 5-FU. This was due to the inherent antioxidant activity of alpha lipoic acid.

Intraperitoneal injection of 50 $mgkg^{-1}$ of 5-FU was associated with significant ($p \leq 0.05$) increase in the Phenobarbital-induced sleeping time in the toxicity model control when compared to the untreated control rats (Figure 3). However, daily oral post-treatment with 400 $mgkg^{-1}$ ALA after 5-FU injection significantly ($p < 0.05$) attenuated increase in the Phenobarbital-induced sleeping time when compared to the toxicity model group. The restoration of phenobarbitone-induced sleeping time suggested the normalization of liver cytochrome P450 enzymes. The 5-FU-induced hepatic injury decreased the activity of cytochrome P450 enzymes and thereby the metabolic functional activity of the hepatocytes. This caused delay in the barbiturate metabolism and slowed down the excretion of phenobarbitone, thereby prolonging the sleeping time.

Table 1: Effect of alpha lipoic acid on Liver Function Tests (AST,ALP,ALT ,Albumin and Total Protein) of 5-fluorouracil induced Toxicity in Wistar Rats

Groups	AST (IU/L)	ALT (IU/L)	ALP (IU/L)	TP (mg/L)	ALB (mg/L)
NS (1 mg/kg)	122.5 ± 10.45	62.87 ± 7.231	98.17 ± 5.618	55.83 ± 2.8	26.03 ± 0.6360
NS (1 mg/kg) + 5FU (50 mg/kg)	599.4 ± 32.7[a]	254.2 ± 11.2[a]	333.3 ± 24.42[a]	23.62 ± 3.6[a]	18.50 ± 0.544[a]
5FU (50 mg/kg) + ALA(400mg/kg)	357.3 ± 18.1[b]	357.3 ± 18.4[ab]	152.0 ± 4.59[b]	63.67 ± 3.6[b]	34.90 ± 0.826[ab]

Values are presented as mean ± SEM. Data was analysed using Oneway ANOVA followed by tukey post-hoc test. [a]$p \leq 0.05$ significant difference as compared to the negative control group (Normal Saline - 1 mL/kg). . [b]$p \leq 0.05$ significant difference as compared to the model or toxicity control group (Normal Saline - 1 mL/kg + 5-Fluorouracil-50 mg/kg).
Key: NS = Normal Saline, ALT = Alanine transaminase, AST = Aspartate Transaminase, ALP = Alkaline Phosphatase.

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Table 2: Effect of alpha lipoic acid on Lipid profile (Triglycerides,Total Cholesterol,HDL and LDL) of 5-fluorouracil induced Toxicity in Wistar Rats

Groups	T (mg/dL)	TC (mg/dL)	HDL (mg/dL)	LDL (mg/dL)
NS (1 mg/kg)	7.883 ± 1.302	8.067 ± 0.5631	9.333 ± 0.4883	3.067 ± 0.4631
NS (1 mg/kg) + 5FU (50 mg/kg)	47.00 ± 2.523[a]	37.67 ± 2.703[a]	3.667 ± 0.2603[a]	26.10 ± 2.097[a]
5FU (50 mg/kg)+ ALA (400mg/kg)	18.17 ± 1.013[b]	18.05 ± 0.4918[b]	11.13 ± 0.7329[b]	14.50 ± 0.4733[ab]

Values are presented as mean ± SEM. Data was analyesd using Oneway ANOVA followed by tukey post-hoc test. [a]$p \leq 0.05$ significant difference as compared to the negative control group (Normal Saline-1 mL/kg). . [b]$p \leq 0.05$ significant difference as compared to the model or toxicity control group (Normal Saline-1 mL/kg + 5-Fluorouracil-50 mg/kg).
Key: T = Triglycerides (mg/dL), TC = Total Cholesterol, HDL = High Density Lipoprotein, LDL = Low Density Lipoprotein.

Post-treatment with ALA and silymarin restored the phenobarbitone-induced sleeping time. This is an indicator of normalization of cytochrome P450 and related hepatic mixed function oxidase enzymes system.[29]

Intraperitoneal injection of 50 $mgkg^{-1}$ of 5-FU was associated with significant ($p \leq 0.05$) increase in the NF-κB level in the toxicity model control when compared to the untreated control rats (Figure 1). However, daily oral post-treatment with 400 $mgkg^{-1}$ ALA after 5-FU injection significantly ($p < 0.05$) attenuated increase in the NF-κB level when compared to the toxicity model group. Nuclear factor kappa B (NF-kappa B) is a transcription factor that promote tumorigenesis after being activated by inflammatory agents, carcinogens and tumor promoters. These are indicative of the ameliorative potential of alpha lipoic acid in 5-FU-induced hepatic injury of Wistar rats. This could be due to the anti-inflammatory, antioxidant, antifibrotic and anti-apoptotic effects of alpha lipoic acid. Intraperitoneal injection of 50 $mgkg^{-1}$ of 5-FU was associated with significant ($p \leq 0.05$) increase in the TNF-α level in the toxicity model control when compared to the untreated control rats (Figure 2). However, daily oral post-treatment with 400 $mgkg^{-1}$ ALA after 5-FU injection significantly ($p < 0.05$) attenuated increase in the TNF-α level when compared to the toxicity model group. Proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 have been the focus of investigations of inflammatory organ injury because the uncontrolled and prolonged action of these proteins is potentially harmful.

Considerable evidence suggests that TNF-α and IL-1β contribute to the pathogenesis of liver inflammatory diseases by activating the NF-κB signaling pathway, suggesting that it may be important to monitor proinflammatory cytokines when studying liver injury.[30] NF-kB and its associated pathways are intricately involved in liver physiology and disease. Selective and even cell type-specific inhibition of NF-kB signaling may offer new ways to treat a variety of liver diseases including cancer. Inhibition may be achieved by targeting either atypical signaling proteins responsible for NF-kB activation in specific types of cells, as well as individual downstream effectors used by NF-kB to achieve some of its numerous effects. Inflammation is an important pathological mechanism that propagates liver injury. As an essential process for subsequent fibrogenesis, the inflammatory response is known to participate in collagen synthesis and accumulation. Therefore, decreasing the levels of pro-inflammatory cytokines may be beneficial in liver fibrosis. Histological examination of the liver showed that 5-FU treatment caused abnormal ultrastructural alterations in these organs. Plates (I-III) depict architecture of normal rat liver showing normal hepatocytes (Plate I). However, intraperitoneal injection of 50 $mgkg^{-1}$ of 5-FU was associated with remarkable necrosis of the hepatocyte (Plate II) when compared to architecture of the liver (Plate I). Rat livers post-treated with 400 $mgkg^{-1}$ ALA Showed slight hepatocellular necrosis (Plate III).This was ameliorated by post treatment with ALA which is consistent with the findings above.

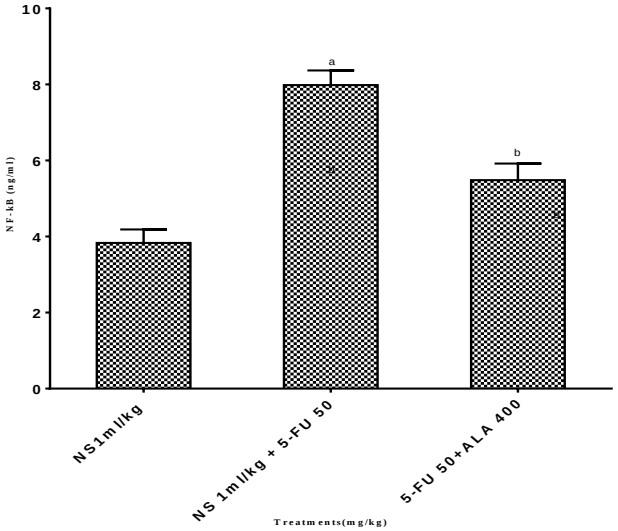

Figure 1: Effect of alpha lipoic acid on NF-κβ of 5-fluorouracil induced Toxicity in Wistar Rats.
Values are presented as mean ± SEM. Data was analyesd using Oneway ANOVA followed by tukey post-hoc test. [a]$p \leq$ 0.05 significant difference as compared to the negative control group (Normal Saline-1ml/kg). . [b]$p \leq$ 0.05 significant difference as compared to the model or toxicity control group (Normal Saline-1 mL/kg + 5-Fluorouracil-50 mg/kg).

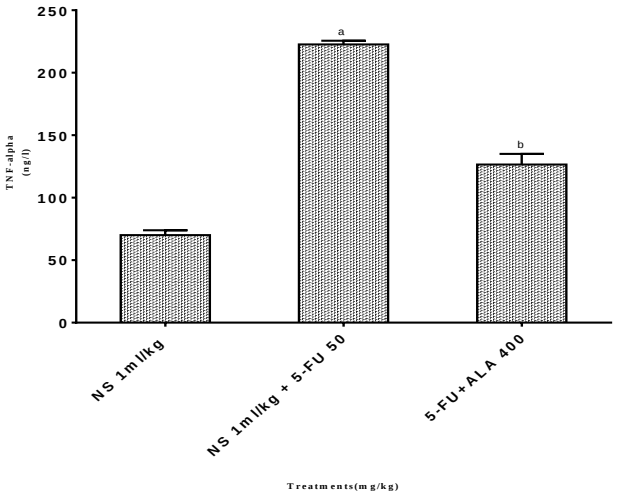

Figure 2: Effect of alpha lipoic acid on TNF-α of 5-fluorouracil induced Toxicity in Wistar Rats.
Values are presented as mean ± SEM. Data was analyesd using Oneway ANOVA followed by tukey post hoc test. [a]$p \leq$ 0.05 significant difference as compared to the negative control group (Normal Saline-1 mL/kg). [b]$p \leq$ 0.05 significant difference as compared to the model or toxicity control group (Normal Saline-1 mL/kg + 5-Fluorouracil-50 mg/kg).

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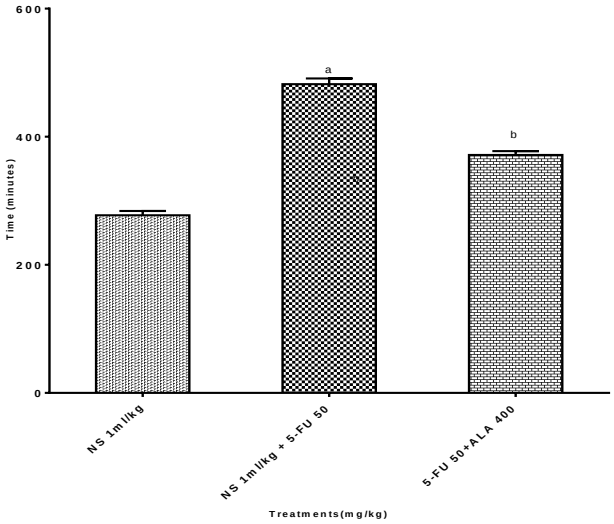

Figure 3: Effect of alpha lipoic acid on Phenobarbital induced Sleeping Time of 5-fluorouracil induced Toxicity in Wistar Rats.

Values are presented as mean ± SEM. Data was analyesd using Oneway ANOVA followed by tukey post hoc test. $^{a}p \leq 0.05$ significant difference as compared to the negative control group (Normal Saline-1 mL/kg). $^{b}p \leq 0.05$ significant difference as compared to the model or toxicity control group (Normal Saline-1ml/kg + 5-Fluorouracil-50mg/kg).

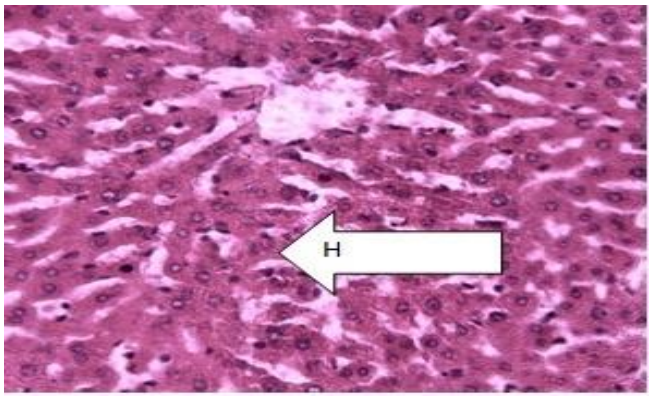

Plate I: Rat liver administered normal saline showing normal hepatocyte (H)

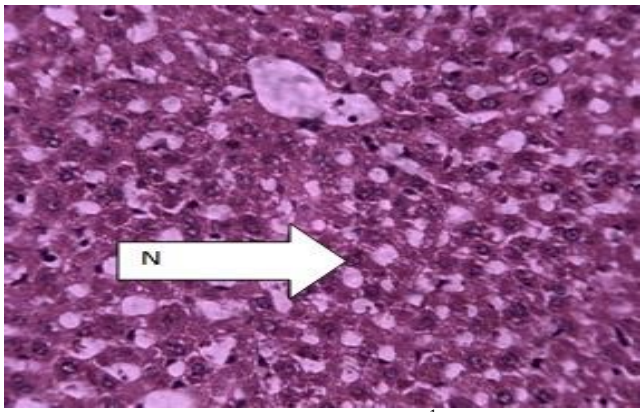

Plate II: Rat liver administered 50 mgkg^{-1} of 5-FU showing necrosis (N) of the liver

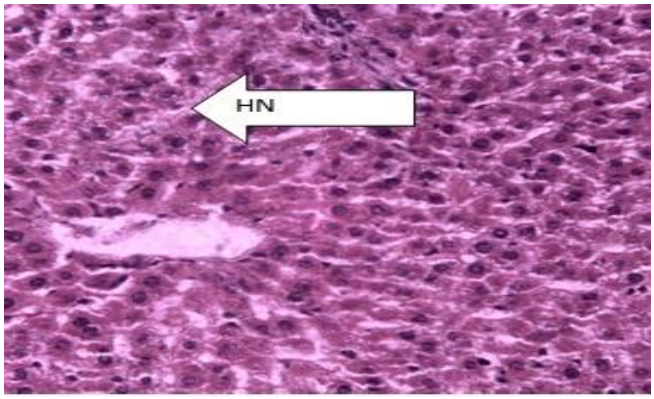

Plate III: Rat liver administered 50 mgkg-1 of 5-FU + 400 mgkg-1 ALA showing bridging necrosis with mild protection from 5-FU (HN)

Conclusion

The present study indicated that ALA downregulated the activation of NF-$\kappa\beta$ Signaling pathway and the pro-inflammatory cytokine TNF-α. It restored lipid peroxidation, liver destruction and antioxidants. These data points strongly to the hepatocurative potential of ALA in 5-FU-induced hepatic damage in Wistar rats.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

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

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