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HEPATOPROTECTIVE ACTIVITY OF ROOTS OF *LAWSONIA INERMIS* AGAINST PARACETAMOL AND ANTI-TUBERCULAR DRUGS INDUCED HEPATOTOXICITY IN RATS

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ABSTRACT

The present study was carried out to evaluate the hepatoprotective activity of roots of *Lawsonia inermis (LI)* ethanolic extract in paracetamol and anti tubercular drugs induced hepatotoxicity in Wistar rats. Roots of *LI* were extracted with alcohol and water which has given ethanolic extract of *LI* & aqueous extract of *LI*. Preliminary phytochemical tests were done. The *in vitro* hepatoprotective activity of the ethanolic extract of *lawsonia inermis* (LIALC) and aqueous extract of *lawsonia inermis* (LIAQ) were assessed. The *in vivo* hepatoprotective activity of LIALC was investigated against Paracetamol and Anti-TB drugs induced hepatotoxicity in Rats. Phytochemical analysis revealed presence of lactones, flavonoids, sterols, terpenes, carbohydrates, tannins compounds, which have been known for their hepatoprotective activities. In both *in vivo* and *in vitro* hepatoprotective models, the levels of cytosolic enzymes, a marker of oxidative damage to hepatocytes, were significantly reversed to almost to normal in dose dependent manner. Both the extracts significantly increased the levels of endogenous antioxidant enzymes: superoxide dismutase (SOD), catalase and glutathione (GSH) as compared to control. *LI* possesses marked hepatoprotective activity against paracetamol and anti tubercular drugs induced hepatotoxicity in rats as evidenced by both *in vivo* and *in vitro* results. The activity may be attributed to the individual or combined action of phytoconstituents present in it.

Keywords: Isoniazid, Pyrazinamide, Paracetmol, Silymarin, Rifampicin

INTRODUCTION

The liver is the key organ regulating homeostasis in the body. It is involved with almost all the biochemical pathways related to growth, fight against disease, nutrient supply, energy provision and reproduction. [1] The liver is expected not only to perform physiological functions but also to protect against the hazards of harmful drugs and chemicals. [2] Hepatic injury is associated with distortion of these metabolic functions. Paracetamol, a well-known antipyretic and analgesic agent, causes hepatotoxicity by its reactive metabolite N-acetyl-p-benzo quinoneimine (NAPQI). At higher doses of paracetamol the reactive metabolite, NAPQI, formed by Cytochrome p-450(CYT P450) causes oxidative stress and glutathione (GSH) depletion leading to hepatic necrosis. [3-5] Drug - induced hepatotoxicity is a potentially serious adverse effect of the currently used anti tubercular chemotherapeutic regimens containing isoniazid (INH), rifampicin and pyrazinamide. [6, 7, 8] Adverse effects of anti tubercular therapy are sometimes potentiated by multiple drug regimens. The drugs INH, Rifampicin and Pyrazinamide *per se* are potential hepatotoxic agents, but when they are given in combination their toxicity is further enhanced. The conversion of monoacetyl hydrazine, a metabolite of INH, to a toxic metabolite via Cytochrome p-450(CYT P450) leads to hepatotoxicity. Patients on concurrent

Rifampicin therapy have an increased incident of hepatitis. This has been postulated to be due to Rifampicin-induced CYT P450 enzyme induction, causing an increased production of toxic metabolites from Acetyl hydrazine (AcHz). [9] In view of this, the present study was undertaken to investigate the hepatoprotective activity of root of *LI* ethanolic extract in Paracetamol and anti tubercular drugs induced hepatotoxicity in Wistar rats. [10]

LI (Henna) is a plant which grows wild in abandoned areas [11] and commonly known as 'Inai' in Sumatra or 'Pachar kuku' in Java. This plant is a worldwide known cosmetic agent used to stain hair, skin and nails. [12] Alcoholic extracts of *LI* leaves showed mild antibacterial activity against *Micrococcus pyrogenes* and *Eschericia coli.* [13] *LI* was also reported to have tuberculostatic activity. [14] The leaves are used as a prophylactic against skin diseases. They are used externally in the form of paste or decoction against boils, burns, bruises and skin inflammations. A decoction is used as gargle against sore throat. [15] The roots of this plant are useful in burning sensation, leprosy, strangury and premature greying of hair. [16] Roots of *LI* were found to possess potent antioxidant and hepatoprotective activity against CCl_4 induced hepatotoxicity. [17] So in the present study the roots of *LI* were selected to assess the hepatoprotective activity against Paracetamol and anti tubercular drugs induced hepatotoxicity in rats.

EXPERIMENTAL

Collection of Plant Material: The plant material (Roots of *Lawsonia inermis*) was collected from Srirangapatnam taluk of Mysore district and was authenticated by a Dr. Maruthi and Dr. K. Mruthunjaya, Assistant Professor, Dept. of Pharmacognosy, JSSCP, Mysore. The roots were cleaned and cut into small pieces.

Animals: The experiments were carried out on Wistar rats of either sex weighing 200 g. Animals used in the study were procured from a registered breeder. The animal care and handling was carried out in accordance to guidelines issued by the Institutional Animal Ethics Committee, JSS College of Pharmacy, Mysore, and Karnataka. Animals were acclimatized to the experimental room for one week prior to the experiment. Animals were maintained under controlled conditions of temperature (27 ± 2^oC) and were caged in sterile polypropylene cages containing sterile paddy husk as bedding material with maximum of four animals in each cage. The rats were fed on standard food pellets and water ad libitum. The studies conducted were approved by the Institutional Ethical Committee, JSS College of Pharmacy, Mysore, and Karnataka.

Extraction Procedure:

***Alcoholic extract of** LI **(LIALC)*:** The crude drug was shade dried for 20 days and then coarse powdered. Soxhlet extraction method was used for extraction. The coarse powder was loaded in thimble made of Whatman filter paper no: 1 and extracted in the Soxhlet extraction column with 1 L of 95% alcohol. Each batch was extracted for 30 cycles in the soxhlet extraction column. The extract so obtained was thick and syrupy with characteristic odour. The excess alcohol was back distilled and the remaining was evaporated on a water bath. The final extract was thick and was stored separately in a dessicator.

Aqueous extract of** LI **(LIAQ): The coarsely powdered shade dried roots of the plant *LI* (150 g) was extracted with Chloroform- water (1:99) by cold maceration process for 7 days. After completion of extraction the marc was filtered through muslin cloth and concentrated in vacuum. Preliminary phytochemical screening was carried out for LIALC and LIAQ for the presence of lactones [18,19], flavonoids [20], sterols [18,21,22], terpenes [18,21], carbohydrates [18,22,23,24], tannins [18,22] compounds, which have been known for their hepatoprotective activities.

***In vitro* hepatoprotective activity:**

Isolation of hepatocytes: [31]

***Step I –Perfusion of liver*:** Overnight fasted male Wistar rat weighing 200 g was used. Animal was anaesthetized by using ketamine and 1% of sod.citrate was injected (i.p.) to prevent blood clotting. Dissection of the animal was carried out under aseptic conditions using sterilized instruments. When the liver was thoroughly perfused (*i.e.* has turned white), the flow of Hank's Balanced Salt Solution (HBSS) was stopped and the needle was removed. The liver was dissected and placed in a beaker containing HBSS.

***Step II- Processing of liver*:** The liver was transferred to a sterile petridish containing Ca^{2+}-Mg^{2+} free HBSS and minced into small pieces, which were transferred to a conical flask containing 2.5 ml of 0.75% collagenase in HBSS and kept in contact with collagenase for 5 min with continuous mixing.

***Step-III- Isolation of hepatocytes*:** The cell suspension thus obtained was centrifuged at 2000

rpm for 10 min. The supernatant was aspirated and the cells washed twice with Ca^{2+}-Mg^{2+} free HBSS and suspended in DMEM media supplemented with serum. Viability of the cells was checked by trypan blue dye which stains the dead cells. The experiment was conducted on hepatocytes if the viability of isolated hepatocytes was above 97%.

***In vitro* hepatoprotective activity of extracts on isolated hepatocytes:** Primary culture of hepatocytes was used to study hepatoprotective activity of the extract. The effect of the plant extract on toxin challenged hepatocytes was evaluated by trypan blue exclusion assay and by biochemical estimations as given below. The trypan blue assay directly measures the viability of toxin challenged heptocytes whereas biochemical estimation indirectly measures the viability of the toxin challenged heptocytes by the levels cytosolic enzymes in the medium. An increased levels of cytosolic enzymes viz. GPT, GOT and LDH in the medium indicates hepatotoxicity.

Trypan blue exclusion assay: [32] Fixed number of hepatocytes (1×10^6) was incubated in the DMEM media with toxin, CCl_4 (20 mM) and with or without extract of *LI* (at conc.10, 100 and 200 µg/ml) at 37°C for 3 h. Hepatoprotective activity was assessed by checking the viability of the cells after 3 h of incubation using trypan blue dye.

Biochemical estimations: After 3 h of incubation cell suspension was centrifuged at 200 rpm and the supernatant was collected to measure cytosolic enzymes such as GPT, GOT and LDH. The enzymes were estimated using respective kits by semiautoanalyser.

Acute toxicity study: [33] Acute toxicity studies were conducted to determine the safe dose by OECD guidelines 425, 2006. According to the guideline female Wistar rats were used for the study. The suspension of LIALC was prepared by suspending in 0.25% CMC and administered by orally. One animal was dosed at the test dose (2000 mg/kg). Animal was observed for the 24 hrs for mortality and it survived. Then another four animals were dosed sequentially so that total five animals were tested. All animals were survived.

***In vivo* hepatoprotective activity of root extract of *LI* against Paracetamol & Anti-TB drugs induced hepatotoxicity:** [5] Wistar rats weighing between 130 and 150 were randomly divided into five groups of six each. The suspension of LIALC was prepared just prior to the treatment by suspending in 0.25% CMC and the treatment was started as given below (Table 1 & 2).

Parameters: After 48 h of toxin administration, on 9^{th} day for paracetmol & 16^{th} day for Anti-TB drugs the following parameters were determined

1. **Estimation of biochemical parameters:** Blood samples were collected in micro centrifuge tubes without an anticoagulant and were allowed to clot at room temperature for 30 min followed by centrifugation at 2500 rpm for 8-10 min to separate the serum. The various serum enzymes, SGOT[34], SGPT[34], ALP[35, 36] and LDH, Total Protein[37] and Total Bilirubin[38] were estimated in semi-auto analyzer using the commercially available (MERCK) kits.

2. **Estimation of liver endogenous antioxidant enzymes:** After the blood withdrawal, the animals were sacrificed by cervical dislocation and were perfused transcardially with an ice-cold saline. The whole liver was then perfused *in situ* with ice-cold saline, dissected out, blotted dry and immediately weighed. A 10% liver homogenate was prepared with 150 mM KCL using Teflon– glass homogenizer (Yamato L.S. G. L.H-21, Japan). The tissue homogenate was centrifuged at 14,000 rpm for 1 h at 4°C. Following endogenous antioxidant enzymes like Superoxide dismutase[25], Catalase assay[26], Glutathione[26], Lipid peroxidation[27] were measured.

Statistical Analysis: Data were analyzed by one-way ANOVA followed by post-hoc Tukey's multiple comparison tests using Graph Pad prism ver. 5.0 software. $P<0.05$ were considered as significant.

RESULTS

Preliminary phytochemical screening of *Lawsonia inermis (LI):* Confirms the presence of sterols, triterpenes, carbohydrates, tannins, flavonoids and lactones in alcoholic fraction and presence carbohydrates, tannins, flavonoids, lactones aqueous fraction.

***In vitro* hepatoprotective activity on isolated hepatocytes:** In the *in-vitro* hepatoprotective study, CCl_4 treatment significantly reduced the percentage viability of hepatocytes when compared to normal. Incubation of toxin challenged hepatocytes with LIALC & LIAQ for 3 h significantly reversed the

CCl_4 induced hepatotoxicity. Further CCl_4 caused significant increase in the GOT, GPT and LDH levels in the media when compared to normal. Both the extracts normalize the increased levels of GPT, GOT, LDH compare to normal in a dose dependent manner. From the acute toxicity study it was observed that oral administration of LIALC at 2000 mg/kg did not cause mortality in the first animal.

The extract was found safe in four more animals dosed at 2000 mg/kg indicating the LD_{50} above 2000 mg/kg. From *in-vivo* study paracetamol & anti tubercular drugs treatment significantly elevated the levels of SGOT, SGPT, LDH, ALP and total bilirubin, while Total Protein level was significantly decreased when compared to normal. Silymarin (100 mg/kg) significantly reversed SGOT, SGPT, LDH, ALP, Total Bilirubin and Total Protein levels when compared to positive Control. Treatment with LIALC at both the tested doses (200 and 300 mg/kg) significantly reversed SGOT, SGPT, LDH, ALP, Total Bilirubin and Total Protein levels when compared to positive Control. The LIALC at 300 mg/kg was similar to standard Silymarin in reducing the elevated LDH levels and less effective in reversing the other biochemical parameters. Paracetamol & anti tubercular drugs treatment significantly decreased liver endogenous antioxidant enzymes such as SOD, CAT and GSH while lipid peroxidation was significantly enhanced when compared to Normal. Silymarin treatment (100 mg/kg) significantly reversed SOD, CAT and GSH levels with a subsequent reduction in lipid peroxidation when compared to Control. LIALC treatment (200 and 300 mg/kg) significantly reversed SOD, CAT and GSH levels with a subsequent reduction in lipid peroxidation when compared to Control. LIALC at 300 mg/kg was similar to standard Silymarin in reversing the SOD levels and lipid peroxidation to normal & is less effective in normalizing the CAT and GSH levels.

DISCUSSION

The present study was undertaken to study the possible hepatoprotective and antioxidant role of ethanolic & aqueous extract of roots of *LI* in CCl_4, Paracetmol & anti-tubercular drugs induced liver toxicity rat model. Mruthunjaya, 2008 in his study reported the presence of very high amount of polyphenols and gallic acid, a well known potent antioxidant [28] as an important constituent of the roots of a widely grown plant *LI*. He also reported the antioxidant and *in-vitro* and *in-vivo* hepatoprotective activity of *LI* root extract against CCl_4 induced hepatotoxicity in rat's liver. So in the present study *LI* was selected for investigating the *in-vivo* and *in-vitro* hepatoprotective activity against drugs induced hepatotoxicity. From the in-*vitro* results it was apparent that both LIALC and LIAQ protect the hepatocytes against CCl_4 induced toxicity as evident by the reversal of cell death and inhibition leakage of cytosolic enzymes. As discussed earlier this strong hepatoprotective activity of LIALC might be due to the presence of polyphenols and gallic acid which are potent free radical scavenging agents and antioxidants. [28]

Paracetamol is converted into its reactive metabolite N-acetyl [-p-benzoquinoneimine] (NAPQI) by hepatic cytochromeP-450.The massive production of reactive species would lead to depletion of protective physiological moieties (glutathione and α-tocopherol,etc.),and ensuing wide spread propagation of alkylation as well as peroxidation, causing damage to the macromolecules in vital biomembranes. The lipid peroxidation also damage cell membrane resulting in the leakage of enzymes into the blood stream. Therefore the elevated biochemical levels are treated as biochemical markers of tissue damage. In addition the extent of lipid peroxidation is directly proportional to the tissue damage. [29]

There are certain inbuilt protective mechanisms, tissue enzymes GSH, SOD, CAT etc. which are involved in the process of combating free radical induced tissue damage. Over powering the inbuilt protective mechanism due to excessive generation of free radicals may lead to destruction of the tissues/organs. [30] SOD removes superoxide (O_2) by converting it to H_2O_2, which can be rapidly converted to water by CAT and Glutathione peroxidase. In addition, a large reserve of reduced glutathione is present in hepatocytes and red blood cells for detoxification of xenobiotics or free radicals. However, oxidative stress results in toxicity when the rate of which the ROS are generated exceeds the cell capacity for their removal. Lipid peroxidation is an autocatalytic process, which is a common consequence of cell death. This process may cause peroxidative tissue damage in inflammation, cancer and toxicity of xenobiotics and aging. MDA is one of the end products in the lipid peroxidation process.

In order to elucidate the protection mechanism of LIALC in Paracetamol and anti- tubercular Drugs induced rat liver, lipid peroxide levels and anti-oxidative enzymes activities were analyzed. GSH is widely distributed in cells. GSH is an intracellular reductant and plays a major role in catalysis, metabolism and transport. It protects cells against

free radicals, peroxides and other toxic compounds. GSH is a naturally occurring substance that is abundant in many living creatures. It is CAT is the key component of the anti-oxidant defense system .Inhibition of this protective mechanisms result in enhanced sensitivity to free radical induced cellular damage.

Administration of LIALC increased the activity of catalase in Paracetamol and anti-tubercular drug induced liver damage to prevent the accumulation of excessive free radicals and thus exhibited protection against Paracetamol and anti-tubercular drug. Paracetamol and anti-tubercular drugs cause ROS mediated hepatotoxicity and elevate serum enzymes levels like SGOT, SGPT, ALP, LDH, and Bilirubin content in animals following administration of anti-tubercular drugs or toxic dose of Paracetamol. Also these drugs cause depletion of endogenous antioxidant enzymes like GSH, SOD, CAT etc and abnormal levels of lipid peroxidation products.

The results obtained in the present are in accordance with understanding as discussed above. i.e., the serum enzymes levels like SGOT, SGPT, ALP, LDH and Bilirubin contents were significantly increased, GSH, SOD, CAT levels in liver depleted and abnormal levels of lipid peroxidation was observed in Paracetamol and anti-tubercular drugs treated animals. Treatment with LIALC markedly reversed these changes. This shows LIALC can scavenge reactive free radicals that might lessen oxidative damage to the liver tissue and improve the activities of the hepatic antioxidant enzymes. LIALC reversed SGPT, SGOT, ALP enzyme levels indicating stabilization of cell membrane by preventing the damage due to free radicals generated by Paracetamol and anti-tubercular drugs. Significant reversal of elevated bilirubin level in Paracetamol and anti-tubercular drug treated animals by LIALC indicated the strong hepatoprotective activity of LIALC. A reduction in total serum protein observed in the Paracetamol and anti-tubercular treated control rats may be associated with the decrease in the number of hepatocytes which in turn might result in decreased hepatic capacity to synthesize protein. But, when the LIALC was administered along with Paracetamol or anti-tubercular drugs a significant increase in protein content was observed indicating the hepatoprotection of LIALC.

The observed increase of SOD, CAT and GSH activity in liver suggests that the LIALC extract have an efficient protective mechanism in response to ROS. And also, these findings indicate that LIALC may be associated with decreased oxidative stress and free radical mediated tissue damage. In our study, elevation in the levels of end products of lipid peroxidation in liver of rat treated with Paracetamol and anti- tubercular drugs were observed. The increase in MDA level in liver suggests enhanced lipid peroxidation leading to tissue damage and failure of anti-oxidant defense mechanism to prevent formation of excessive free radicals. Treatment with LIALC significantly reversed these changes. Hence it may be possible that the mechanism of hepatoprotection of LIALC is due to its antioxidant effect.

CONCLUSION

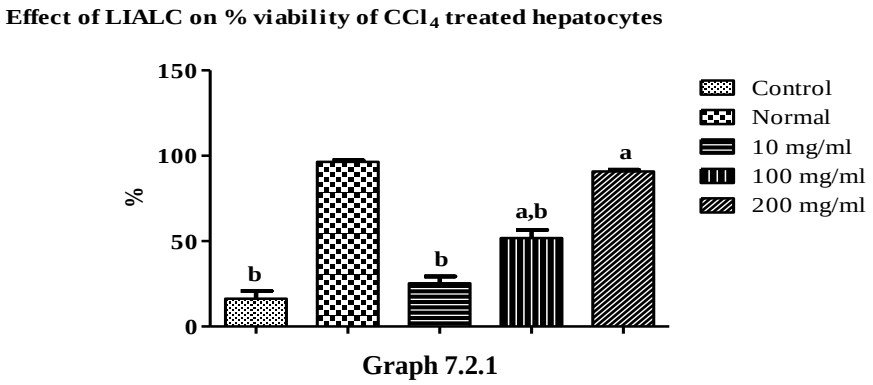
From the results obtained, it can be concluded that the ethanolic extract of *lawsonia inermis* (LIALC) roots possess hepatoprotective activity in rats.

Table 1: TREATMENT SCHEDULE FOR PARACETAMOL MODEL

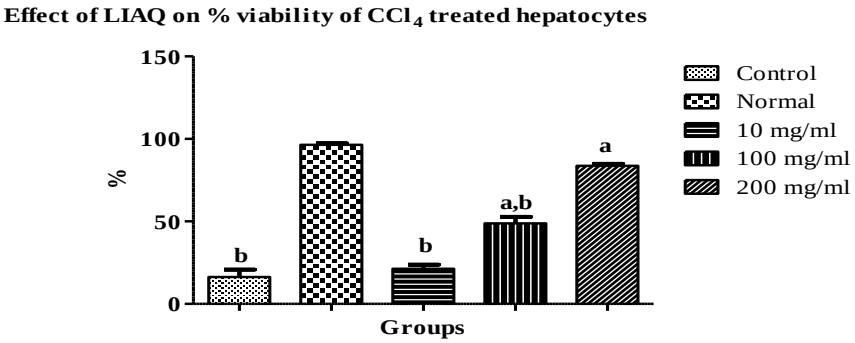
GROUPS (N=6)	TOXIN ON DAY 7TH (P.O.) AFTER 2H OF TREATMENT	TREATMENT FOR 7 DAYS (P.O)
Toxin Control	Paracetamol (2 gm/kg),	Saline
Normal Control	-------	----------
Test (i)	Paracetamol (2 gm/kg)	LIALC 200 mg/kg
Test (ii)	Paracetamol (2 gm/kg)	LIALC 300 mg/kg
Standard	Paracetamol (2 gm/kg)	Silymarin 100 mg/kg.

Table 2: TREATMENT SCHEDULE FOR ANTI- TUBERCULAR DRUGS MODEL

GROUPS (N=6)	TOXIN TREATMENT FOR 14 CONSECUTIVE DAYS (DOSE MG/KG)	TREATMENT FOR 14 CONSECUTIVE DAYS (DOSE MG/KG. P.O)
I Toxin Control	Combination of INH 50 + RMP100 i.p and PYZ 350 p.o	Saline
II. Normal Control	--------	----------
III. Test (i)	Combination of INH 50 + RMP100 i.p. and PYZ 350 p.o	LIALC 200 mg/kg
IV. Test (ii)	Combination of INH 50 + RMP100 i.p. and PYZ 350 p.o	LIALC 300 mg/kg
V. Standard	Combination of INH 50 + RMP100 i.p. and PYZ 350 p.o	Silymarin 100 mg/kg.

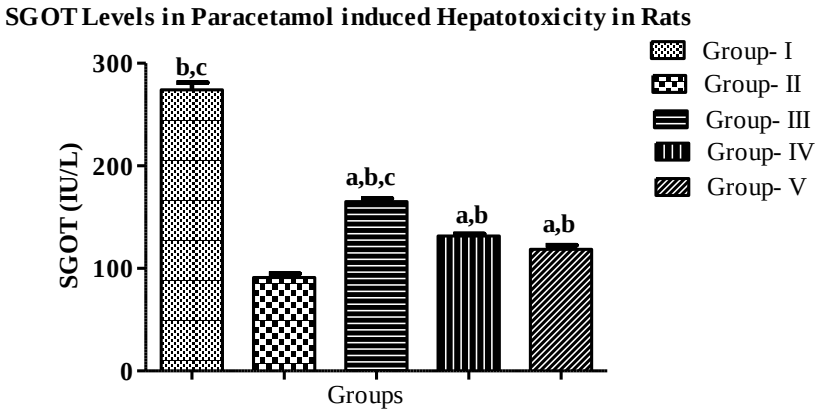


Graph 7.2.1



Graph 7.2.2

Values are mean ± SEM. [a]P<0.05 as compared with Control. [b]P<0.05 as compared with Normal



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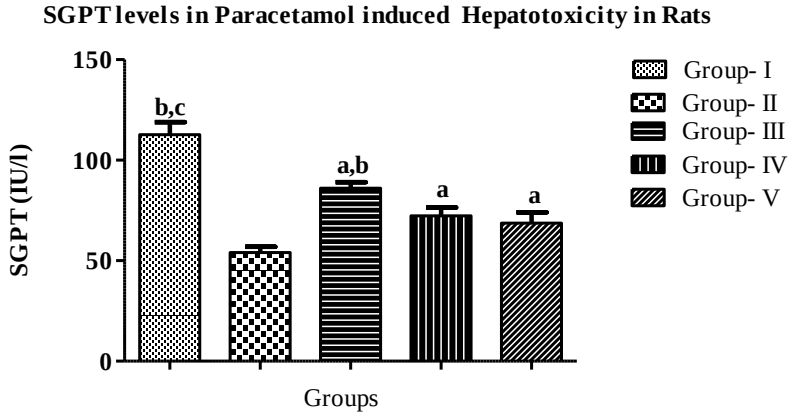

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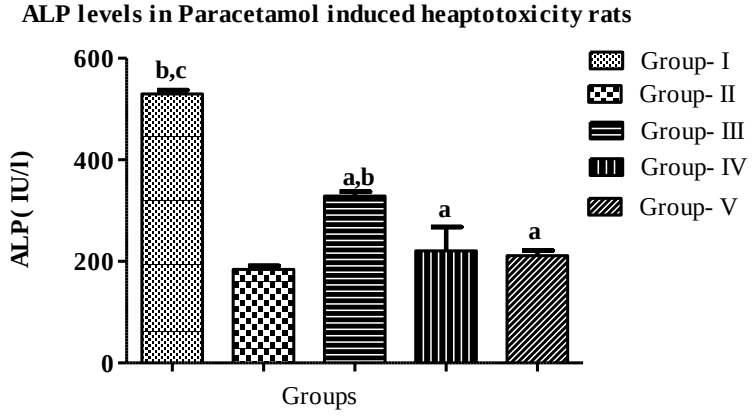
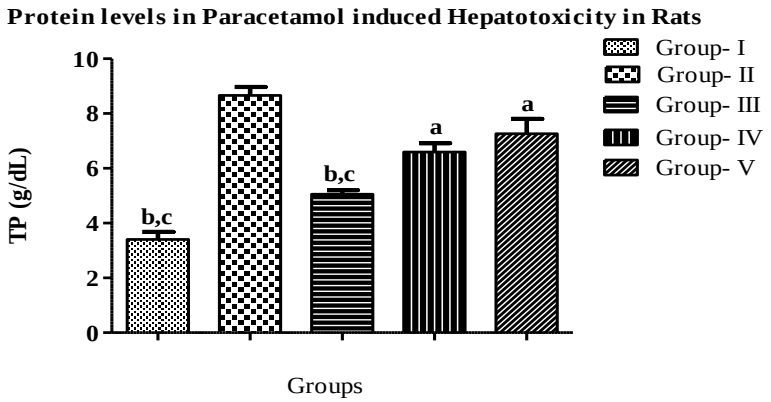


Graph 7.2.5

Values are mean ± SEM. [a]P<0.05 as compared with Control. [b]P<0.05 as compared with Normal.



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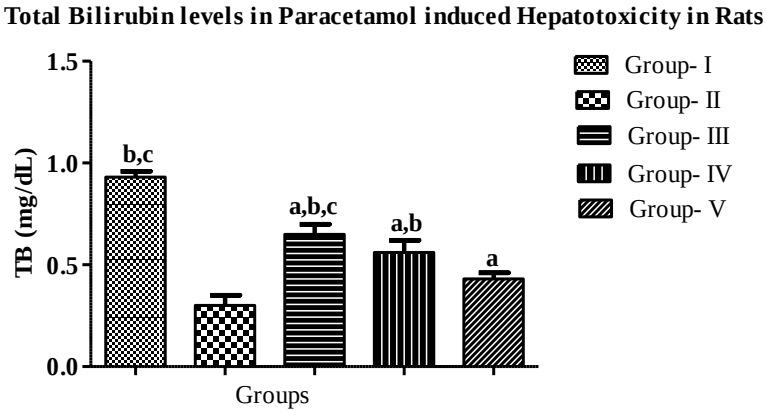

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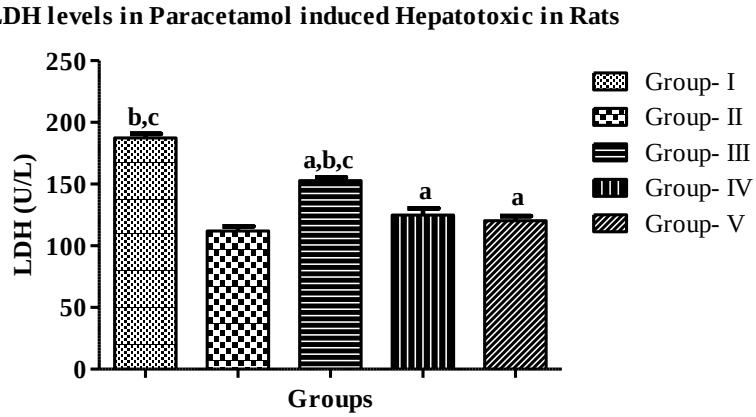
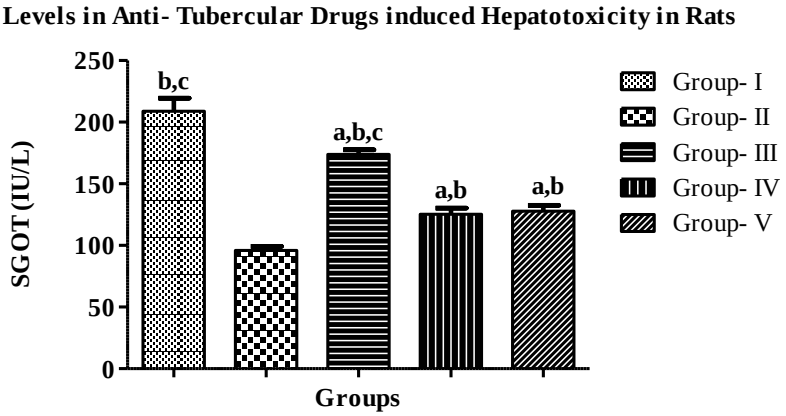


Graph 7.2.8

Values are mean ± SEM. [a]P<0.05 as compared with Control. [b]P<0.05 as compared with Normal.



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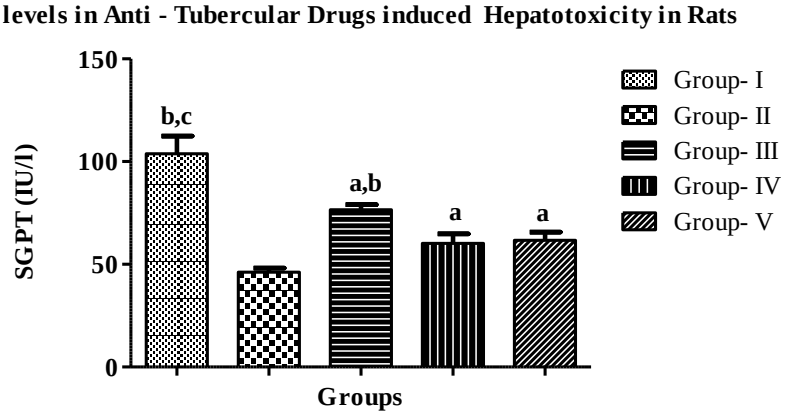
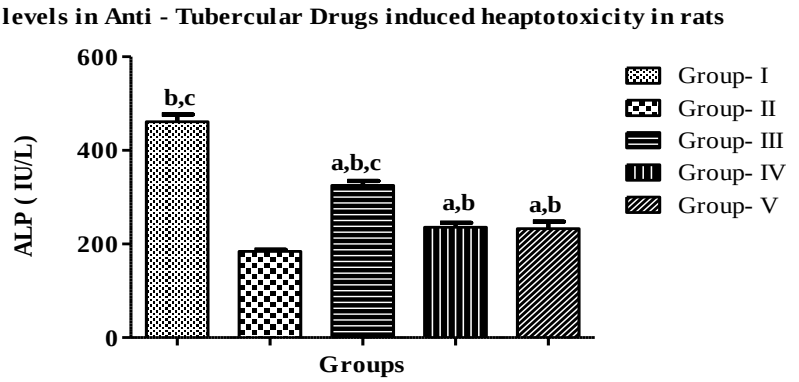
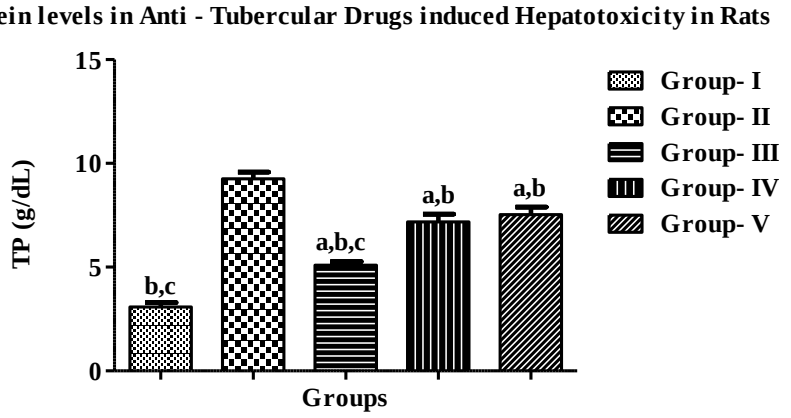


Graph 7.2.10



Graph 7.2.11

Values are mean ± SEM. [a]P<0.05 as compared with Control. [b]P<0.05 as compared with Normal.



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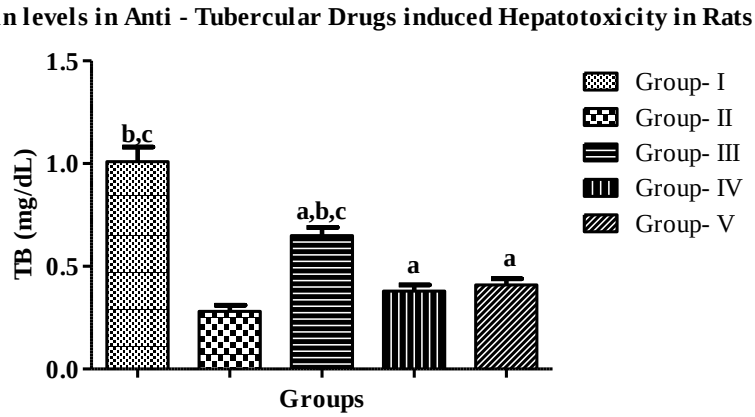
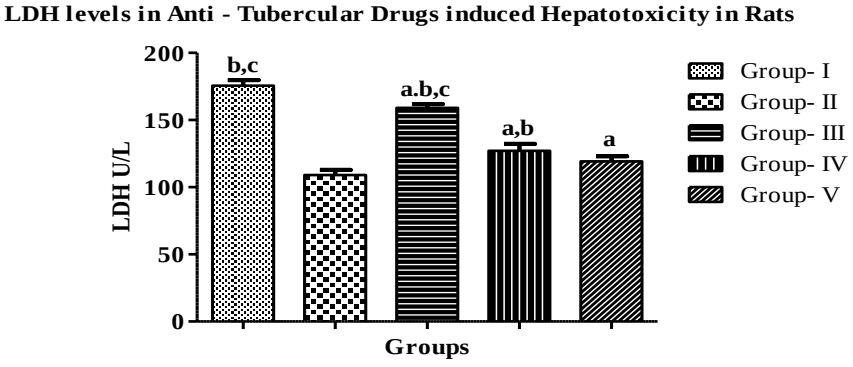


Graph 7.2.13



Graph 7.2.14

Values are mean ± SEM. [a]P<0.05 as compared with Control. [b]P<0.05 as compared with Normal.

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