Protective Effects of Aqueous Extract of *Centella asiatica* Against Cyclophosphamide Induced Toxicity in Experimental Rats

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ABSTRACT

Background: Cyclophosphamide (CPA) is an alkylating agent and is widely used in cancer chemotherapy. Several studies have also shown that long term administration of cyclophosphamide has the ability to produce various toxicities in individuals. Popularity of *Centella asiatica* (CA) or pegaga as one of the local medicinal plants is mostly due to its reputation is used as a wound healing agent and brain stimulant. The herb is said to have various medicinal properties, like it has direct effect in lowering blood pressure and is often referred to as a rejuvenating medicament in the Ayurvedic Pharmacopoeia. Objective: This study was designed to investigate the protective effects of aqueous plant extract (PE) of CA against cyclophosphamide induced toxicity in experimental rats. Method: The whole plant aqueous extract of CA was administered to rats orally for 15 days. On day 13th, 14th and 15th cyclophosphamide was also given orally to rats after 30 minutes of administration of aqueous extract of CA. After 15 days animals were sacrificed and were investigated for Melondialdehyde (MDA), Reduced glutathione (GSH), nitric oxide (NO), white blood cells (WBC), red blood cells (RBC), total protein content, creatinine, total cholesterol, triglycerides, body weight, organ weight and were compared relatively among experimental groups. Results: It was found that the group which was pretreated with CA showed the decreased levels of oxidative stress related parameters when compared with the group which was treated with cyclophosphamide alone. Conclusion: Present study has shown that cyclophosphamide which is used as anticancer drug exhibited oxidative stress in experimental rats and CA was found to exert beneficial effects against the toxicity caused by cyclophosphamide upto a certain extent.

1. INTRODUCTION

Popularity of *Centella asiatica* or pegaga as one of the local medicinal plants is mostly due to its reputation as a wound healing agent and brain stimulant [1]. *C. asiatica*, which grows wild in both tropical and sub-tropical countries, is closely related to the species Hydrocotyle and produces characteristic essential oil [2] and various types of flavanoids [3]. The herb is said to have a direct effect in lowering blood pressure and is often referred to as a rejuvenating medicament in the Ayurvedic Pharmacopoeia [4]. In addition, it is also believed to be able to purify blood, cure indigestion and nervousness, treat skin disorders, and as a diuretic, and antihypertensive agent. Further it has been used as a remedy against asthma, leprosy, anaemia and inflammations [5]. Numerous clinical reports verify the ulcer preventive [6], antidepressive and sedative [7] effects of *C. asiatica* preparations, as well as their ability to improve venous insufficiency [8].

Cyclophosphamide is an alkylating agent widely used in cancer chemotherapy. It is an inactive cytostatic, which is metabolized into active metabolites mainly in the liver. During bio-activation reactive oxygen species are also formed, which can modify the components of both healthy and neoplastic cell leading to decreased anti-oxidative capacity [9]. It has been observed that cyclophosphamide causes genotoxicity and oxidative stress in mice [10] and early lung injury in rats [11]. It also produces cardiotoxic effects [12]. Hemorrhagic cystitis is a major dose limiting side effect of cyclophosphamide [13]. Studies have also shown that cyclophosphamide has the ability to produce male germ cell toxicity [14].

There is general agreement that alkylating agents are among the most effective medications for treating autoimmune disease [15]. However, recognition of a variety of toxicities, particularly concerns about the long-term risk of malignancy and gonadal toxicity, often lead clinicians to consider less toxic alternative medications whenever possible [16-18].

In view of the above findings and the ongoing search for antioxidants that may reduce drug induced lipid peroxidation (16-19) the present work has been carried out to evaluate the protective effects of CA against cyclophosphamide-induced toxicity in experimental rats.

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2. MATERIALS AND METHODS

2.1 Animals

Random breed Wistar albino rats (100-150 g body weight) of both sexes were used for this study. Rats were obtained from animal house of School of life science, DAVV, Indore. Animals were housed at temperature 24 ± 2^0C, 12 hour light or dark cycle and fed with standard pellet diet and water. They were kept in wire-bottomed stainless steel cages. The experimental procedure used in this study met the guidelines of the Animal Care and Use Committee of the Devi Ahilya University (Registration Number -770).

2.2 Chemicals

Chemicals were of the highest purity and were obtained from Sigma (USA). The commercial whole plant aqueous extract of CB was used in our study and was obtained from AMSAR private limited, Indore.

2.3 Plant extract

The whole plant aqueous extract of CA was collected from AMSAR private limited, Indore was used for experiments in this study.

2.4 Experimental design

Animals were divided into various treatment groups (minimum 5 animals per group) and the plant extract was suspended in water and was administered orally for 15 days in the following way:

Group I: (Healthy control) received water only.

Group II: (Cyclophosphomide control) This group was given water for initial 12 days and later (on 13th,14th and 15th day) cyclophosphamide was administered   (30 mg/kg/p.o).

Group III: plant extract (100 mg/kg/po) for all 15 days + cyclophosphamide (30 mg/kg/p.o) on 13th, 14th and 15th day.

Group IV: plant extract (200 mg/kg/po) for all 15 days + cyclophosphamide (30 mg/kg/p.o) on 13th, 14th and 15th day.

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Group V: plant extract (400 mg/kg/po) for all 15 days + cyclophosphamide (+30 mg/kg/p.o) on 13th, 14th and 15th day.

2.5 Analytical procedure

After 24 hours of the last dose, animals were sacrificed and their blood was collected in falcons tube containing anticoagulant. For serum collection another vial was used without anticoagulant. The blood cells were allowed to settle and then serum was separated by centrifugation at 3000 g at 37ºC. Thus blood and serum were used for determination of various biochemical and hematological parameters. Several organs from the animal body were also eradicated and were preserved for further studies.

2.6 Tissue homogenate preparation for LPO, GSH assay: 1 g of liver tissue was collected from each experimental rat, washed in normal saline and soaked in filter paper. The tissues were then homogenized in 10 ml of 0.15 M Tris buffer (pH-7.4) and centrifuged at 3000 g at 4°C for 30 min. The supernatant was collected was taken for lipid peroxidation, glutathione assay (19).

2.7 Tissue homogenate preparation for Nitrite concentration: A 10% (w/v) tissue homogenate was prepared in chilled 0.1 M phosphate buffer (pH 7.4) using a homogenizer. The homogenate was centrifuged at 12000 g for 20 min, 4°C for 30 min. The supernatant was collected and was used for estimation of nitrite (19).

2.8 Lipid Peroxidation

Lipid peroxidation (LPO) was assayed according to the method of Okhawa *et al* [19]. To 1 ml of tissue homogenate, 1 ml of normal saline (0.9% w/v) and 2.0 ml of 10% TCA was added and was mixed well. The mixture was then centrifuged (3000 rpm) at room temperature for 10 minutes to separate proteins. Then 2 ml of supernatant was taken and 0.5 ml of 1.0% TBA (Tri butyric acid) was added to it, followed by heating at 95°C for 60 min to generate the pink colored MDA. Absorbance of the samples was measured at 532 nm using spectrophotometer. The levels of lipid peroxides were expressed as nM of MDA/mg wet tissue using extinction co-efficient of 1.56 x105 M^{-1} cm^{-1}.

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Calculation:

Malondialdehyde (nM/ml)

= Absorbance of test × total volume ∕ Nanomolar extinction coefficient × sample volume × 100

2.9 Reduced glutathione

Reduced glutathione (GSH) activity was assayed according to the method of Ellman (1959). Reduced Glutathione was estimated spectrophotometrically by determination of DTNB (Dithiobis-2-nitrobenzoic acid) reduced by SH-groups, expressed as µg/mg protein.

To 0.1 ml of tissue homogenates, 2.4 ml of 0.02 M EDTA solution was added and kept on ice bath for 10 min. Then 2 ml of distilled water and 0.5 ml of 50 %w/v TCA were added to it. The mixture was kept on ice bath for 10-15 min, then centrifuged at 3000 rpm for 15 min. To 1 ml of supernatant, 2.0 ml of Tris buffer (0.4M), 0.05 ml of DTNB solution (Ellman's reagent; 0.01M DTNB in methanol) were added and vortexed thoroughly. Absorbance was measured (within 2-3 min after the addition of DTNB) at 412 nm in spectrophotometer against blank solution. Appropriate standards were run simultaneously [20].

2.10 Determination of Nitric oxide content

Nitric oxide plays an important role in neurotransmission, vascular regulation, immune response and apoptosis. Since NO is rapidly converted to nitrite (NO2-) and nitrate (NO3-), the total concentration of nitrite and nitrate is used as a quantitative measure of NO production. A 10% (w/v) tissue homogenate was prepared in chilled 0.1 M phosphate buffer (pH 7.4) using a potter Elvehjem homogenizer. The homogenate was centrifuged at 12000 rpm for 20 min, 4^0C for post mitochondrial supernatant. Nitrite level was estimated using Griess reagent, which serves as an indicator of nitric oxide production. Briefly 1.0 ml of Griess reagent (1:1 solution of 1% sulphanilamide in 5% phosphoric acid and 0.1 % napthylethylene diamine dihydrochloride in water) was added to 1.0 ml of homogenate and absorbance was read at 546 nm. Nitrite concentration was calculated using a standard curve for sodium nitrite and nitrite levels were expressed as µg/ml tissue [21].

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2.11 Determination of RBC and WBC [22]

The RBC count denotes how many red blood cells per cubic millimeter of blood. Blood was diluted using Hayem's fluid in ratio 1: 200 and counting was done using haemocytometer counting chamber.

Calculation was done using the following formulae:

No. of RBC per cubic mm of blood= Total no. of cells counted $\times$ dilution$\times$ 4000/ no. of small squares in which counting has been done.

Where,

Dilution=200

Each smaller square has blood= 1/4000 cu. Mm

No. of small squares = 80

2.12 Determination of Triglycerides

The triglyceride colorimetric assay uses the enzymatic hydrolysis of triglycerides by lipase to glycerol and free fatty acids. The glycerol released is subsequently measured by a coupled enzymatic reaction system. The glycerol formed in reaction 1 is phosphorylated to glycerol-3-phosphate in a reaction catalyzed by glycerol kinase. The glycerol-3-phosphate is oxidized by glycerol phosphate oxidase producing dihydroxyacetone phosphate and hydrogen peroxide. Peroxidase catalyzes the redox-coupled reaction of H_2O_2 with 4-aminoantipyrine (4-AAP) and N-Ethyl-N-(3-sulfopropyl)-m-anisidine (ESPA), producing a brilliant purple color. Determination of triglycerides was done by following the protocol mentioned in Autospan liquid Gold triglyceride kit. The absorbance was measured at 540 nm.

Calculation:

Triglycerides (mg/dl) = Absorbance of test $\times$ 200 /Absorbance of standard

For glycerol free triglycerides

= Triglycerides – 10 mg/dl

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2.13 Determination of Glucose

Glucose oxidase oxidizes glucose to gluconic acid and hydrogen peroxide. In the presence of enzyme peroxidase, released hydrogen peroxide is coupled with phenol and 4-aminoantipyrine (4-AAP) to form coloured quinoneimine dye. Determination of glucose was done by following the protocol mentioned in Autospan liquid Gold Glucose kit. Absorbance of coloured dye was measured at 505 nm and was directly proportional to glucose concentration in the sample.

Calculation

Serum/plasma glucose (mg/dl)= Absorbance of test $\times$ 100/Absorbance of standard.

2.14 Determination of Creatinine

Determination of creatinine was done by following the protocol mentioned in Autospan liquid Gold creatinine kit. Creatinine reacts with picric acid in an alkaline medium to form an orange colored complex. The rate of formation of this complex was measured by reading the change in absorbance at 505 nm in a selected interval of time which was proportional to the concentration of creatinine in the serum. The reaction time and the concentration of picric acid sodium hydroxide have been optimized to avoid interference from ketoacids.

Calculation:

Serum/Plasma Creatinine (mg/dL) = (AT2-AT1/AS2-AS1)$\times$2

Where,

AT1: Initial O.D. of Test

AT2: Final O.D. of Test

AS1: Initial O.D. of Standard

AS2: Final O.D. of Standard

2.15 Determination of Total Cholesterol

Cholesterol esters are hydrolyzed by Cholesterol Esterase (CE) to give free Cholesterol and fatty acids. In subsequent reaction, Cholesterol oxidase (CHOD) oxidizes the 3-OH group of free cholesterol to liberate Cholest-4-en-3-one and Hydrogen Peroxide. In presence of Peroxidase

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(POD), Hydrogen Peroxide couples with 4-Aminoantipyrine (4-AAP) and Phenol to produce Red Quinoneimine dye. Determination of cholesterol was done by following the protocol mentioned in Autospan liquid Gold cholesterol kit. Absorbance of colored dye is measured at 505 nm and is proportional to amount of Total Cholesterol concentration in the Sample.

 Calculation:

Total cholesterol concentration (mg/dL) = Absorbance of Test/Absorbance of Standard $\times$ 200

2.16 Determination of protein

"Protein by Folin Reaction"[23] has been the most widely used method to estimate the amount of protein (already in the solution or easily soluble in dilute-alkali) in biological samples. The amount of protein in the sample was estimated via reading the absorbance (at 750 nm) of the end product of the Folin reaction against a standard curve of a selected standard protein solution (Bovine Serum Albumin-BSA-solution).

2.17 Determination of body weight and organ weight

Body weight was measured on 1st day before the treatment and also after the completion of 15 days of treatment protocol. After 15 days of treatment protocol, the animals were humanely sacrificed by ether anaesthetization and the rats were carefully dissected, and the whole liver, kidney, heart, lungs and the spleen were excised, freed of fat, blotted with clean laboratory tissue paper and then weighed.

2.18 Statistical Analysis: The values were represented as mean $\pm$ S.D for every group. Analysis of variance (ANOVA) test used Prism Pad Software (version 3.0) for determination of level of significance. The values of $p \leq 0.05$ were considered as statistically significant.

3. RESULTS & DISCUSSION

3.1 Lipid Peroxidation: When compared with cyclophosphamide control it has been observed that MDA content is greatly reduced in the groups which were orally administered with the plant extract. MDA content is directly related with the extent of lipid peroxidation and its content was found to be reduced in groups treated with plant extract. This reduction in MDA content was found to be dose dependent as it was 14.2 nM/mg in group treated with PE (100 mg/ml), 11.8

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nM/mg in group treated with PE (200 mg/ml) and in the last group which was administered the highest dose of plant extract (400 mg/ml) the MDA content was very low at a value of 08.2 nM/mg.
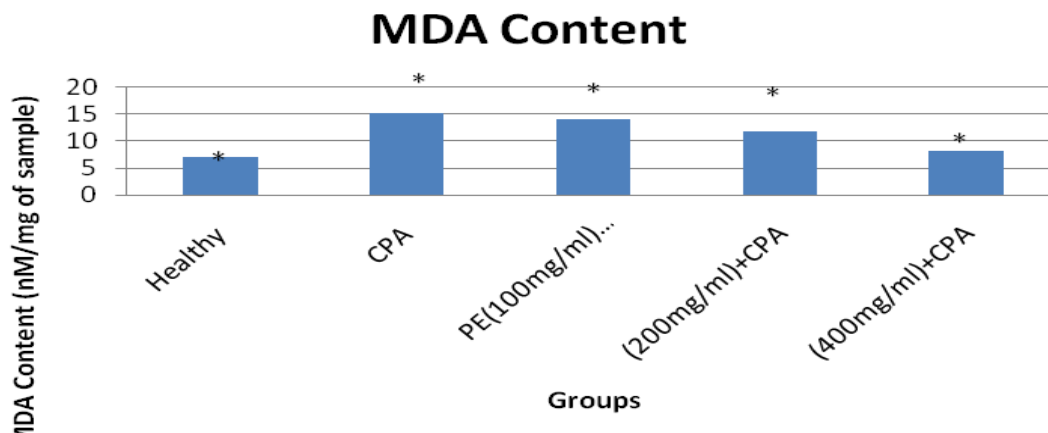

Figure 1: Effect of *C. asiatica* on liver LPO (MDA nmole/ mg of protein) against cyclophosphomide induced toxicity in rats (p <0.05).

3.2 Reduced Glutathaione (GSH): It was observed that reduced glutathaione was greatly increased in the groups which were pretreated with plant extract at different doses before administration of cyclophosphamide as compared to group which was given only cyclophosphamide. This increase in GSH content was in a dose dependent manner i.e. 0.28 in group treated with PE (100 mg/ml), 0.57 in group treated with PE (200 mg/ml) and in the group which was administered the highest dose of plant extract PE (400 mg/ml) its content was very high at a value of 0.92 comparable with healthy group but could not reach up to the limits of significance (P> 0.05).
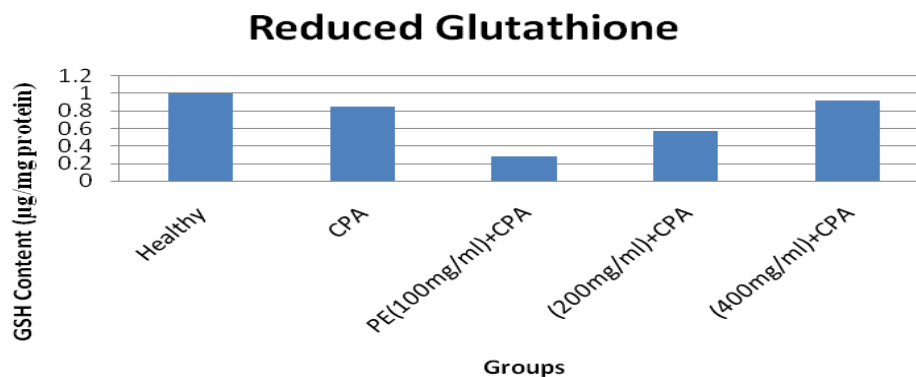

Figure 2: Effect of plant extract on GSH content (P> 0.05)

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3.3 Nitric Oxide Content: When compared with cyclophosphamide control it was observed that NO content was greatly increased in the groups which were orally administered with the plant extract. This increase in NO content is dose dependent as it was 66.00 in group treated with PE (100 mg/ml), 85.75 in group treated with PE (200 mg/ml) and in the group which was administered the highest dose of plant extract (400 mg/ml) the NO content was very high at a value of 105.6 comparable with that of the healthy control. These values were found to be significant when compared statistically, $p < 0.05$.
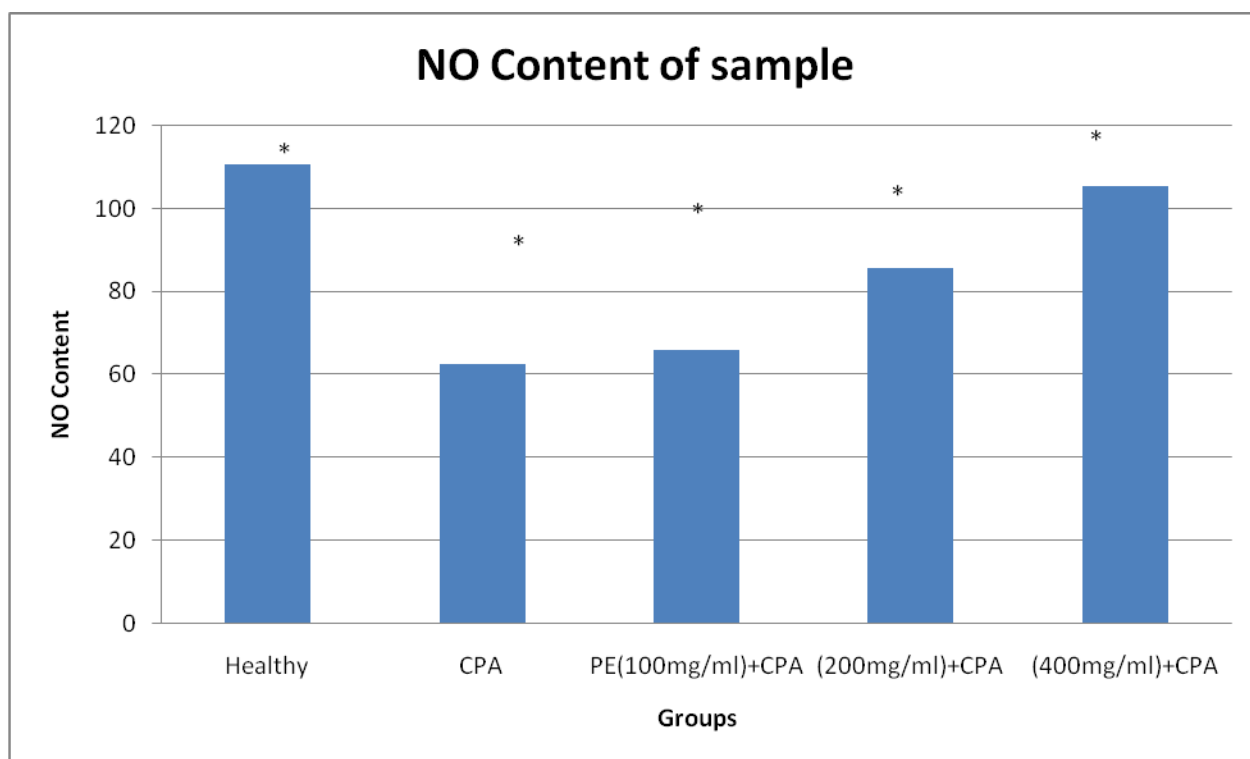
Figure 3: Effect of plant extract on NO content (* indicates p< 0.05)

3.4 RBC Count: RBC Count is greatly increased in the groups which were orally administered with the plant extract as compared to cyclophosphamide. This increase in RBC is dose dependent as it is 6.48×10^6 mm^3 in group treated with PE (100 mg/ml), 6.75×10^6 mm^3 in group treated with PE (200 mg/ml) and in the last group which was administered the highest dose of plant extract PE (400 mg/ml) the WBC Count was very high 7.41×10^6 mm^3 .

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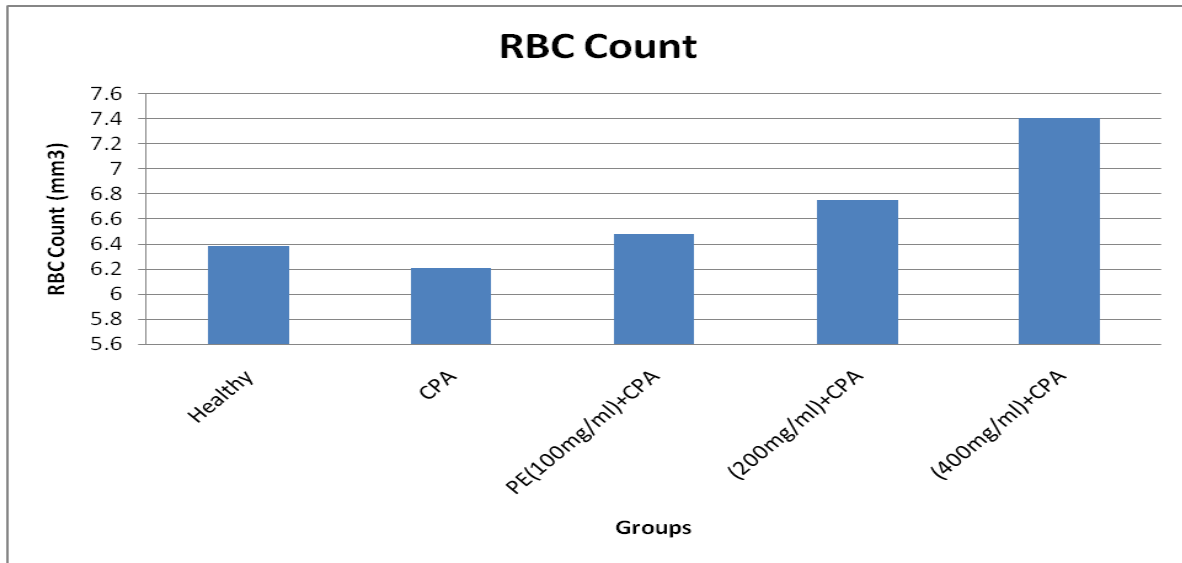
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RBC Count

Figure 4: Effect of plant extract on triglyceride content (P>0.05)

3.5 WBC Count: WBC Count is greatly increased in the groups which were orally administered with the plant extract as compared to cyclophosphamide. This increase in WBC is dose dependent as it is 7925 mm³ in group treated with PE (100 mg/ml), 8472 mm³ in group treated with PE (200 mg/ml) and in the last group which was administered the highest dose of plant extract PE (400 mg/ml) the WBC Count was very high 8530 mm³. These values were found to be significant when analysed statistically, p<0.05.
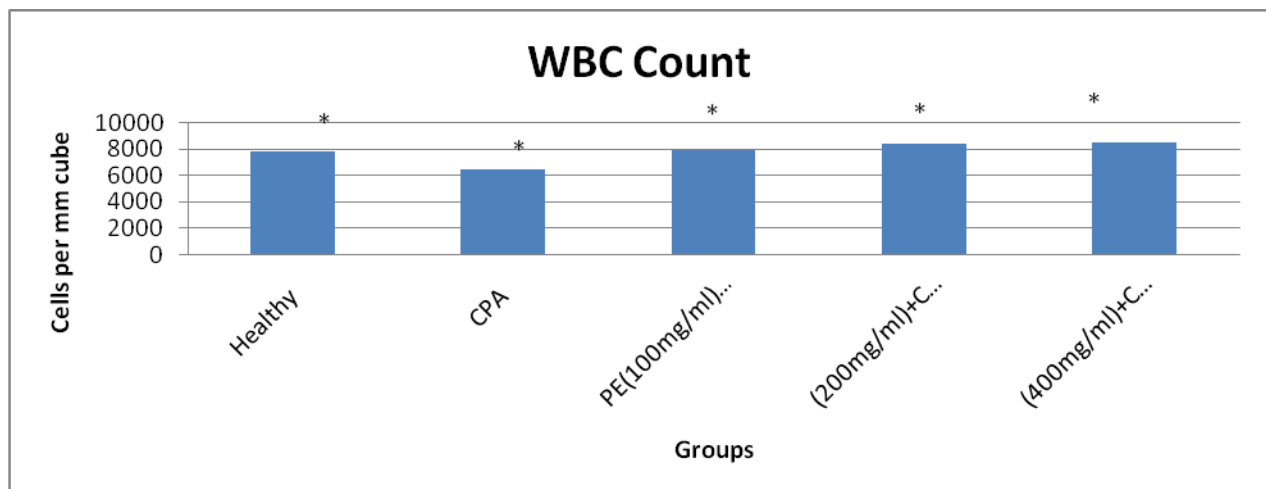
WBC Count

Figure 5: Effect of plant extract on WBC Count. (* indicates p<0.05)

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3.6 Triglyceride: It has been observed that triglyceride content is greatly increased in the groups which were orally administered with the plant extract as compared with cyclophosphamide control. This increase in triglyceride content is dose dependent as it is 89.68 mg/dL in group treated with PE (100 mg/ml), 102.2 mg/dL in group treated with PE (200 mg/ml) and in the last group which was administered the highest dose of plant extract PE (400 mg/ml) the creatinine content was same as healthy control is 132.7 mg/dL. These difference could not reach up to the limits of significance ($p > 0.05$).
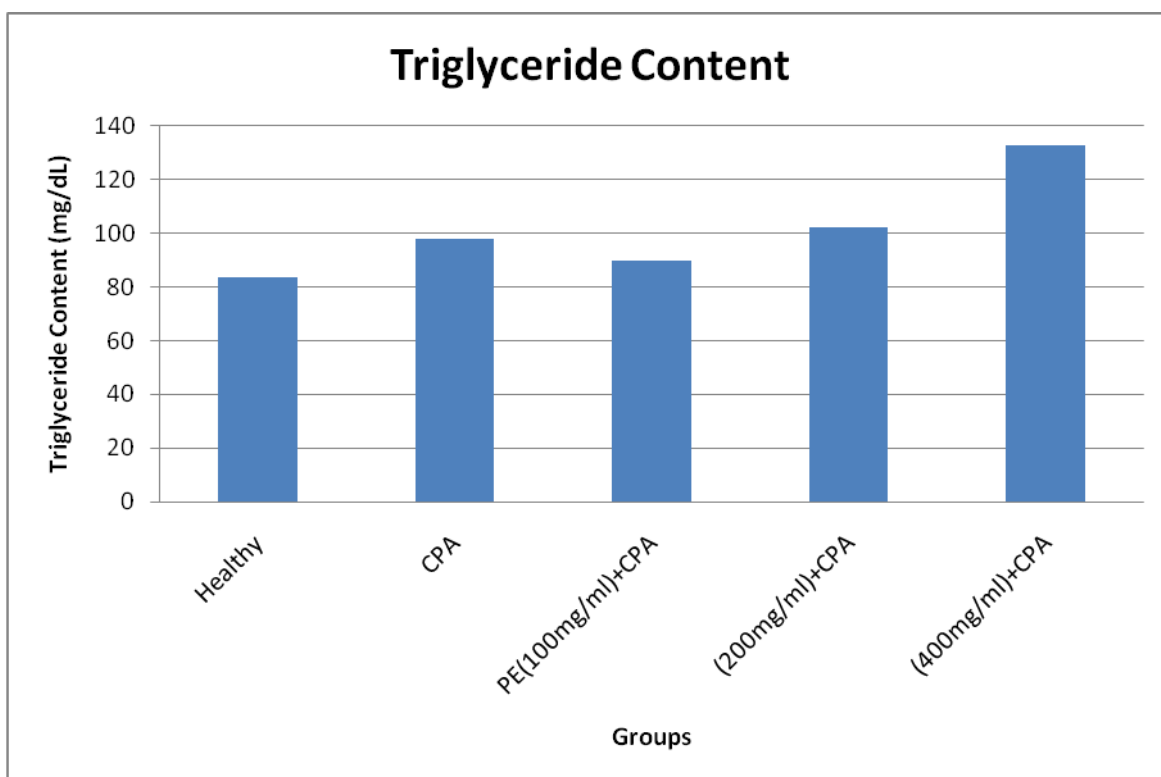

Figure 6: Effect of plant extract on triglyceride content ($p > 0.05$).

3.7 Glucose Level: On comparing that glucose content in groups treated with cyclophosphamide alone it has been observed that there is no change in glucose level of healthy control. There is no activity in group treated with low concentration of plant extract.
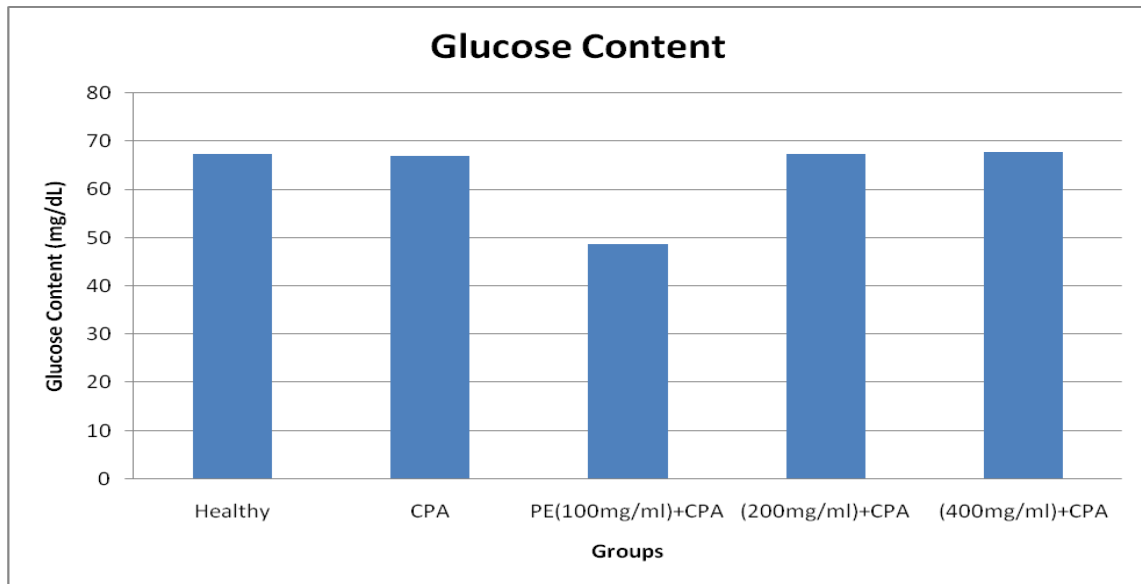
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Figure 7: Effect of plant extract on glucose level. No significant (p>0.05).

3.8 Creatinine content: It has been observed that creatinine content is greatly increased in the groups which were orally administered with the plant extract as compared with cyclophosphamide control. This increase in creatinine content is dose dependent as it is 0.16 mg/dL in group treated with PE (100 mg/ml), 0.21 mg/dL in group treated with PE (200 mg/ml) and in the last group which was administered the highest dose of plant extract PE (400 mg/ml) the creatinine content was same as healthy control is 0.27 mg/dL. But these values could not reach the limits of significance, (p>0.05).
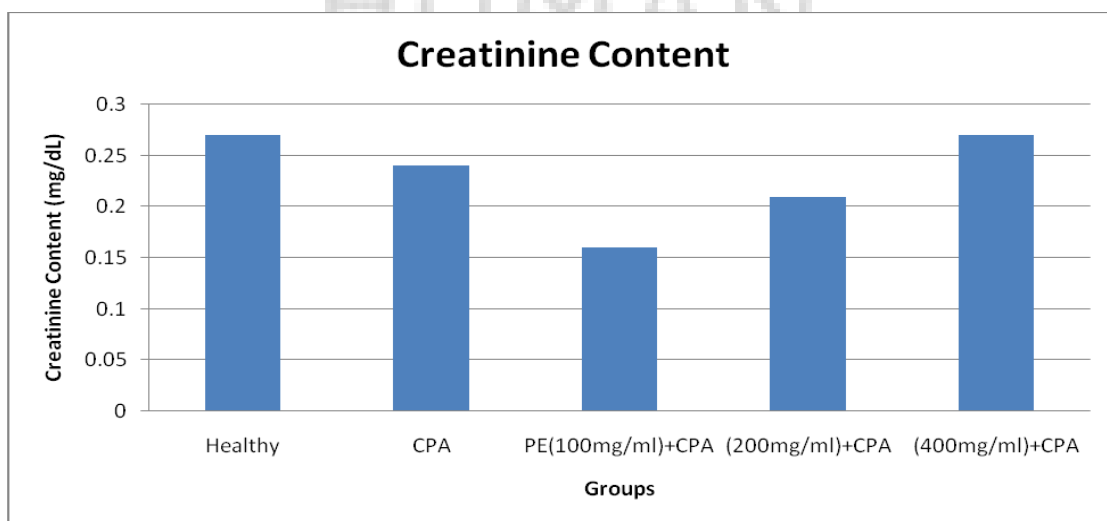

Figure 8: Effect of plant extract on creatinine content (p>0.05)

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3.9 Total Cholesterol: When compared with cyclophosphamide control it was observed that total cholesterol content is greatly reduced in the groups which were orally administered with the plant extract. This reduction in cholesterol content is dose dependent as it is 252 mg/dL in group treated with PE (100 mg/ml), 231 mg/dL and in the last group which was administered the highest dose of plant extract (400 mg/ml) the cholesterol content was very low178 mg/dL. But these values could not reach the limits of significance, (p>0.05).
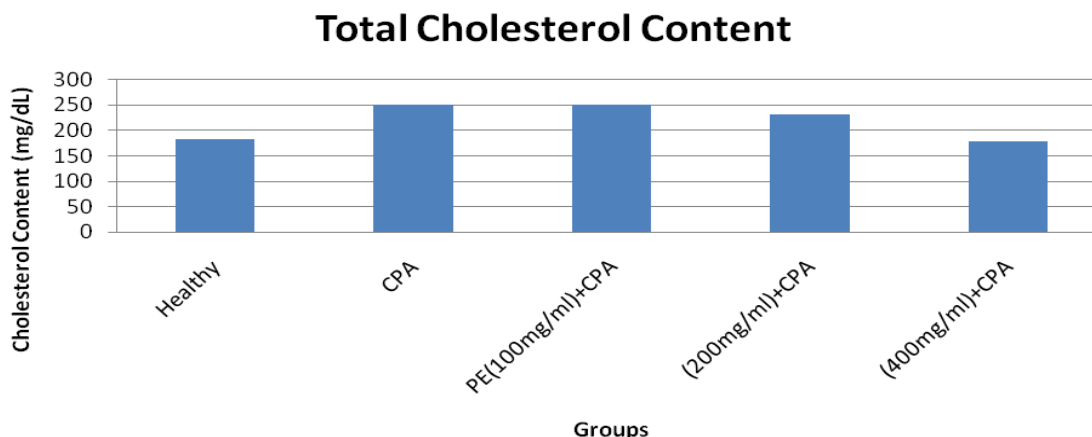
Total Cholesterol Content

Figure 9: Effect of plant extract on total cholesterol content (non-significant value, p>0.05)

3.10 Total Protein Content: Total protein content is greatly increased in the groups which were orally administered with the plant extract as compare to cyclophosphamide. This increase in protein content is dose dependent as it 57.6 µg/µl in group treated with PE (100 mg/ml), 64.5 µg/µl in group treated with PE (200 mg/ml) and in the last group which was administered the highest dose of plant extract PE (400 mg/ml) the protein content was very high74.1 µg/µl. These values were found to be significant when analysed statistically, p<0.05.
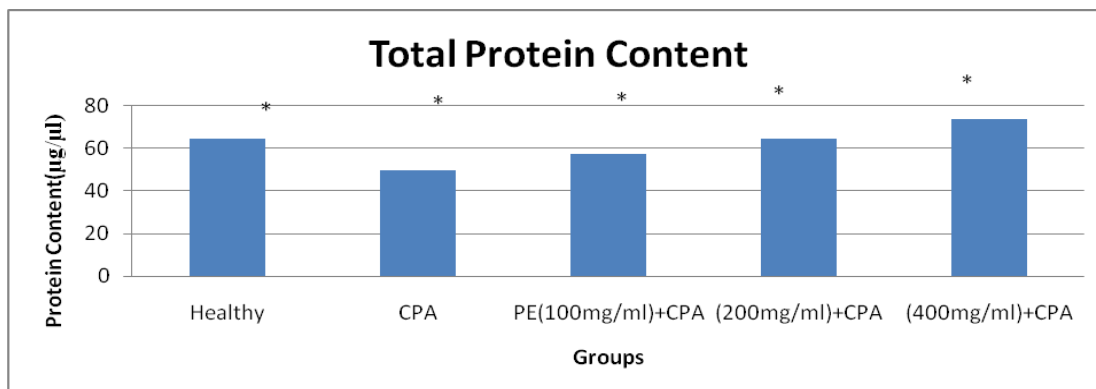
Total Protein Content

Figure 10 (a): Effect of plant extract on total protein content. (* significant value, p<0.05).

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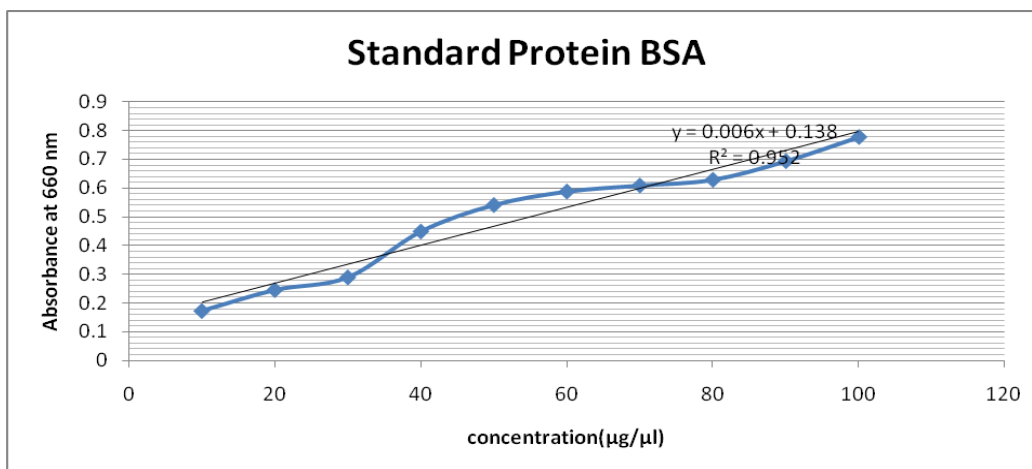
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Figure 10 (b): Estimation of total protein content in various experimental groups using BSA as standard

Through regression analysis with the equation y= 0.006x + 0.138, it has been observed that total protein content in healthy group is higher as compared to cyclophosphamide treated group.

As dose of plant extract increases total protein content is also increases as compare with standard BSA.

3.11 Body weight: It was observed that body weight is increased in cyclophosphamide control as compare to healthy control. But it is greatly increased in the groups which were orally administered with the plant extract. This increase in body weight is dose dependent as it is 123 gm in group treated with PE (100 mg/ml), 127 gm in group treated with PE (200 mg/ml) and in the last group which was administered the highest dose of plant extract PE (400 mg/ml) is 131 gm.
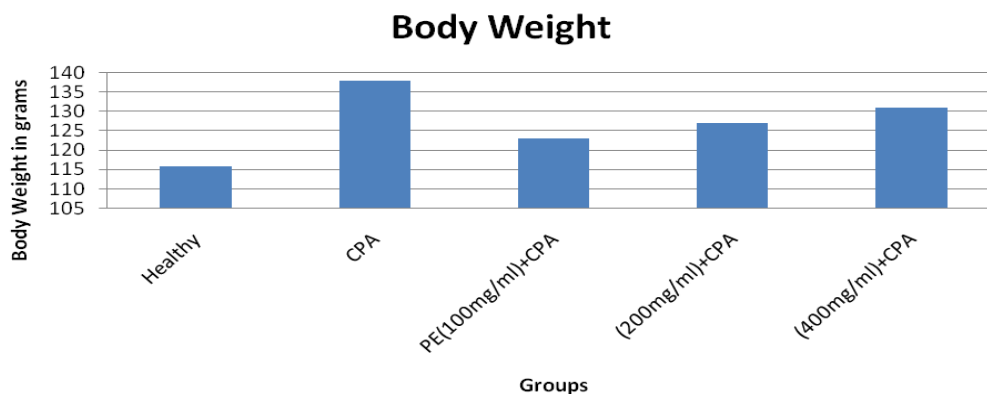

Figure 11: Effect of plant extract on body weight of rats. No significant results were obtained.

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3.12 Histopathological examination

The biochemical observations reported in this study were supplemented by histopathological examination of liver sections of rats. The obtained results showed that examination of livers of the healthy control group which was fed on the basal diet had normal histological picture of hepatic lobule that consists of central vein surrounded by normal hepatocytes [Figure 12(a)]. Examination of liver of rats treated with cyclophosphamide showed severe fatty degeneration of the hepatocytes [Figure 12(b)]. Examination of livers obtained from rats which were pretreated with aqueous extract of *C. asiatica* (400 mg/kg) before cyclophosphamide administration revealed almost normal hepatic lobules, as illustrated in [Figure 12(c)].
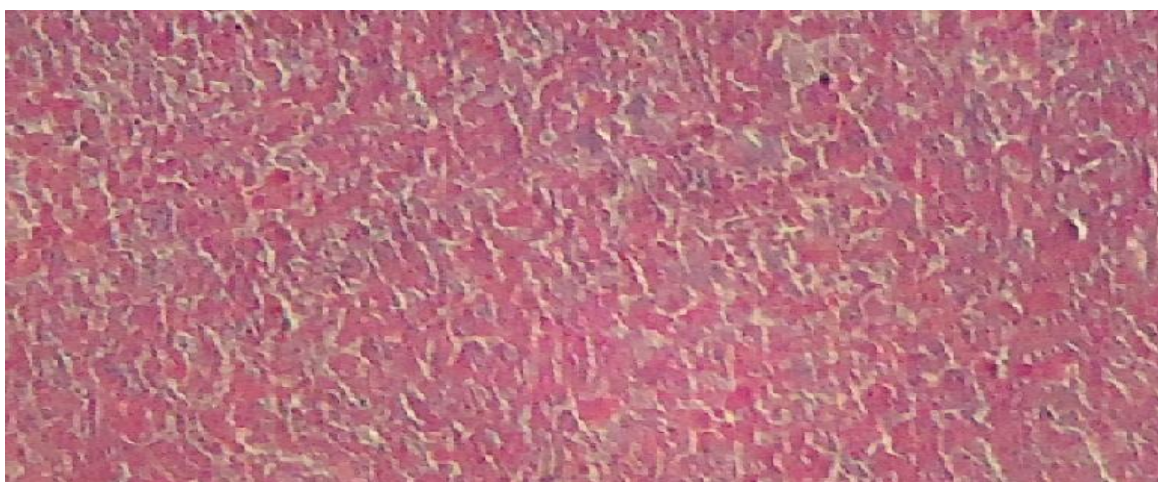

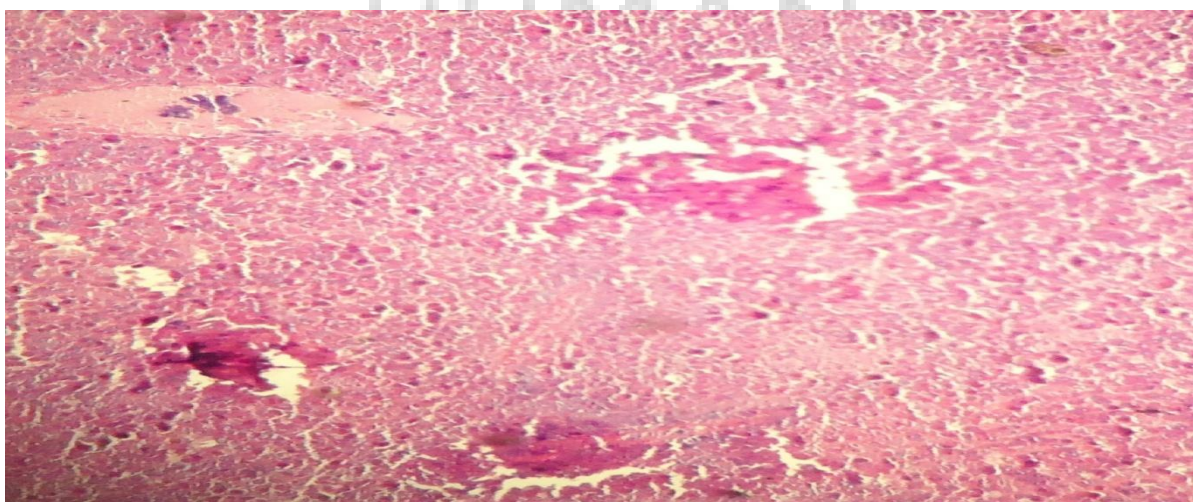**Figure 12 (a): liver of control rats showing normal histology of hepatic lobule.**

Figure 12 (b): Liver of cyclophosphamide rats showing severe fatty degeneration of hepatocytes
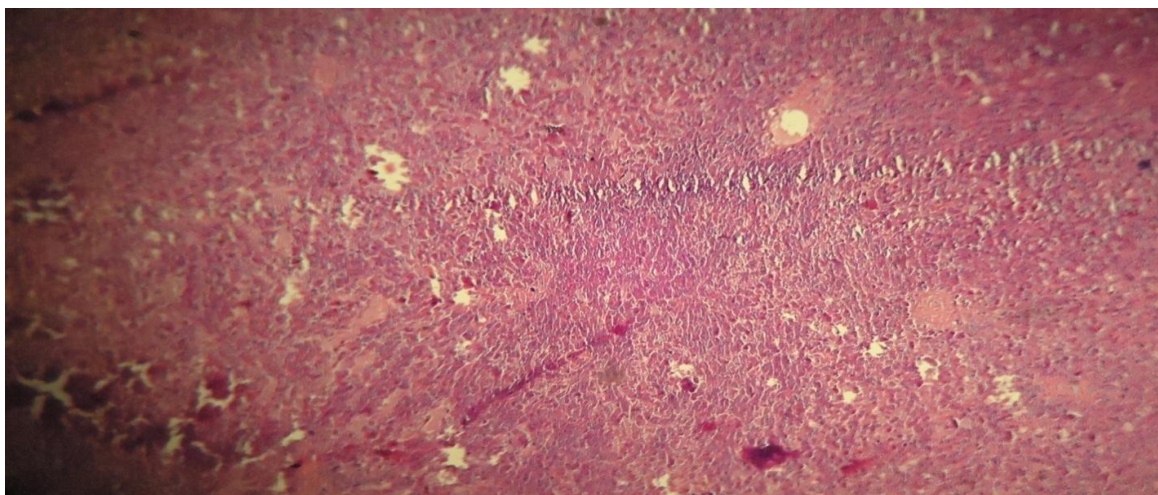
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Figure12 (c): Liver of rat treated with cyclophosphamide and higher dose of plant extract (400 mg/kg), revealed almost normal hepatic lobule.

4. CONCLUSION

These findings from both *in vitro* as well as *in vivo* models indicate the lipid peroxidation induction potential of cyclophosphamide which may be related to its toxic potential. The results also suggest antiperoxidative effects of *C. asiatica* and demonstrate its potential to reduce cyclophosphamide induced lipid peroxidation and thus to increase therapeutic index.

ACKNOWLEDGEMENT

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