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Hepato-protective capacity of *Morus (Morus macroura)* fruit extract against Lead treated mice: A histopathological study

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Abstract

Present research work focuses on hepatotoxicity of lead exposure and hepato-regenerative potential of *Morus macroura* Black (MMB) fruit extract post treatment. 15 male mice were distributed into three groups (n=5) that is, 1: CO group (without any treatment), 2: Pb group (50 ppm lead ion in lead acetate dissolved in drinking water), 3: Pb+MMB group (exposure to lead as in the Pb group + 5 days treatment with 0.2ml MMB fruit extract once per 24 hours). Animals in all three groups were recovered on 21st day. Animal and organ weight and various other parameters (CSA of hepatocytes, number of hepatocytes per unit area, number of oval cells per unit area, Mean CSA of central hepatic vein, CSA of hepatic nuclei) were assessed. Result has shown severe liver damage indications such as shrinkage of sinusoidal spaces, swelling and enlargement of hepatocytes and presence of debris around the marginal hepatic portal venules as significant alterations in lead exposure. Significant recovery of above said parameters and their histological signs are indicated by presence of oval cells, large numbers of macrophages, peri-central area containing normal hepatocytes and enlargement in the size of both marginal & central hepatic vein were observed in MMB post treatment in Pb+MMB group. Lead is highly toxic to the animal liver and morus fruit extract has shown excellent recovery potential from lead exposure related toxicity. Present study has highlighted the need to further explore the potential of morus fruit extract in hepatotoxicity in particular and rest of body in general so as its medicinal capacity may be understood for the benefit of humanity.

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Introduction

Heavy metals are naturally present in environment at various levels. Industrial wastes are mixture of different pollutant such as hydrocarbons and heavy metals (Oyewo and Don-Pedro, 2002). Heavy metals have density above 3.5-5g/cm^3 and atomic number greater than 20 (Duffus, 2002). All heavy metals naturally occur with high atomic weight and denser than water (Fergusson, 1990). Heavy metals including mercury, cadmium and lead are widely distributed in the environment (Pohl, 2011), have adverse health effect in human. Large proportions of lead poisoning cases include ingestion by mouth and absorption from intestine (Papanikolaou *et al.*, 2005). Lead is heavy metal with symbol Pb and atomic number 82. Freshly cut metallic lead has bluish-white color, but it change in a dull grayish color when exposed to air. It is heaviest non-radioactive element (Thurmer *et al.,* 2002). Among the heavy metals, Lead is a toxic metal and its widespread use can cause many health problems in different parts of the world. It is cumulative, toxic and adversely effects on nervous, intestinal, circulatory and renal systems (Fewtrell, 2003). Acute exposure to lead may cause gastrointestinal disturbances such as nausea, pain in abdomen, retching and loss of appetite. It can cause hepato-nephric damage, hypertension and neurological defects that may lead to death. Long term occupational exposure to lead may contribute to development of cancer. The International Agency for Research on Cancer has classified inorganic lead compound as carcinogenic to human (IARC, 2006). Liver is heterogeneous and second to brain in its complexity. Liver has thousands of vital function including efficient uptake of amino acids, carbohydrates, bile acids, cholesterol, proteins, lipids, vitamins for storages and metabolism, subsequently release into bile and blood (Burt and Day, 2002). Mice and rats have four liver lobes: median (middle), left, right and caudate. All lobes except left are further sub divided into two or more parts (Kogure *et al.*, 1999).

Lead has many different effects in hepatobiliary system such as catalysis and peroxidation of unsaturated fatty acid (Yiin and Lin, 1995), reduction of N-oxide production (Krocova *et al.*, 2000) and the formation of hydroxyl radical (Ding *et al.*, 2000).

Each effect may promote stone formation. Lead induces liver injury as a result increase in serum bilirubin content along with increase in serum marker enzymes (Akilavalli *et al.*, 2011). Lead also simulates intercellular signaling between hepatocytes and Kupffer cell which are enhanced in the presence of low lipopolysaccharide (Milosevic and Maier 2000). Lead acetate induces biochemical and histological abnormalities in liver (Ozsoy *et al.*, 2011). Hepatotoxicity and nephrotoxicity can occurs in person with exposure to heavy metals. Liver regeneration after loss of cell population is complex phenomenon (Ankoma-Sey, 1999; Michalopoulos, 2007). Autopsy studies of human lead exposure indicate that liver tissues is the major repository 33% of lead among the soft tissue present in kidney medulla and cortex (Goswami *et al.*, 2005 ; Patrick, 2006). Heavy metals produce reactive oxygen species which enhance lipid peroxidation, decrease the saturated fatty acid and increase the unsaturated fatty acid contents. ROS is highly reactive to protein and DNA.

It is major factor of injuries and lead to rapid cellular damage (Afify *et al.*, 2011). ROS has not only negative role but it also has some positive effects. Negative effect includes apoptosis and its positive effects are induction of host defense and mobilization of ion transport system (Rada, 2008). Platelets involved in wound repair release ROS to recruit additional platelets to site of injury. ROS is a double edged sword. At low level, ROS facilitates survival of cancer cell (Ramsey and Sharpless, 2006), while on other hand high level of ROS can suppress tumor growth (Takahashi *et al.*, 2006).

Plants are source of medicines and several drugs are derived directly or indirectly from them. *Morus* is genus of flowering plants belong to family Moraceae. *Morus* has 24 species. Fruits of this plant are used in fruit juice, liquor, natural dyes and cosmetics industry

(Ozan *et al.*, 2008). Mulberry trees are predominantly dioecious, monecious plants are not common. It is also reported that mulberry changes its sex depending on environmental conditions (Tikader *et al.* 1995). Its fruit is a multiple fruit because fruiting bodies formed from a cluster of fruiting. Immature fruits are white, green, or pale yellow. Mulberry is the most commonly used medicinal plant. It is used for many medical purposes such as to nourish the skin, benefit the liver and kidney.

It also helps in treatment of many serious diseases like diabetes mellitus, arthrosclerosis and hypertension etc (Venkatesh and Seema, 2008). Mulberry plant plays their role in various ways such as reduction in food intake and reduces absorption of blood glucose (Lee *et al.*, 2002). *Morus macroura* is commonly known as "king white". It is medium sized spreading tree with weeping habit. Ripe fruit is white, pink or red, and is described as honey-sweeten (Akram and Aftab, 2012).

Aims of study

The pupose of present study was to evaluate the effects of *Morus macroura* fruit against lead (Pb) exposure.

Materials and methods

Animal rearing maintenance and feeding

Albino laboratory mice, *Mus musculus which is a swiss Webster strain*, were used. Animals were reared in the animal house. For the experiment fifteen adult male *Mus musculus* weighing between 28- 30gm and aged 3-4 months were used.

Dose Groups

15 Animals were divided into three groups (5 animals each) randomly.

1. Control group (CO): This group was given regular drinking water only.

2. Lead treated group (Pb): These animals were provided with 50ppm lead acetate in drinking water for 15 days, followed by 5 days distilled water.

3. *Lead treated+ Morus extract group* (Pb+MMB): 50ppm lead acetate solution was administered for 15 days. For next 5 days, 0.2ml *Morus* fruit extract treatment was given regularly every 24 hrs along with water.

Dose preparation

For getting 50ppm Pb ion solution a $Pb(CH_3COO)_2$ 1000ppm stock solution was prepared by dissolving 1.56 g of $Pb(CH_3COO)_2$ in 1L of water. The dose (50ppm) was then prepared by adding 950ml of water to 50ml stock solution.

Fruit extracts dose

Fresh fruits (Mulberry) were taken from market and pulp was removed from fruit. Then pulp was grinded and juice was obtained by using electric blender. Later on juice was centrifuged to remove fibrous content and thus only watery supernatant was used for animal treatment.

Organ recovery

On 21th day cervical dislocation was carried out upon all groups (CO, Pb and Pb+MMB) and livers were recovered surgically which were fixed for supplementary work.

Histological Preparations and studies:

Recovered livers were wax embedded. Initially, dehydration was achieved by immersing in 50%, 70%, 90% and absolute alcohol for 3-5 hours. After achieving dehydration, these liver pieces were immersed in xylene for 5-6 hours.

Wax embedding was achieved by placing them in molten wax (at 56-58^{o}C) for 3-5 hours. Paraffin rectangular blocks were prepared. Serial transverse sections (5µ thick) were prepared through rotary microtomes (ERMA TOKYO 42) which were stretched on albumenized glass slides. Hematoxyline and Eosin staining was carried out followed by Canada balsam mounting.

Digital photography and processing

Photomicrographs of the selected histological sections

from liver of different groups (CO, Pb, Pb+MMB) were obtained using digital camera of 7.2 mega pixel mechanically fitted on Labomid CXR2 trinocular microscope at 100x and 400x magnifications. The soft copies of the above mentioned photomicrographs were processed in Corel Draw 11. Microsoft word was used for labeling and printing after pasting digitally improved images in it.

Micrometry

Digital micrometry of histological sections of liver was done by the help of a computer assisted technique inCorel Draw11. Soft images (100x and 400x) of 10 randomly selected sections of liverfrom each group were used. Measurements of cross-sectional area of hepatocytes etc. were taken from ten randomly selected areas from each section using digital scales (pre-calibrated) in CorelDraw11.

The micrometric data obtained was used to calculate group means ± SEM values. Thus measurement was made for mean Cross Sectional Area (CSA) of hepatocytes, CSA of central hepatic vein, number of hepatocytes per unit area, CSA of hepatic nuclei and number of oval cells per unit area. The mean CSAs were calculated using following formula:

$$CSA = (Length \times width \div 4) \times \Pi$$

Data analysis

The data obtained was then analyzed statistically based on single factor ANOVA and further on the basis of Tukey's Multiple Range test for the comparison of the groups.

The obtained data has been presented in the form of histogram. These statistical analyses were carried out by using IBM SPSS Statistics 23 software.

Results

Histological result

The histological section of control group liver shows normal architectural distribution of the hepatic microanatomy, containing central vein, marginal lobule vein, and hepatic cord containing uni and binucleated hepatocytes and sinusoidal spaces separating adjacent hepatic cord (Fig. A1).

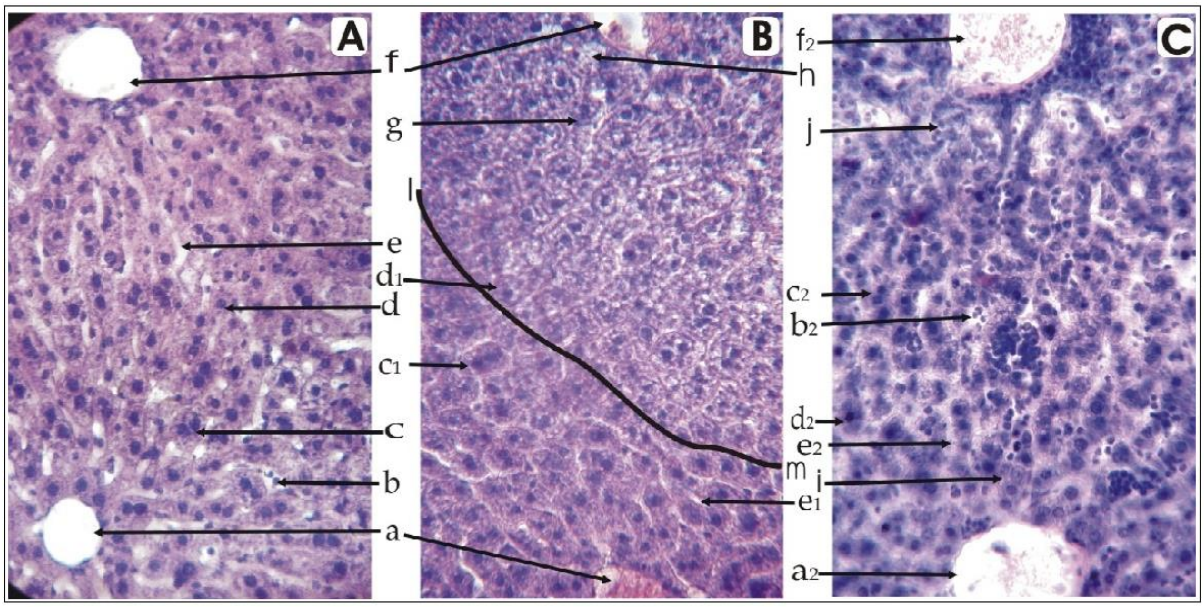

Fig. 1. Hematoxylin and Eosin stained histological sections (400x) of mice liver: A: Control group, B: Pb treated group, C: P b+ MMB group: a: central hepatic vein, b & b_2: oval cells, c: normal binucleated hepatocytes, d: normal uninucleated hepatocytes, e: sinusoidal spaces, f: marginal hepatic vein, c_1: swollen binucleated hepatocytes, d_1: swollen uninucleated hepatocytes, e_1: shrunk sinusoidal spaces, g: cell necrosis, h: debris, a_2: enlarged central hepatic vein, e_2: widening of sinusoidal space, f_2: enlarged marginal hepatic vein, i: formation of new hepatocytes, j: macrophages, k: formation of new cord in figure B, upper portion of the line l, m shows the necrosis in hepatocytes.

In Pb exposure group peri-lobular area has shown various sign of hepato-pathology such as enlargement of individual hepatocytes with obvious indication of cytoplasmic infestation simultaneously obliteration of sinusoid debris of the dead hepatocytes etc.

were seen whereas centri-lobular hepatocytes were somewhat normal although they give impression of slight enlargement which is obvious by the shrunken of sinusoidal spaces of the structure from the center to margin of lobules show most interiorly normal hepatocytes and slightly swollen and enlarge hepatocytes were present slightly away from central vein while binucleated hepatocytes present in center and hepatocytic debris all around the marginal hepatic portal venules (Fig. B1).

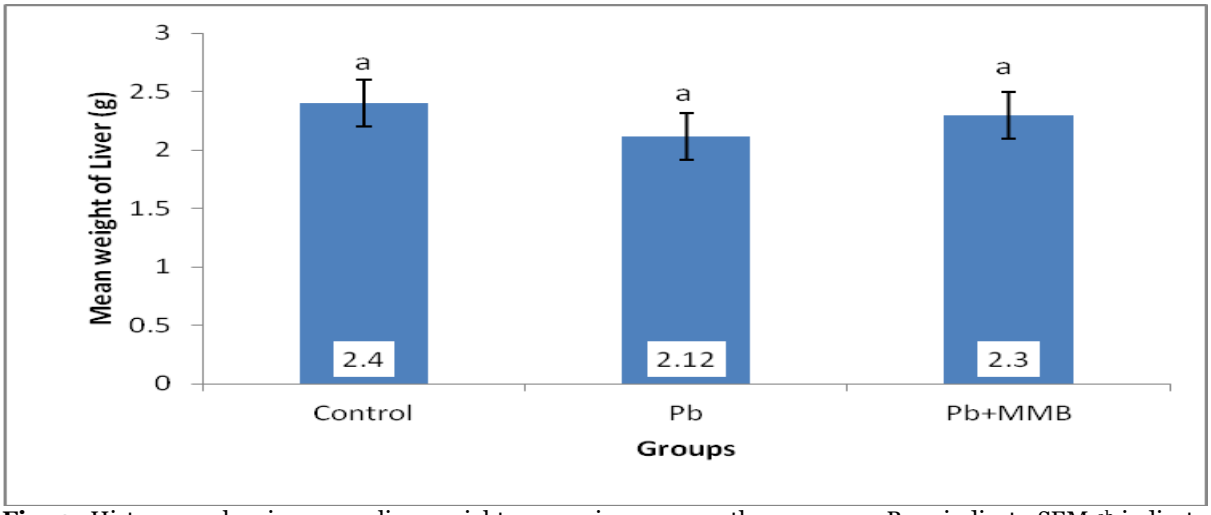

Fig. 2. Histogram showing mean liver weight comparison among the groups, ± Bars indicate SEM;[ab] indicate significant difference between the groups not shearing a common lower case letter.

A large number of oval cells clearly visible along the marginal hepatic portal venules. Sizes of both marginal and central hepatic vein were excessively enlarged in Pb+ MMB group as compared to lead or control group. A large number of macrophages are also visible which do form aggregation in middle of the lobule. Peri-central vein area contains normal hepatocytes.

The structures of hepatic cord were irruptive however regeneration of liver in term of transformation of oval cell nescient hepatic cords also seen (Fig.C1).

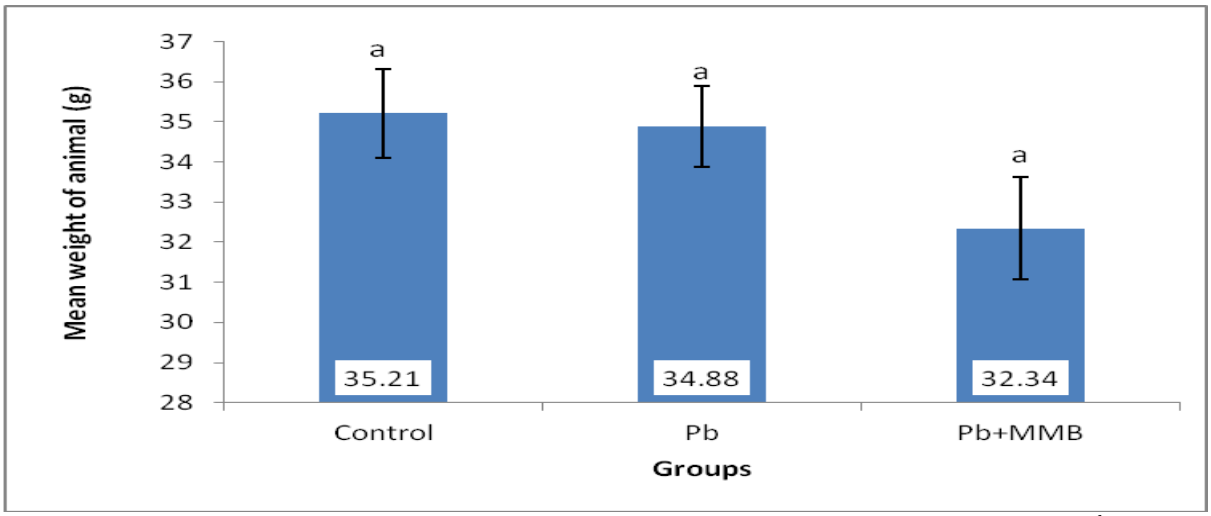

Fig. 3. Histogram showing mean body weight comparison among the groups, ± Bars indicate SEM;[abc] indicate significant difference between the groups not shearing a common lower case letter.

Morphometric and micrometric result

Mean animal weight + mean weight of liver

With slight variations in the mean final weight of liver CO (2.4g ±0.2), Pb (2.12g ±0.2), Pb+MMB (2.3g ±0.2) (Fig. 2). Weight of animals CO (35.21g ±1.1), Pb (34.88g ±1), Pb+MMB (32.34g ±1.27) did not show any significant variation ($p<0.05$) among the three groups (Fig. 3).

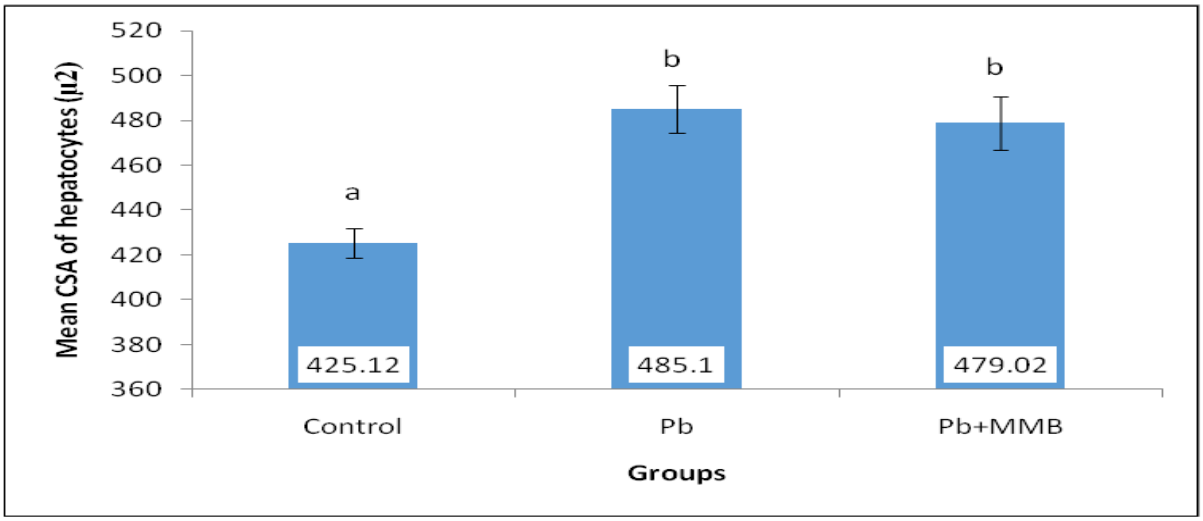

Fig. 4. Histogram showing mean CSA of hepatocytes (µ²) comparison among the groups, ± Bars indicate SEM;[abc] indicate significant difference between the groups not shearing a common lower case letter.

Mean cross sectional area of hepatocytes (CSA)

Histometry of the liver show very high significant ($p\leq0.0001$) variation in mean CSA of hepatocytes, post hoc comparison of three groups show significantly lower value (425.12µ²±6.63) in CO group than that of Pb (485.1µ²±10.5) and Pb+MMB (479.02µ²±11.9) groups (Fig. 4).

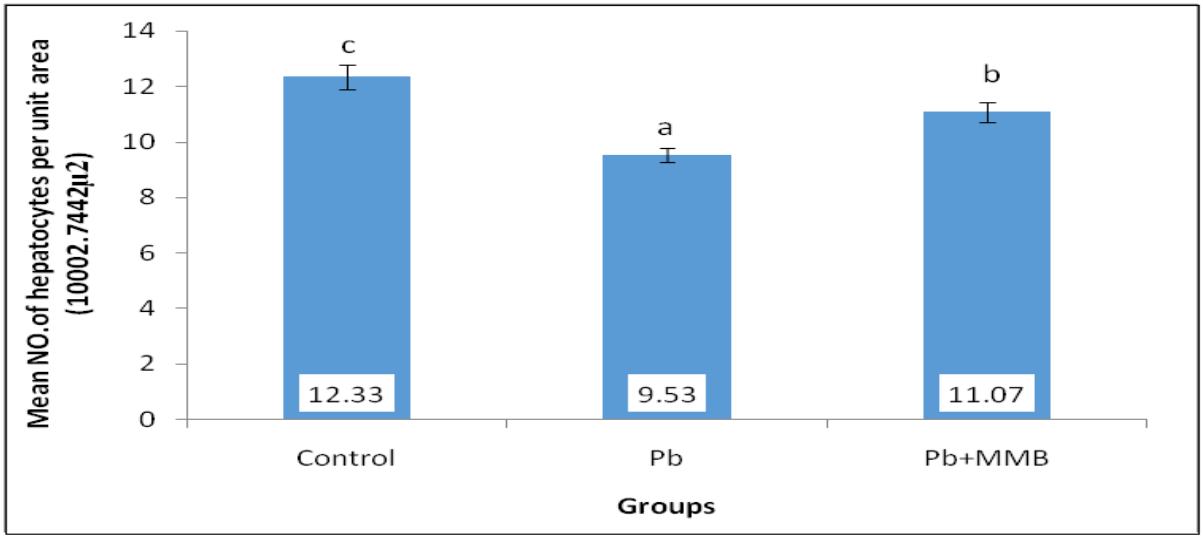

Fig. 5. Histogram showing mean numbers of hepatocytes per unit area (µ²) comparison among the groups, ± Bars indicate SEM;[abc] indicate significant difference between the groups not shearing a common lower case letter.

Number of hepatocytes per unit area

Highly significant variations ($p\leq0.0001$) among the groups were also noted for mean numbers of hepatocytes per unit area (10002.7442µ²) with significant higher mean value (12.33µ²±.45) were shown in the control than the other two groups. However the mean value (11.07µ²±.380) of Pb+MMB group significantly higher than the mean value

(9.53μ²±.253) of Pb group as indicated by post hoc analysis (Fig. 5).

Number of oval cells per unit area

The number of oval cell for the data of number of oval cells per unit area (10002.7442μ²) in liver section has shown highly significant variation (p≤0.0001) among the groups whereas Tukey post hoc analysis show higher value (7.567μ²±0.38) Pb+MMB group then CO (1.94μ²±0.21) and Pb (2.933μ²±0.28) groups (Fig. 6).

Mean cross sectional area of central hepatic vein (CSA)

In similar way above parameter the data for mean CSA for central hepatic vein showed highly significant variations (p≤0.0001)among the groups, and the post hoc analysis show significant variations among the values CO (1205.1μ²±98.6), Pb (2671.1μ²±156.6) and Pb+MMB (3566.94μ²±209) of three groups (Fig. 7).

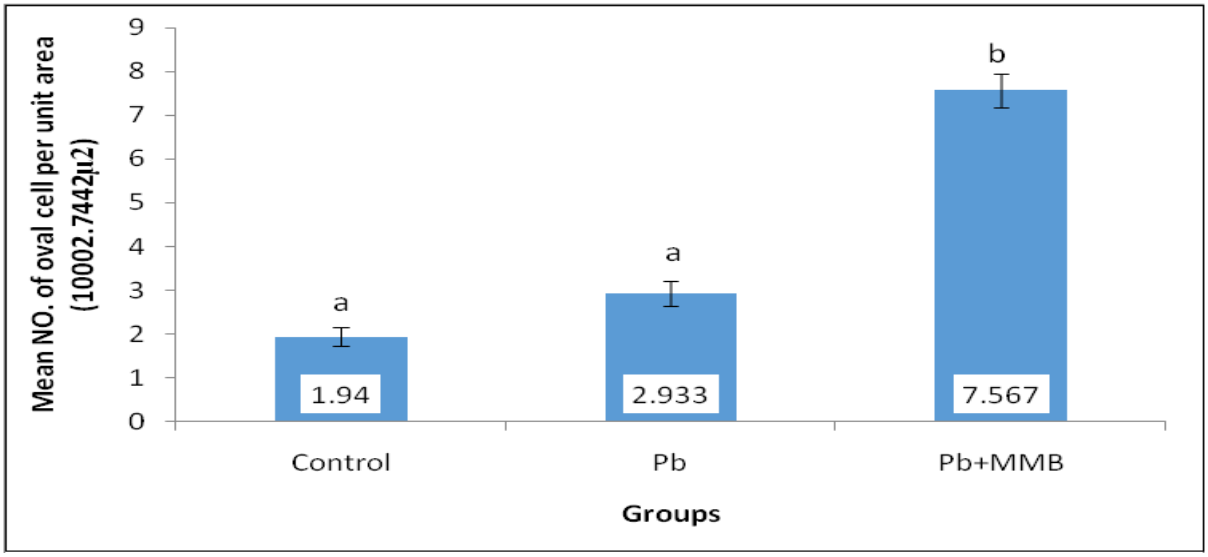

Fig. 6. Histogram showing mean number of oval cells per unit area (μ²) comparison among the groups, ± Bars indicate SEM;[abc] indicate significant difference between the groups not shearing a common lower case letter.

Mean cross sectional area of hepatic nuclei (CSA)

Mean CSA of the hepatic nuclei data analysis has shown highly significant variations (p≤0.0001) among the groups.

Whereas post hoc analysis indicates significantly higher mean values (78.95μ²±1.955) in Pb than the mean values (64.42μ²±1.6), (69.87μ²±1.762) of CO and Pb+MMB groups (Fig. 8).

Discussion

Hepatotoxicity of chronic Pb exposure has been reported in multiple studies. By virtue of functionality, liver receives absorbed nutrients, drugs and toxic substances (including lead) that can alter its function by causing injury through acute or chronic exposure (Bersenyi *et al.*, 2003; Garg *et al.*, 2007; Liu *et al.*, 2012; Shalan *et al.*, 2005). In multiple studies, it has been established that Pb, Hg and Cd exposure can cause severe liver insult in laboratory animals (Bersenyi *et al.*, 2003; Liu *et al.*, 2012).

It has been reported that lead destroy the normal architecture of the hepatocytes. In lead treated animals, hepatic-nuclear size was increase, indicating the megakaryocytic. Lead modifies the replication and transcriptional processes (Ahmed *et al.*, 2012).

Lead acetate causes general disturbances in the normal physiological function of the liver. Similarly hepato histopathological signs have also been reported in earlier hepatic injury in the lead acetate treated group suggesting the adverse effect of the chemical in the liver tissue that results in the hepatic necrosis, as well as increases the number of binucleated hepatocytes (Kerr *et al.*, 1972).

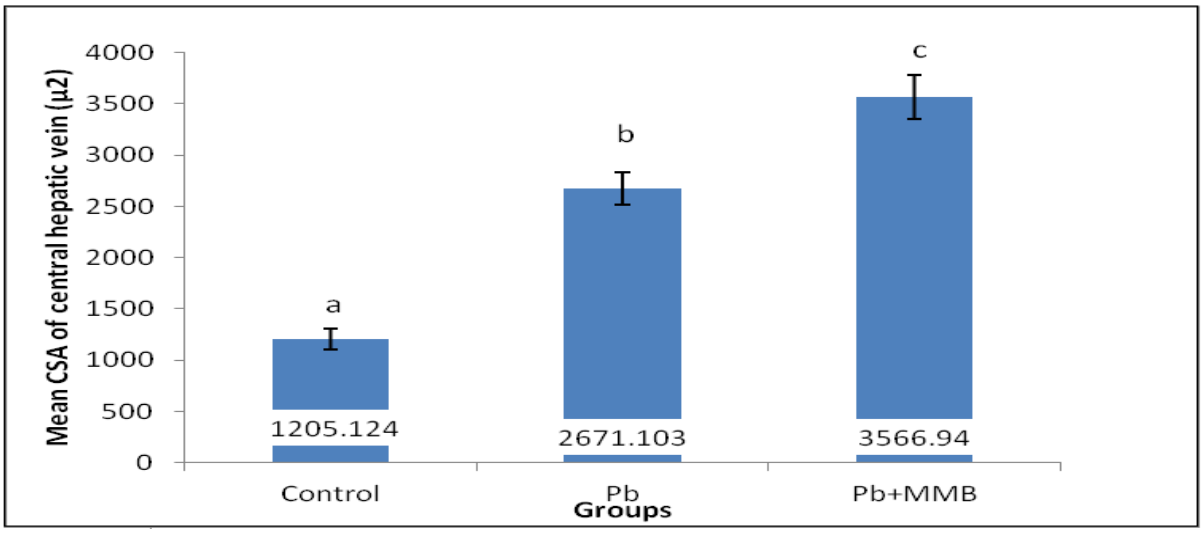

Fig. 7. Histogram showing mean CSA for central hepatic vein (µ²) comparison among the groups, ± Bars indicate SEM;[abc] indicate significant difference between the groups not shearing a common lower case letter.

In present study, we found different prominent histopathological indication such as swelling and enlarged hepatocytes, presence of debris around the marginal hepatic venules, necrosis and shrinkage of sinusoidal spaces in Pb group of male mice. Histopathological alterations were most prominent in the peri-lobular areas, while all around the central vein almost mid-way to the margin of the lobules the pathological sign were not very much prominent, that is histological distribution of hepatocytic cord were almost similar to control group while peri central area where above mentioned histopathological alterations were observed.

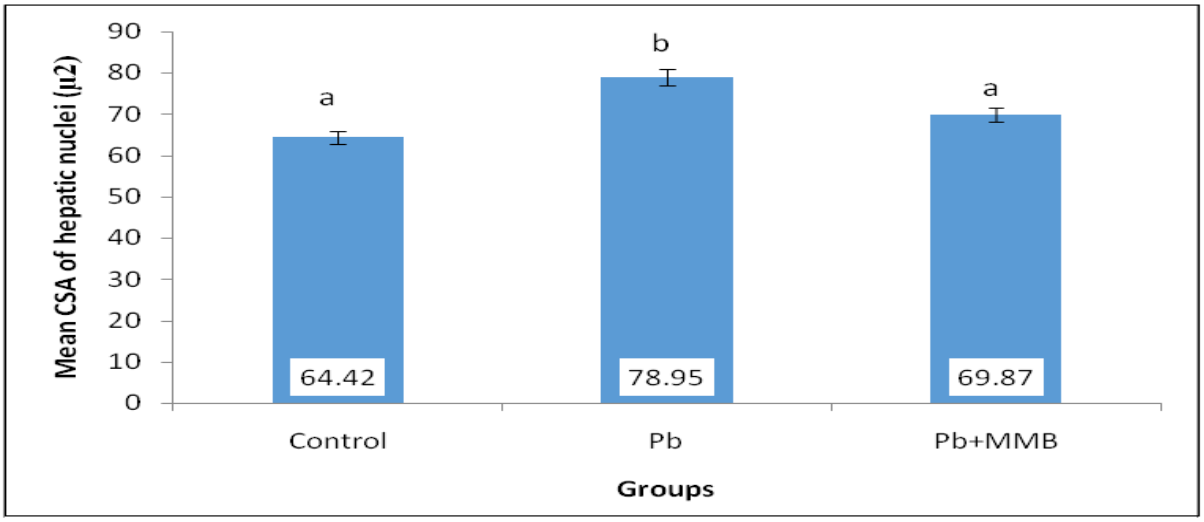

Fig. 8. Histogram showing comparison of mean CSA of hepatic nucleiamong the groups, ± Bars indicate SEM;[abc] indicate significant difference between the groups not shearing a common lower case letter.

The hepatocytic sinusoidal spaces were almost completely obliterated. This observation indicates that hepatocytes under lead exposure in the peri-central area of hepatic lobules caused the hepatic sinusoid to squeeze completely leaving very little blood to enter into centri-lobular vein through inner half of the lobules, lesser flow of blood in this region saved the hepatocytes from the signs of histopathology. Thus, a clear dichotomy between most effected peripheral and least effected center lobular hepatocytes was seen. The Pb+MMB group slides show profuse infestation of macrophages and

obvious appearance of a large number of oval cells. In the infected lobules again in this slide the macrophage infestation is greater in peripheral area, indicating rapid removal of debris and simultaneously repair work in the lobule architecture in this area. This is indication of a very obvious increase in the repair activities in hepato-lobular architecture after their damage by lead exposure on MMB extract treatment. The micrometric observation in the term of CSA of hepatocytes, numbers of hepatocytes per unit area, number of oval cells per unit area etc. also support the histo-regenerative observation of hepato-regenerative ability treatment. Based upon the results and this discussion, it is concluded that chronic Pb exposure at 50 ppm or higher concentration in drinking water consumed *ad libitum* for 15 days or above duration can lead to sever histopathological alteration in hepato-lobular architecture. Whereas the fresh fruit extract from ripened fruit of MMB are helpful to rescue such histo-pathologies, indicating the nutraceutical and medicinal common fruit plant of Pakistan.

Conclusion

The results shows that *Morus macroura* (Black) fruits used in the present research are found to harbor alleviative and ameliorative capacities against lead inflicted hepatic histopathologies, indicating its medicinal neutral-ceuticals importance for all the possible human benefits. The findings also suggest promoting similar investigations on other wild and folklore medicinal plants for the discovery of their potential nutraceuticals benefits.

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