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Protective effect of alcoholic fruit extract of *mallotus philippensis* muell.Arg in aniline induced spleen toxicity in rats.

G.Sumithira*, V.Kavya, Emil Mariya Shaji, V. Safrooq, V. Sakthivel, P. Sangeetha Mol, T. Shyamjith.

The Erode College of Pharmacy, Erode, Tamilnadu.

Corresponding Author: G. Sumithira

ABSTRACT

This study was designed to evaluate the protective effect of *Mallotus philippensis* on aniline induced spleen toxicity in rats. Wister albino rats of either sex were used and divided into 5 different groups. Spleen toxicity was induced by aniline hydrochloride (100 ppm) in drinking water for a period of 30 days. Control group received 1% carboxy methyl cellulose orally as vehicle. Treatment groups (III, IV &V) received ascorbic acid (40mg/kg b.w p.o), *EEMP* (200mg/kg b.w p.o) and *EEMP* (400 mg/kg b.w p.o) respectively followed by aniline hydrochloride for 30 days. At the end of treatment period haematological parameters and tissue parameters were evaluated. Aniline hydrochloride treated rats showed significant alteration in Body weight and spleen weight, Haematological parameters (Hb, RBC&WBC) and tissue parameters (LPO, GSH &NO) compared to control group. Treatment of *EEMP* (200mg/kg) and *EEMP* (400mg/kg) respectively showed a significant recovery in aniline hydrochloride induced spleen toxicity.

Keywords: Aniline hydrochloride, Spleen toxicity, Ascorbic acid, *Mallotus philippensis.*

INTRODUCTION

The spleen is the largest lymphoid tissue, it acts primarily as a blood filter. The spleen plays important roles in regard to red blood cells (also referred to as erythrocytes) and the immune system [1]. It removes old red blood cells and holds a reserve of blood, which can be valuable in case of hemorrhagic shock, and also recycles iron. The spleen synthesizes antibodies in its white pulp; the red pulp of the spleen forms a reservoir that contains over half of the body's monocyts[2]. These monocytes, upon moving to injured tissue (such as the heart after myocardial infarction), turn into dendritic cells and macrophages while promoting tissue healing. [2, 3, 4]

Aniline, a toxic aromatic amine, is widely used in industry for the manufacturing of dyes, resins, varnishes, perfumes, pesticides, explosives, isocyanates, hydroquinone, and rubber chemicals [5]. Chronic exposure to aniline leads to the development of splenomegaly, increased erythropoietic activity, hyper pigmentation, hyperplasia, and fibrosis [6, 7, 8]. Earlier studies have shown that aniline hydrochloride (AH) exposure leads to the formation of oxidative and nitrosative stress which are due to iron overload and induction of lipid peroxidation. AH enhance the production of reactive oxygen/nitrogen species (ROS/RNS) which attacks proteins and nucleic acid leading to the structural and functional changes in the spleen [9].

Antioxidant compounds in food play an important role as a health protecting factor. Most of the antioxidant compounds in a typical diet are derived from plant sources and belong to various classes of compounds with a wide variety of physical and chemical properties.

In addition, they have a potential for substantial saving in the cost of healthcare delivery [10]. *Mallotus philippinensis Muell*. It is one of the common plants used in Indian system of medicine.

According to Ayurveda, leaves are bitter, cooling, and appetizer. All parts of plant like glands and hairs from the capsules or fruits are used as heating, purgative, anthe0lmintic, vulnerary, detergent, maturant, carminative, anti-diabetic, and alexiteric and are useful in treatment of bronchitis, abdominal diseases, and spleen enlargement, and if taken with milk or curd (yoghurt), it can be quite useful for expelling tapeworms [11]. Kamala or Kampillakah is also used as an oral contraceptive. It also shows anti-oxidant[12], insectidal /pestecidal, anti-microfilaria, anti-lithic, heptoprotctive activities. [13]

The powder and a few other parts of Kamala are also used in external applications to promote the healing of ulcers and wounds[14]. They are used to treat parasitic affections of the skin like scabies, ringworm, and herpes.

Literature showed that there are no works carried out to explore the protective effects of *Mallotus phillipensis* in AH induced spleen toxicity, so the present study was initiated to assess the effect of *Mallotus philipensis* on aniline exposure-induced spleen toxicity in rats by evaluating different biochemical parameters.

MATERIALS AND METHODS

All the experimental procedures and protocols used in the study were reviewed by the Institutional Animal Ethics Committee (IAEC Register number: XLVIII/04/CLBMCP/2016) and were in accordance with the Committee for the purpose of Control and Supervision on Experiments on Animals (CPCSEA) Guidelines, Government of India.

Animals

Healthy albino rat (100-200 g) of either sex of wistar strain were obtained from a disease free animal house of king's institute, Guindy, Tamilnadu (India). Animals were kept in standard polypropylene cages and maintained under controlled standard conditions of temperature (25± 5ºC), relative humidity (55± 5%), with 12/12 h light/dark cycle. They were fed with commercially available rat feed and had free access to water maintenance and use of animals as per the experiment was approved by the institutional Animal Ethics Committee.

Drugs and Chemicals

Aniline hydrochloride (AH); 2, 2'-dipyridy l, 5,5'-dithiobis-(2-nitrobenzoic acid); and N-(1-Napthyl) ethylenediamine dihydrochloride were purchased from S.D fine chemicals, India. Ascorbic acid were purchased from sigma and all the other chemicals used in the study were of analytical grade and procured from standard supplier.

Plant material

The Fruits of *Mallotus Philippensis* Muell Arg. were collected in the month of December from, Cherpulassery, Palakkad (Dist), Kerala South India. The plant material was taxonomically identified and authenticated by Dr. A. Balasubramanian, Director, ABS Botanical conservation, Research & Training center, Kaaripatti, Salem.

Extraction method

The Fruits of *Mallotus Philippensis* Muell Arg were dried under shade and then powdered with a mechanical grinder. The powder was passed through sieve No 40 and stored in an airtight container for further use. Thus the obtained coarse Fruit powder was extracted with ethanol by continuous hot percolation using soxhlet apparatus. After completion of extraction, extract was filtered and the solvent was removed by under reduced pressure. The dried extract was stored in desiccators

Preliminary phytochemical study

The ethanolic extract of dried fruits of crude *Mallotus Philippensis* was analyzed for the presence of various phytoconstituents [15].

Acute toxicity study

Acute toxicity study of ethanolic extract of the fruits of *Mallotus philipensis* Mull. Arg was determined in wister albino rats (150-200gm) according to OECD guidelines no:423 [16]. Based on performed toxicity tests the $LD_{50.}$ Dose were selected as 200 and 400mg/kg.p.o.

Experimental procedure

The Animals were divided into five groups and each group contains six animals (n=6).

- Group I - served as normal control and received 1% carboxymethyl cellulose (CMC) orally for 30 days.
- Group II – Animals, received Aniline hydrochloride (100 ppm) in drinking water for 30 days.
- Group III - Animals, received Aniline hydrochloride (100 ppm) in drinking water and

standard drug Ascorbic acid co treatment at the dose of 40mg/kg b.wt, orally for 30 days.

- Group IV - Animals, received Aniline hydrochloride (100 ppm) in drinking water and plant extract co treatment at the dose of 200mg/kg b.wt, orally for 30 days.
- Group V - Animals, received Aniline hydrochloride (100 ppm) in drinking water and plant extract co treatment at the dose of 400mg/kg b.wt, orally for 30 days.

ASSESSMENT OF SPLEEN TOXICITY

Estimation of general parameters and biochemical evaluation

General parameters like body weight and spleen weight were noted. At the end of treatment period, blood was withdrawn from retro orbital plexus using glass capillary and serum was separated. Blood sample was used for the estimation of hemoglobin (Sahli's hemometer method), and red blood cell (RBC) and white blood cell (WBC) using hemocytometer; [17] and serum sample was used for the estimation of iron content[18] and total protein (Lowry et al., 1951[19].

Estimation of Heamoglobin

The heamoglobinometer tube was filled with N/ 10 HCl up to the marking 10, to this 20µl of blood was added with the help of pipette. Wipe off the first drop of blood and suck the blood from the second drop in Hb pipette 0.2µl up to the mark 20 cu mm. Fill the Hb pipette by capillary action. Wipe the tip of the pipette with the help of cotton to remove sticked blood around the tip. Extra blood is removed by filter paper/ tissue paper. The contents in the tube were mixed by stirring, and allowed to stand for 10 minutes. A clear brown colour solution was formed due to the formation of acid hematin. Then distilled water was added drop by drop to dilute. The colour of diluted fluid was compared with the standard; dilution was continued until the colour of the fluid exactly matches the standard. The lower meniscus of the fluid was noted and reading was noted directly from the graduated tube and reading is expressed as g/ percentage of haemoglobin.

Estimation of RBC

The RBC pipette was filled with blood up to the mark 0.5, immediately RBC diluting fluid. (Hayem's fluid) was filled up to the mark 101. Pipette was rolled between the palms to ensure thorough mixing of blood with diluting fluid and kept aside for a while. The counting chamber was placed and the RBC squares were focused under low power first, when markings were identified then turn to high power. The first 3-4 drops of blood mixture was discarded and it was mixed once again, the counting chamber was charged with the mixed blood. After charging mount the slide, allow the fluid to settle then using a 45X lens the RBC. The values of RBC were expressed as 10^6/cells.

Estimation of WBC

The WBC pipette was filled with blood up to the mark 0.5, immediately RBC diluting fluid (Hayem's fluid) was filled up to the mark 11.Pipette was rolled between the palms to ensure thorough mixing of blood with diluting fluid and kept aside for a while. The counting chamber was placed and the WBC squares were focused under low power first, when markings were identified then turn to high power .The first 3-4 drops of blood mixture was discarded and it was mixed once again, the counting chamber was charged with the mixed blood. After charging mount the slide, allow the fluid to settle then using a 10X lens the WBC were counted uniformly in corner squares. The values of WBC were expressed as 10^3/cells.

Estimation of total protein

For total protein estimation the serum was prepared in 5% trichloroacetic acid. The precipitated protein was dissolved in sodium hydroxide and used as aliquots for the estimation of total proteins. Soluble and insoluble fractions of the protein were estimated by preparing homogenate in double distilled water. The water soluble supernatant was used for estimation of soluble protein while the residue dissolved in sodium hydroxide was used for the estimation of insoluble protein. The protein content of the samples was determined by the method of Lowry et al using bovine serum albumin as the standard.

Estimation of Iron content

The iron content in the serum was estimated by Ramsay method. Equal volume of serum, 0.1M sodium sulphide, and 2,2′-dipyridyl reagent were mixed in glass stopper centrifuge tubes. The tubes were heated in boiling water both for 5 min, The content was cooled and 12ml of chloroform was added in each tube. The tube was mixed vigorously

for 30 seconds and centrifuged for 5 min at 1,000 rpm. The colour intensity was measured at 520nm. Standard iron solution: 498 mg of ferrous sulphate was dissolved in distilled water and 0.1 ml of conc.H_2SO_4 was added and the final volume was made up to 1 L (5-20ml of the standard iron) [18].

Assessment of Markers of Oxidative Stress

The animals were euthanasiously sacrificed and isolated spleen was quickly transferred to ice-cold tris hydrochloric buffered saline (pH 7.4). It was blotted free of blood and tissue fluids, weighed on electronic balance WENSAR (Model PGB200). The spleen was cross-chopped with surgical scalpel into fine slices, suspended in chilled 0.25 M sucrose solution, and quickly blotted on a filter paper. The tissue was then minced and homogenised in chilled tris hydrochloride buffer (10 mM, pH 7.4) to a concentration of 10% w/v. The homogenate was centrifuged at 10,000 rpm at 0°C for 15 minutes using Remi C-24 high speed cooling centrifuge. The clear supernatant was used for the determination of lipid peroxidation, reduced glutathione, and nitric oxide.

Estimation of Lipid Peroxidation (LPO)

Lipid peroxidation (LPO) was assayed by the method of Ohkawa et al [20] to 0.2 ml of tissue homogenate, 0.2 ml of 8.1% SDS, 1.5 ml of 20% acetic acid (pH 3.5) and 1.5 ml of 0.8% TBA were added. The mixture was made up to 4 ml with water and then heated in a water bath at 95.8ºC for 60 min using glass ball as a condenser. After cooling, 1 ml of water and 5 ml of n-butanol: pyridine (15:1 v/v) mixture were added and shaken vigorously. After centrifugation at 4000 rpm for 10 min, the organic layer was taken and its absorbance was measured at 532 nm. The level of lipid peroxides was expressed as nmoles of MDA formed/mg of protein.

Estimation of Reduced glutathione (GSH)

Estimation of GSH[21] the procedure to estimate the reduced glutathione (GSH) level followed to the method as described by Ellman (1959)15. The homogenate (in 0.1 M phosphate buffer, pH 7.4) was added with equal volume of 20 % trichloroacetic acid (TBA) containing 1 mM EDTA to precipitate the tissue proteins. The mixture was allowed to stand for 5min prior to centrifugation for 10 min at 200 rpm. The supernatant (200µl) was then transferred to a new set of test tubes and added 1.8ml of the Ellman's reagent (5,5'-dithio bis2-nitrobenzoi acid) (0.1mM) was prepared in 0.3 M phosphate buffer with 1% of sodium citrate solution). Then all the test tubes make up to the volum of 2ml. After completion of the total reaction, solutions were measured at 412 nm against blank. Absorbance values were compared with a standard curve generated from standard curve from known GSH. The glutathione level in spleen was calculated as micromol/g spleen.

Estimation of nitric oxide (NO) contents

Nitric oxide radical scavenging activity was measured by using Griess reagent (Green et al., 1982) [22] [0.1% N-(1 -Naphthyl) ethylenediamine dihydrochloride, 1% Sulfanilamide, 2.5% Phosphoric acid] i.e. 0.01 gm NEDA, 0.1 gm sulphanilamide, and 250 µl of phosphoric acid was mixed and volume was made to 10.0 ml. Equal volume of Griess reagent and tissue homogenate were treated with regents 1mM NADPH, Nitrate reductase 1 U/ml and 1 M Zinc acetate and incubated at 37°C for 10 min followed by absorbance reading at 542 nm wavelengths spectrophotometrically

Histopathology of Spleen

After decapitation, spleen was rapidly dissected out and washed immediately with normal saline and fixed in 10% buffered formalin. Small section of tissue was cut stained with haematoxylin and eosin (H&E) for general morphological evaluation.

Statistical analysis

The data of all the results were represent as means ±S.E.M on statistically analyzed by one-way ANOVA followed by Tukey's multiple comparison test was used for statistical analysis $p<0.05$ was considered significant.

RESULTS

Preliminary phytoconstituents

Preliminary phytochemical screening revealed the presence of carbohydrate, protein, steroids. Phenols, tannins, flavonoids, glycosides and terpenoids.

Acute toxicity study

Extract treated mice showed no lethality or any discernible behavioral changes up to 2000 mg/kg by oral route. No mortality was observed at this dose during 24 h observation period.

Results of the effect of *EEMP* Body weight and Spleen weight

At the end of treatment period body weight, spleen weight from all the groups were monitored. It was found that *EEMP* showed a significant recovery in altreration of spleen weight; bodyweight compared to aniline hydrochloride treated rats. Combination of aniline hydrochloride +ascorbic acid (40mg/kg) showed better result as compared to aniline hydrochloride alone (Table 1).

Table 1: Effect of EEMP on Body weight and spleen weight:

Parameter	Control	AH	AH+Ascorbic acid (40mg/kg)	AH+EEMP (200mg/kg)	AH+EEMP (400mg/kg)
Body weight(g)	250.16±4.400	180.83±2.483*	245.5±6.090**	194.33±2.581**	240.16±5.419**
Spleen weight(g)	3.76±0.078	7.758±0.086*	3.645±0.029**	4.193±0.069**	3.65±0.039**

Values are expressed as mean ± SEM.
Level of significance is considered as p < 0.05.* compared to control group and ** compared to AH-treated group.

Effect of EEMP on RBC, WBC, and Haemoglobin Level

RBCs count and haemoglobin level were significantly (*P*< 0.05) decreased and WBC count was significantly (*P*< 0.05) increased in AH-treated rats as compared to control rats. The chronic (30 days) treatment with ascorbic acid (40 mg/kg day, p.o.) , EEMP (200 mg/kg day,p.o) and(400 mg/kg day,p.o) were showed a significant (*P*< 0.05) increase in RBC count and haemoglobin level and significant (*P*< 0.05) decrease in WBC count compared to aniline-treated rats. (Figures1 and 2).

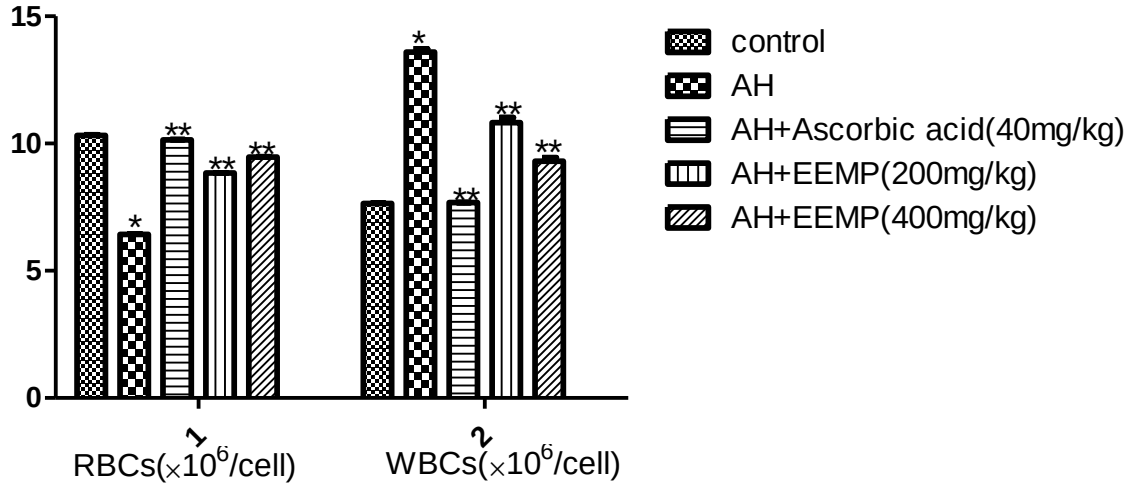

Figure 1: Effect of EEMP on RBCs and WBCs in AH-treated rats.

Values are expressed as mean ± SEM.
Level of significance is considered as p< 0.05.*compared to control group and ** compared to AH-treated group.

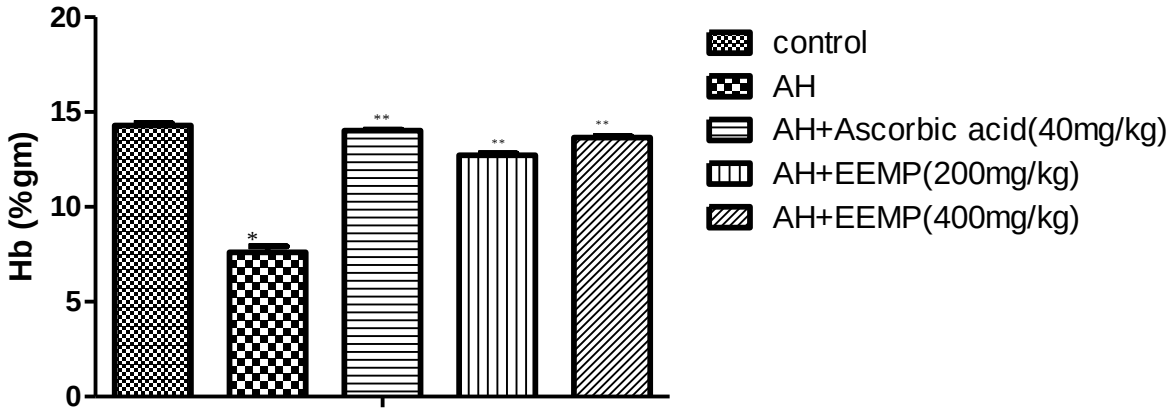

Figure 2: Effect of EEMP on Haemoglobin in AH-treated rats.

Values are expressed as mean ± SEM.
Level of significance is considered as p < 0.05.* compared to control group .and* compared to AH-treated group.

Effect of EEMP on Serum total protein and Iron contents

Total protein contents and serum iron content were monitored and shown in Figure 3 and 4 A significant (*P*< 0.05) decreased in the level of serum protein and a significant (*P*< 0.05) increased in total iron content was observed in aniline-treated group as compared to control group. Treatment with EEMP (200 and 400 mg/kg) for 30 days showed significant (*P*< 0.05) increased in total protein contents and significant (*P*< 0.05) decreased in total iron content as compared to aniline-treated rats.

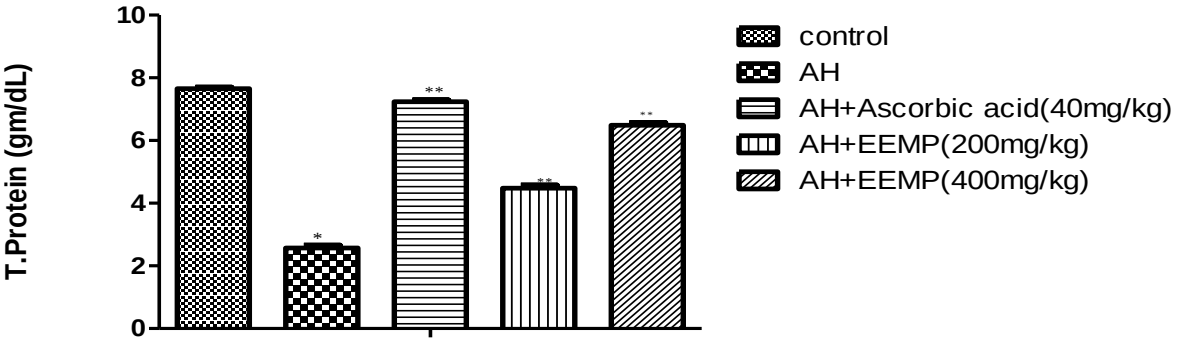

Figure 3: Effect of EEMP on Total protein in AH-treated rats.

Values are expressed as mean ± SEM.
Level of significance is considered as p < 0.05.* compared to control group. and ** compared to AH-treated group.

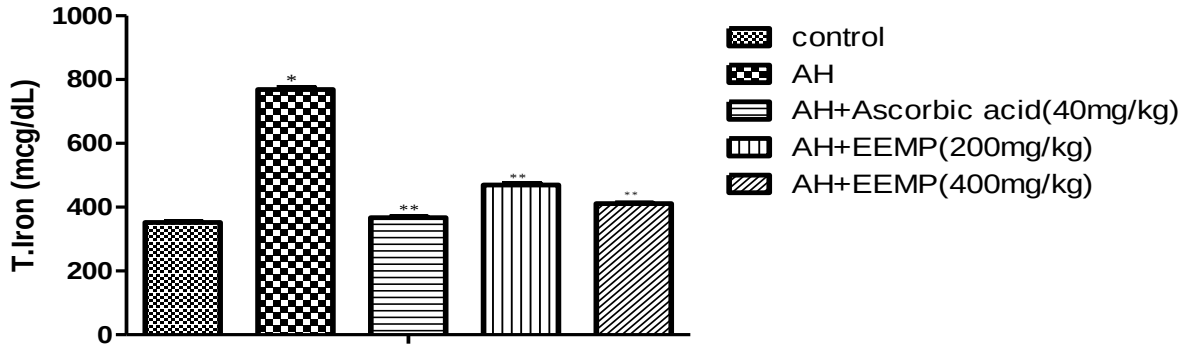

Figure 4: Effect of EEMP on Serum iron in AH-treated rats.

Values are expressed as mean ± SEM.
Level of significance is considered as p< 0.05.* compared to control group and**compared to AH-treated group.

Effect of EEMP on Tissue Lipid Peroxidation, Reduced Glutathione Content, and Serum NO Levels

The level of endogenous antioxidants such as LPO, GSH, and NO was measured in spleen tissue homogenate. LPO and NO levels were found to be significantly (*P*< 0.05) increased and GSH level was significantly decreased in spleen of AH-treated rats as compared to control group. Chronic treatment with Ascorbic acid (40 mg/kg), EEMP (200mg/kg) and EEMP (400mg/kg) were showed significant (*P*< 0.05) decrease in LPO and NO levels and significant (*P*< 0.05) increased in GSH level as compared to aniline-treated group. EEMP (400 mg/kg) was found to be more effective in maintaining the antioxidant status in aniline-treated rats [Figure: 5]

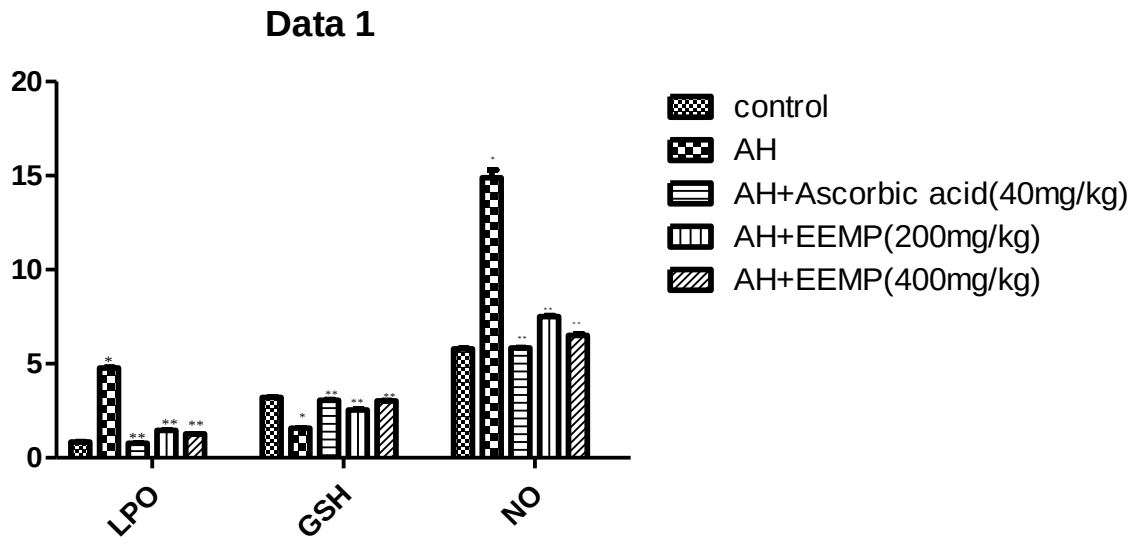

Figure 5: Effect of EEMP on LPO, GSH. and NO in AH-treated rats.

Values are expressed as mean ± SEM.
Level of significance is considered as p< 0.05.*compared to control group. And **compared to AH-treated group.

Effect of EEMP on Histopathology of Spleen

The section of control rat (Figure6 (a) showed the normal architecture, vascular organization, and cellular composition of white pulp and red pulp of spleen. AH-treated group (100 ppm in drinking water) showed congested red pulp, severe depletion of white pulp lymphocytes, extensive hemorrhages in the red pulp, and hyaline degeneration of the wall of splenic arterioles with edema and increased red blood cells (Figure6(b). The section of AH+Ascorbic acid treated rats spleen showed marked restoration of spleen architecture (Figures 6(c)). AH+EEMP(200mg/kg) showed mild restoration of spleen architecture (Figures 6(d)) while AH+EEMP(400mg/kg) showed comparatively more protection by effective restoration of spleen architecture as compared to AH-treated group (Figure 6(e

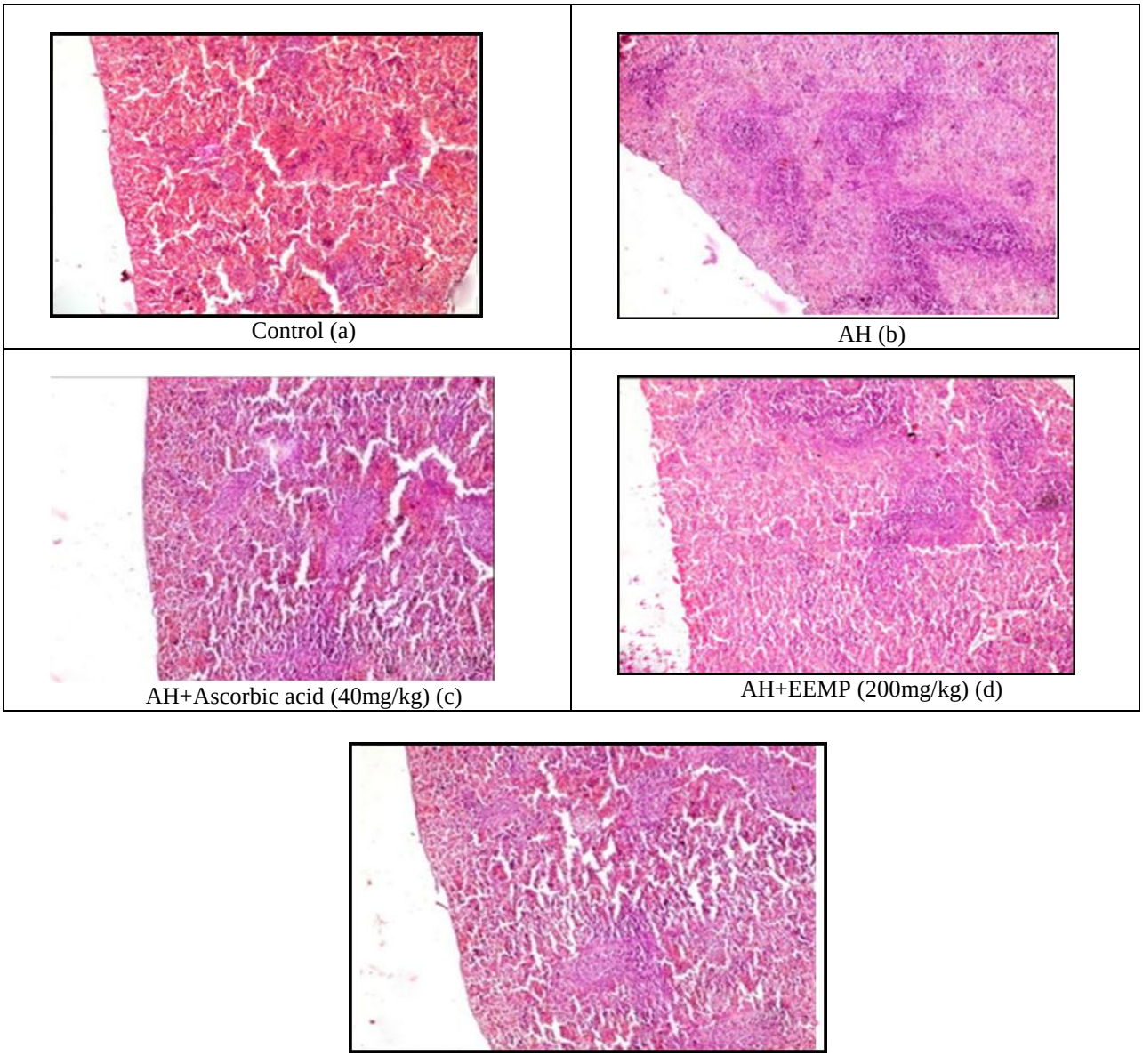

Figure 6: Histopathology of spleen stained with hematoxylin-eosin staining

DISCUSSION

All parts of plant like gland and hairs from the capsule (or) fruits are used as Heating, Purgative, Anthelmintics, Vulnerary, Detergent, Maturant, Carminative, Anit diabetic, and Alexiteric and are useful in treatment of Bronchitis, Abdominal, and Spleen Enlargement. The ethanolic extract of *Mallotus Philippensis Muell* Arg. was evaluated for its splenotoxic activity. The ethanolic extract of *Mallotus Philippensis muell* Arg. Shown the presence of the phyto chemical constituents such as phenols, diterpenoids, steroids, flavonoids, cardinolides, coumarin, and isocoumarins. Aniline exposure leads to the development of splenic toxicity in rats. Previous studies show that exposure to aniline produces increases in total iron content and oxidative stress in rats, and it leads to enlargement of spleen (splenomegaly) due to excess deposition of damaged RBC [23, 24, 25]. The present study shows the splenoprotective effect of *EEMP*. Splenic toxicity in rats was induced by chronic supplementation of AH (100 ppm) via drinking water. Significant increase in the weight of spleen (splenomegaly) Toxicity of spleen was confirmed by evaluating the haemoglobin level, RBC and WBC count on 30th day. The haemoglobin level and RBC count were significantly decreased where as WBC was increased indicating the development of spleen toxicity in AH-treated rats indicated the deposition of damaged RBCs in the spleen [23, 24]. *EEMP* is reported to play a major role in the treatment of various conditions due to its strong antioxidant property. In the present study, *EEMP* treatments reverse the changes in Body weight and spleen weight in AH-treated animals. This alteration of general parameters (Body weight & spleen weight) suggested the positive effect of *EEMP* in AH toxicity.

In the present study AH exposure in rats showed significant rise in the level of haemoglobin, RBC, and WBC when compared to normal control rats. These changes might be due to the excessive generation of oxidative and nitrosative stress [26, 27]. Treatment with *EEMP* showed significant alteration of hemoglobin level and RBC and WBC content, which might be due to the strong antioxidant/free radical scavenging activity of *EEMP* [28]. Aniline administered rats showed a significant increase in iron load and decrease in protein contents. Iron plays a significant role as a mediator of aniline-induced splenotoxicity [3, 9]. AH- treatment causes remarkable accumulation of iron which may catalyze excessive formation of reactive oxygen species, which react with and damage proteins, nucleic acids, and lipids; leading to cellular dysfunction [29]. The AH exposure leads to iron overload and induction of lipid peroxidation (oxidative stress) in the spleen. Lipid peroxidation and protein oxidation are at least two important early biochemical events in AH-induced splenic toxicity. In the present study AH induced group showed a significant increase in lipid peroxidation and NO content and a significant decrease in GSH level in spleen. These alterations in oxidative stress markers produced structural modification of native proteins and their function which might lead to splenic toxicity [6]. Treatment with *EEMP* and Ascorbic acid preserves the intracellular LPO, NO, and GSH level. GSH may react with nitric oxide to form S-nitro glutathione that is far more potent than nitric oxide itself [30].

The morphological changes are always supported with histopathological alteration. The histopathological changes in the AH-treated rat spleen include vascular congestion and increased red blood cells [31]. These changes are closely associated with increased iron deposition in the red pulp of the spleen. The vascular congestion and marked iron deposition in the spleen with the increasing AH exposure are consistent with scavenging of damaged red blood cells in the red pulp. This, in conjunction with the accumulation of aniline metabolites within the spleen, could lead to the transformation of mesenchymal cells of the spleen [27]. The present study is associated with increased iron deposition and development of fibrotic lesions in the AH-treated rats[32], due to iron-mediated production of ROS which might act as a stimulus for increased collagen production in splenic tissue, leading to fibrosis. An increase in collagen gene transcription and collagen production occurred when cultured human fibroblasts were subjected to iron-induced lipid peroxidation or exposed to malondialdehyde [33]. The histoarchitecture of the spleen supports the biochemical findings in the present study. Free radicals damage RBCs which might be the reason for observed changes in spleen histology. Treatment with *EEMP* showed the attenuation of splenic toxicity induced by aniline which might be due to its inhibitory potential of reactive oxygen species as well as potent free radical scavenging activity.

CONCLUSIONS

Ethanolic fruit extract of *Mallotus phillipensis* exhibited significant protective effect in AH induced spleen toxicity in rats. The extract showed improvement in various biochemical and antioxidant parameters an as well as restoration of spleen architecture and so might be of value in spleen toxicity. However, further phytochemical investigations are required to isolate and identify the ameliorative effect of active principle in the plant as well as elucidating their mechanism of action.

Conflict of interest

We declare that we have no conflict of interest.

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