Trop J Nat Prod Res, March 2021; 5(3):559-563 ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Tropical Journal of Natural Product Research

Available online at https://www.tjnpr.org

Original Research Article

Acute Toxicity and Liver Protective Effects of *Elephantopus mollis* H.B.K

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ARTICLE INFO

Article history:
Received 01 February 2021
Revised 17 February 2021
Accepted 20 March 2021
Published online 01 April 2021

ABSTRACT

Elephantopus mollis (EM) is a wild species and is commonly used in folk medicine as remedy for various ailments. Some uses of this plant has been documented in the scientific literature. However, studies on the medicinal properties of this specie remain rarely published and the specie has not been recorded in the Vietnamese pharmacopeia. The present study therefore assessed the acute toxicity and liver-protective effects of *Elephantopus mollis* (EM). The acute toxicity of the aqueous extract of EM in mice was assessed following the method stipulated by the Organization for Economic Cooperation and Development (OECD), while the hepatoprotective effect was assessed following previously reported method. Whole plant extract of EM was not toxic to the tested mice (at a dose of 9,000 mg/kg) and showed good liver protection. The plant extract had the ability to reduce the degradation of liver cells by reducing the amount of liver enzymes. At a dose of 400 mg/kg EM, the reduction efficiency of AST and ALT in tested mice were 71.43 ± 11.36% and 63.82 ± 19.43%, respectively, when compared with the negative control group after 2 weeks of treatment. Mice liver morphology and histopathology treated with EM extract showed a decent improvement. The liver cells size of mice treated with EM extract decreased significantly (28.02 ± 6.33 µm) compared to the mice in the negative control group (34.82 ± 6.36 µm). The results suggest the potential effect of this extract as a hepatoprotective agent towards liver damage without any oral acute toxicity effect.

Keywords: *Elephantopus mollis*, Folk remedies, Acute toxicity, Liver protection.

Introduction

Vietnam, a fast-developing country,[1] has a high prevalence of non-communicable diseases, poor socio-economic status, problem with conservation of natural resources and environmental pollution.[2,3] The increase in environmental pollution, frequent exposure to toxic chemicals, and unhealthy eating habits have become major causes of outbreaks of non-communicable diseases such as diabetes, cancer, or obesity.[4,5] The economic burden on patients and their families has been enormous when using modern treatments.[6] Natural products, which account for approximately over half of the pharmaceuticals in clinical use today, have been receiving more attention.[7] The focus on the use of herbs has increased due to their accessibility, affordability, and the high cost of synthetic medicines with their attendant side effects.

Elephantopus mollis H.B.K. is a wild species distributed in many different habitats. It is a medicinal plant used in many folk remedies to treat diseases such as liver disease, skin disease, and cancer.[8] There have been many studies on the value of *EM* in the treatment of non-communicable diseases.[9,10] However, research on the botanical and bioactive characteristics with respect to the soil conditions of *EM* in the south of Vietnam is still being very limited. In addition, the hepatoprotective effects of *EM* have only been verified *in vitro*. The present study therefore evaluated the hepatoprotective effect of *EM* on CCl_4 - induced liver injury in mice.

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Citation: Phan TD and Nguyen THP. Acute Toxicity and Liver Protective Effects of *Elephantopus mollis* H.B.K. Trop J Nat Prod Res. 2021; 5(3):559-563. doi.org/10.26538/tjnpr/v5i3.23

Official Journal of Natural Product Research Group, Faculty of Pharmacy, University of Benin, Benin City, Nigeria.

Materials and Methods

Preparation of E. mollis extract.

Samples of *EM* were collected from different altitudes (ranging from 9 m to 700 m) and habitats (residential areas, trails, forests, gardens, valleys, and ravines) in An Giang during the summer season (from June to August 2020) and examined for morphology. The morphological features coincided with the identifiable descriptions in an Illustrated Flora of Vietnam and Flora of Vietnam – Asteracease Dumort.[11,12] The collected *Elephantopus mollis* plants were dried and an herbarium specimen prepared and stored in Plant Laboratory (Code: *Elephantopus mollis*.2020.WE.001-012), School of Education, Can Tho University. The whole EM plant was dried in the shade before being ground into a fine powder. Twenty-five (25) grams of the powder sample was macerated in 750 mL of water and boiled for 30 minutes in a conical flask. The extract was filtered using filter paper and then evaporated using a rotary evaporator (RV 10 digital V-C, IKA, Germany).[13]

Experimental animals.

Seventy-six **(**76) Male Swiss mice *Mus musculus* (20-25 g) obtained from Pasteur Institute (Ho Chi Minh City, Vietnam) were randomly distributed into 10 groups (4 groups for toxicity test and 6 groups for hepatoprotective test) and acclimatized to the laboratory condition for 7 days. Ethical approval was granted by the Animal Welfare committee with Assessment No. AWA2020-02/KSP.

Acute toxicity study of EM aqueous extract.

EM extract at doses of 0 mg/kg, 3,000 mg/kg, 6,000 mg/kg and 9,000 mg/kg were administered orally to four groups of mice comprising of ten male Swiss mice (30-35 g) in each group following the guidelines stipulated by the Organization for Economic Cooperation and Development (OECD).[14] The mice were given the extract orally once daily with a maximum volume of 10 mL/kg of body weight. The animals were then observed for behavioral changes within 72 hours.

Hepatoprotective assay

Experimental design for hepatoprotective activity was carried out following the studies of Kang and Koppula (2014) and Xia *et al.* (2019).[15,16] A total of 36 mice were divided into 6 groups, each comprising 6 mice. Group I served as biological control and received only the distilled water (1 mL/kg/day). Group II served as negative control and the mice in this group received CCl_4 at 1 mL/kg dose (1:4 of CCl_4 in olive oil) once daily for 14 days. Group III served as positive control and the mice in this group received CCl_4 1 mL/kg (1:4 of CCl_4 in olive oil) and silymarin 17 mg/kg orally for 14 days. The mice in Groups IV, V, and VI received CCl_4 1 mL/kg (1:4 of CCl_4 in olive oil) and 200 mg/kg, 400 mg/kg and 800 mg/kg *EM* aqueous extract orally for 14 days. At the end of the experiment, the animals were quickly slaughtered by stretching the spine after diethylether anesthesia. Blood and liver tissue samples, were collected. The livers were fixed in 10% neutral formalin for histological examination. Blood was collected from each animal for red blood cell count (RBC), hemoglobin concentration (Hb), serum alanine aminotransferase (ALT), and aspartate aminotransferase (AST) activity to examine liver function. Liver samples were preserved in 10% buffered formalin and dehydrated in ascending grades of ethanol and cleared in xylene. The specimens were then embedded in paraffin and cut into 5 µm thickness section. The sections were stained with Hematoxylin and Eosin Y (H&E). The degree of necrosis, hepatocyte size, and hepatocyte nuclear size were recorded under the CX23 light microscope (Olympus, Japan) with a digital camera using OptixCam Toupview software (Microscope, USA). Hematological parameters including RBC and Hb were determined using a CELL-DYN Ruby Automated Hematology Analyzer (Abbott, USA).[17] AST and ALT concentration were determined using the Cobas clinical chemistry automatic analyzer (La Roche Ltd., Japan).[18]

Statistical analysis

All data were analyzed using ANOVA with the aid of IBM SPSS 22 for Windows. Data obtained were expressed as mean ± SD. Multiple comparisons of the means were done using the Duncan Multiple Range Test at 5% probability.

Results and Discussion

Acute toxicity test

Oral administration of *EM* extract at a dose of 3,000, 6,000 and 9,000 mg/kg resulted in no mortality or clinical signs of acute toxicity in the mice as observed for the period of 72 hours. All the animals survived till the end of the observation period. Therefore, the LD_{50} value was not identified in the study and *EM* was non-toxic to mice. The results of evaluation of the levels of AST and ALT showed that *EM* extracts were not toxic to the liver at high doses. Similarly, the RBC count and Hb concentration at doses of 3,000, 6,000 and 9,000 mg/kg were not significantly different from the control treatment.

Liver protective effect

CCl_4 is harmful to the liver because it causes the cytochrome P450 of liver cells to become trichloromethyl (CCl_3*). CCl_3* increases liver peroxide products and causes liver damage. Signs of liver damage are shown by increased serum concentrations of AST and ALT.[19] A decrease in serum levels of AST and ALT is an indication of the hepatoprotective effect of a drug.[20] When compromised by CCl_4, levels of AST (1160.00±497.80 U/L) and ALT (1805.00 ± 1303.90 U/L) in mice blood increased significantly compared to the control (AST: 51.00±2.65 U/L; ALT: 31.67±6.66 U/L). Treatment with Silymarin (17 mg/kg) caused reductions in AST and ALT by 63.40±12.62% and 67.90±11.54%, respectively. When using *EM* extract, the AST and ALT concentrations in the plasma tended to decrease. At doses of 200 and 400 mg/kg *EM*, the AST concentrations in blood plasma was comparable to that observed with Silymarin treatment. Reduction in ALT concentration was highest at a concentration of 200 mg/kg (75.32 ± 18.41 U/L). These results can help conclude that *EM* extract is capable of reducing AST and ALT concentrations in mice poisoned with CCl_4. In Figure 1A, RBC count in negative control treatment was 6.26 ± 0.45 million/mm^3, a decrease compared to the biological control (7.22 ± 0.93 million/mm^3). This proved that CCl_4 has the ability to reduce the number of red blood cells in the blood and this result is similar to the results of Ab *et al*. (2010). The group of mice treated with Silymarin had a higher number of erythrocytes (8.14 ± 1.14 million/mm^3) than the mice taking CCl_4 ($p < 0.05$), and equivalent to the biological control group ($p > 0.05$). Silymarin showed an ability to increase red blood cells in the mice. *EM* extracts were unable to increase the number of red blood cells in the mice; however, these extracts were able to increase the Hb content in each cell (Figure 1B).

There were significant differences in the liver morphology of mice between experimental groups for one week. Mouse liver administered with only distilled water (Figure 2.I) had a smooth, glossy, soft, smooth surface and dark red colour. In contrast, the group of mice taking CCl_4 (Figure 2.II) had damaged liver, liver surface roughness, tough fibrosis, pale colour. For the mice given CCl_4 and treated with Silymarin (Figure 2.III), the liver improved morphologically, but the liver surface was still rough compared to that of the biological control. The mice administered with the extracts had a better improvement, the colour turned red, the surface was smooth, reduced roughness compared to that of the negative control (Figure 2. IV, V and VI).

The histology of mouse liver is shown in Figure 3. The mice administered with CCl_4 but untreated had larger cells than those of the biological control (Figure 3.I). The cells were swollen, adhered to each other with empty cytoplasm, containing dimensional fat particles. There were many phagocytic organizations around the vessels and between the liver cells. This result was consistent with studies of Kang and Koppula (2014), Athokpam *et al.* (2017), Singhal and Gupta (2012).[15,21,22] CCl_4 compromised-mice treated with Silymarin had a recovery of liver cells (Figure 3.III). The hepatocytes were arranged in series towards the central vein. The phagocytic organizations were significantly reduced compared to the group of mice administered with CCl_4, concentrating mainly around vascular structures, demonstrating the liver protective effects of Silymarin.[21,23,24]

The hepatocytes of mice with liver damage by CCl_4 treated with *EM* extract at concentrations of 200, 400 and 800 mg/kg were all well improved (Figure 3. IV, V, VI). The cell enlargement were reduced, arranged in series, easy to recognize capillary spokes, reduced phagocytic organizations compared to the group of mice administered with CCl_4, demonstrating the liver recovery ability of *E. mollis* extract. Random measurement of 30 cells diameter and hepatocyte nucleus around the central vein at the edge of the liver on each specimen showed that the histology of the liver cells were different among the treatments ($p < 0.05$). Mice liver cell size in the negative control was the largest with a diameter of 34.82±6.36 µm, compared to the rest of the treatments ($p < 0.05$). CCl_4 caused inflammation of cells, enlarged liver cells. For the group of mice administered with Silymarin and *EM* extract, there was a decrease in liver cell diameter compared to the negative control group, but it was still larger than that of mice administered with only distilled water. In contrast, the diameter of the nucleus of mice challenged with CCl_4 was the smallest (7.67 ± 1.13 µm). CCl_4 caused necrosis or destruction of the nucleus, and therefore the diameter of the cell nucleus decreased. Comparing the ratio of nucleus diameter and cell diameter shows that the biological control group had the highest rate, and the negative control group had the lowest rate. The mice administered with Silymarin and *EM* extracts had equal rates, both greater than that of the mice administered CCl_4 only. The results show the ability to restore damaged liver cells.

Conclusion

The aqueous extract of EM was not toxic to mice at a dose of 9000 mg/kg and has potential for use in humans. The plant also showed potential in the treatment of liver disease through physiological, biochemical, and histological tests on mice with a recovery value of damaged liver tissue equivalent to Silymarin. Further research is needed to determine exactly which compound(s) contained in EM played important roles in the hepatoprotective effect of the plant.

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Table 1: Hematological parameters of tested mice

Treatments	Indices			
	Distilled water	3,000 mg/kg	6,000 mg/kg	9,000 mg/kg
AST (U/L)	92.00 ± 18.36[a]	115.00 ± 62.52[a]	118.67 ± 67.00[a]	78.33 ± 34.44[a]
ALT (U/L)	40.33 ± 3.21[a]	37.67 ± 7.09[a]	48.67 ± 11.68[a]	40.00 ± 14.11[a]
RBC (mil/mm^3)	7.08 ± 0.72[a]	6.90 ± 0.90[a]	7.52 ± 1.20[a]	7.55 ± 1.19[a]
Hb (g/100mL)	12.43 ± 1.28[a]	14.78 ± 3.06[a]	13.85 ± 0.97[a]	13.27 ± 2.51[a]

Means ± SD in rows having the same letter are not significantly different (Duncan, $p > 0.05$)

Table 2: Biochemical parameters and therapeutic efficacy of extracts

Treatments	AST (U/L)	AST-RP (%)	ALT (U/L)	ALT-RP (%)
Distilled water	51.00 ± 2.65[a]	100.00 ± 0.00[d]	31.67 ± 6.66[a]	100.00 ± 0.00[d]
CCl_4	1160.00 ± 497.80[c]	0.00 ± 0.00[a]	1805.00 ± 1303.90[c]	0.00 ± 0.00[a]
CCl_4 + Silymarin	399.67 ± 118.50[a]	63.40 ± 12.62[c]	618.00 ± 174.25[ab]	67.90 ± 11.54[c]
CCl_4 + 200 mg/kg	306.67 ± 129.08[a]	74.40 ± 21.87[cd]	415.67 ± 71.28[ab]	75.32 ± 18.41[cd]
CCl_4 + 400 mg/kg	372.00 ± 54.06[a]	71.44 ± 11.37[cd]	646.33 ± 107.75[ab]	63.82 ± 19.43[bc]
CCl_4 + 800 mg/kg	773.00 ± 90.51[b]	29.65 ± 23.36[b]	1029.50 ± 573.46[bc]	39.19 ± 12.18[b]

Means ± SD in a column have the same letter are not significantly different (Duncan, $p > 0.05$). AST-RP: reduce performance of AST; ALT-RP: reduce performance of ALT.

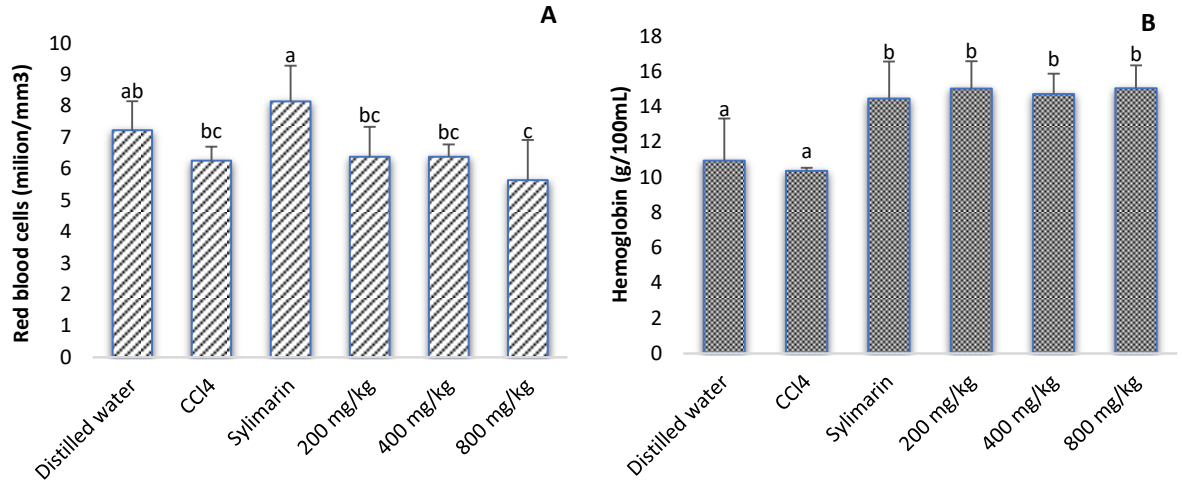

Figure 1: Red Blood Cells count (A) and Hb concentration (B) in mice blood of the treatments. Bars (mean ± SD) have the same letter are not significantly different (Duncan, $p > 0.05$)

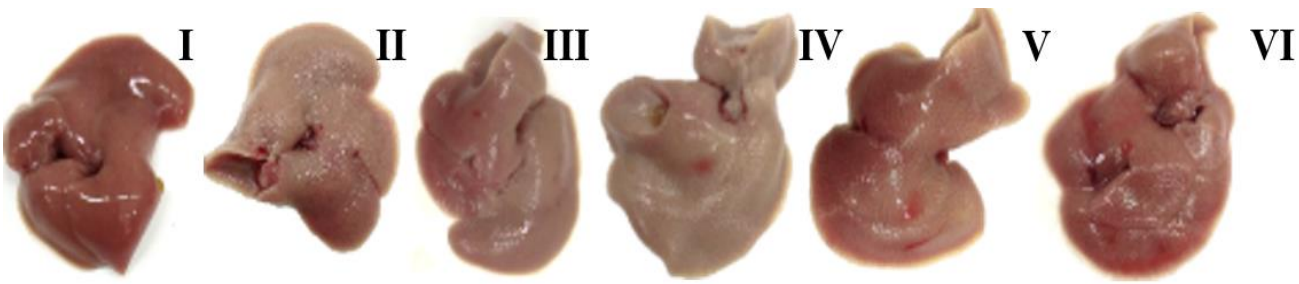

Figure 2: Macroscopic morphology of mice liver in all treatments.

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

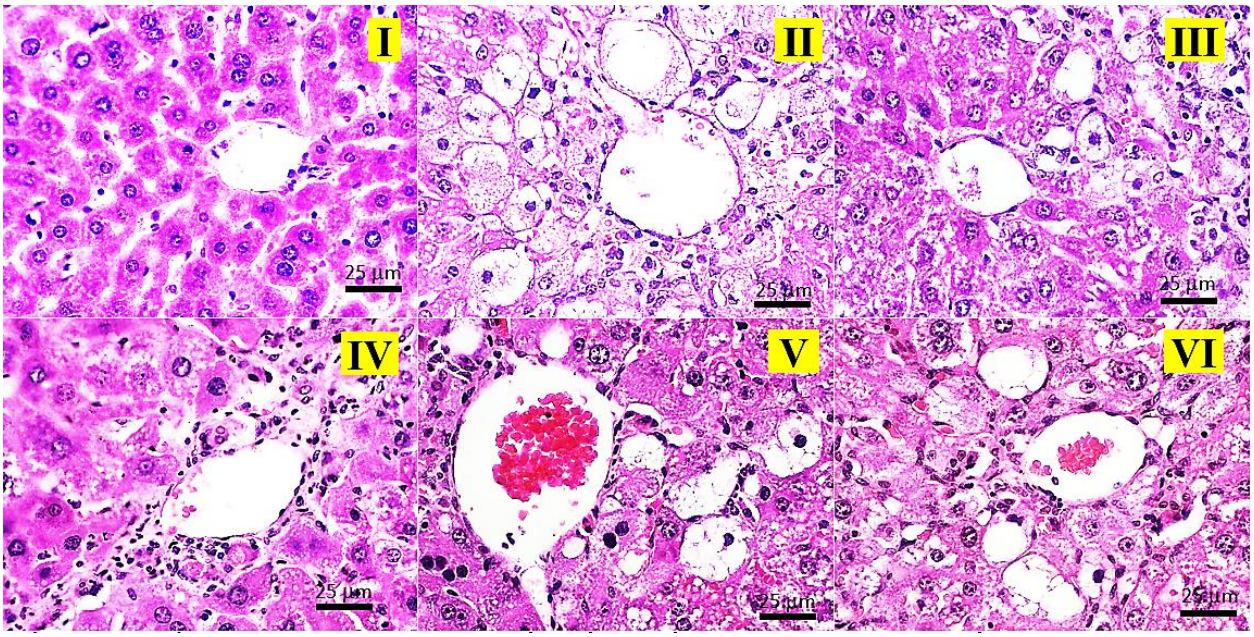

Figure 3: Microstructure of mouse liver disease after 2 weeks of treatment with Silymarin and EM extract (magnification of 400 times).

Table 3: Cellular characteristics of mouse livers under the effects of CCl_4 and EM extracts

Treatment	Cell diameter (µm)	Nucleus diameter (µm)	Nucleus/cells ratio
Distilled water	21.28 ± 2.61^{a}	12.13 ± 1.43^{c}	0.58 ± 0.94^{c}
CCl_4	34.82 ± 6.36^{d}	7.67 ± 1.13^{a}	0.23 ± 0.49^{a}
CCl_4 + Silymarin	25.62 ± 4.17^{b}	11.53 ± 1.50^{b}	0.46 ± 0.10^{b}
CCl_4 + 200 mg/kg	27.82 ± 6.18^{c}	11.89 ± 1.37^{bc}	0.45 ± 0.10^{b}
CCl_4 + 400 mg/kg	28.02 ± 6.23^{c}	11.67 ± 1.94^{bc}	0.44 ± 0.11^{b}
CCl_4 + 800 mg/kg	26.98 ± 4.77^{bc}	11.96 ± 1.49^{bc}	0.43 ± 0.09^{b}

Means ± SD in a column that have the same letter are not significantly different (Duncan, $p > 0.05$).

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

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

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