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***Original Research***
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# Toxicological Evaluation of the Repeated Dose Administration of the Ethanolic Extract of *Azolla microphylla* in Wistar Albino Rats

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## Abstract

*Azolla microphylla* is an easily cultivable aquatic plant with the commendable nutritious property. Recent reports on Azolla species emphasize the therapeutic potential of the plant extracts. Moreover, the same genus of plant also had displayed antioxidant potential owing to its free radical scavenging tendency. Although these attributes were identified, a study investigating the toxicological property of different dosages of ethanolic extract of A. *microphylla* (EAM) is not yet reported. Thus the present study aims for the in vivo toxicological evaluation of the EAM in Wistar strain of rats. Daily doses of 0, 250, 500, 1000 and 2000 mg/kg body weight of EAM were administered orally to group-I, group-II, group-III, group-IV & group-V rats, respectively for 14 days. Biochemical and histopathological studies were established through standard methods. The acute toxicity results suggest the non-toxic nature of the extract supported with the absence of mortality and toxic symptoms until 72 h of observation. The results of sub-acute toxicity study in the extract-treated rats (group-II to group-IV) indicate non-significant changes to the biochemical (total protein, AST, ALT, LDH, albumin, globulin, urea, creatinine, cholesterol, & triglycerides), hematological (Hemoglobin, RBCs, WBCs, platelets, monocytes, lymphocytes, & neutrophils), and histopathological observations when compared to the control group of rats. However, group-V rats were treated with 2000 mg/kg b.w. exhibited statistically significant variations to most of the biochemical and hematological parameters although no mortality/physical toxic signs were reported till the end of the experimental period. Thus, the sub-acute toxicity results suggest that the extracts were non-toxic and safe to rats between 250-2000 mg/kg b.w. concentration under 14 days observational period. Moreover, as there was no mortality upto 2000 mg/kg b.w., 50% lethal dose ($LD_{50}$) could not be determined, and hence it is considered to be greater than 2000 mg/kg/day.

**Keywords:** *Azolla microphylla*, Hematology, Lethal Dose, Toxicology

## 1. Introduction

Although a complex paradigm of allopathic drugs are available in the market for treating chronic diseases like Coronary Heart Disease (CHD), type-2 diabetes and multiple types of myeloma, there is growing interest among the patients to choose folklore-based treatment due to its cost-effectiveness and lessened side effects. The following factors such as economy, swift availability and decreased post-treatment complications are attractive factors in the choice of folklore medicines against numerous diseases[1]. Previous studies indicate that a significant number of Indian populations living in tribal zones and rural areas are dependent on medicinal plant therapy for their primary health care requirement[2]. Accordingly, medicinal plant researchers have leveraged their research in the exploration of the phytochemicals excelling in therapeutic potential against various diseases and disorders. It is to be emphasized that the therapeutic efficiency of the extracts are linked to the antioxidant properties of phytonutrients present in plants.

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Aquatic plants have been widely used for decades in biotechnology and pharmaceutical industries for the benefit of mankind. In the Asian continent, the marine algae serves as a nutritional source owing to its captivating proximate composition. In addition to that, they had been reported to possess antibiotic, antiviral, antifouling, antiproliferative, cytoprotective and anti-inflammatory potential[3].

*Azolla microphylla* is an aquatic water fern falling under to the division pteridophyta. Originally this plant is renowned for its nitrogen-fixing potential and agronomical support. On the other hand, recent reports on the genus Azolla stated that the plant possesses antioxidant[4], antimicrobial[5], feed supplement[6], antifungal[7], hepatoprotective[8], anti-ulcer potential[9] and cardioprotective potential[10]. These properties are attributed partly to the phytochemical constituents such as flavonoids, tannins, and saponins. Phytochemicals are of potential significance; however, over-administration or excessive intake of the phytochemicals could lead towards toxicity or sometimes may turn out to be fatal. Even though previous reports on *A. microphylla* indicated their medicinal properties; a study on the toxicological evaluation of this plant is yet to be investigated as per the best of our literature review. Hence, the present study aims to investigate the possible acute and repeated dose toxic effects of the ethanolic extract of *A. microphylla* in Wistar albino rats.

## 2. Material and Methods

### 2.1 Chemicals

All chemicals used were of analytical grade and were purchased from HiMedia laboratories (India) and Sigma-Aldrich (St. Louis, MO).

### 2.2 Preparation of the Plant Extract

Fresh samples of *A. microphylla* were collected from the local farms of Coimbatore and authenticated by Dr .G. V.S. Murthy from TNAU, Coimbatore (Voucher# BSI/SRC/5/23/2014-15/Tech/623). The collected materials were washed gently with tap water, surface sterilized by dipping in 0.2% Clorox for 30 min and rinsed with distilled water and air dried. The air dried samples were grounded to a fine powder. Pre-weighed plant (50 g) was extracted in ethanol (1000 ml) for 72 h in an orbital shaker. The extract obtained was evaporated to dryness using a rotary evaporator at 40 °C and refrigerated until further use. The dried extract was dissolved in sterile water and pre-treated to rats through oral gavage tube.

### 2.3 Experimental Animals

Adult male Wistar rats weighing in the range of 100-120 grams were obtained from Karpagam Academy of Higher Education animal house. Rats were divided into independent groups and kept in polyurethane cages under hygiene conditions with 12 h light and dark cycle. During the treatment period the rats were allowed free access to feed and water. The study was approved by Institutional Animal Ethical Committee constituted for the purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India **(KU/IAEC/Ph.D./144).**

### 2.4 Experimental Design

The **acute toxicity study** was performed according to the procedure of the Organization for Economic Cooperation and Development (OECD, 2002) guidelines 423[11]. A limit dose of 250 and 2000 mg/kg b.w. of extracts were used for the study. After the rats had fasted for 24 h (with free access to water), each rat was treated with a single oral dose of 250 mg and 2000 mg/kg of the extract. Following the administration of the extract, animals were observed individually for any toxic sign during the first 30 min and then further monitored for every 24 h, 48 h and 72 h for any signs of abnormalities in behavioral pattern, moribund status or for mortality.

The ethanolic extract of *A. microphylla* was then subjected to **sub-acute toxicological study**. Accordingly, four different doses of the extract, i.e. 250 & 500 mg/kg (lower range) and 1000 & 2000 mg/kg (higher range), were administrated orally using oral gavage tube daily one dose for 14 days to 5 different groups of rats with 6 rats in each group[12]. Distilled water was used as carrier to administrate the extract. After feeding the extracts, the entire groups of animals were observed for abnormal clinical signs and mortality daily for 14 days. At the end of the experimental period the animals were fasted for 24 h to minimize any potential interference from dietary sources. Later, the animals were anaesthetized, and the blood samples were collected through cervical decapitation into the Vacutainer tubes added with and without anticoagulant (EDTA), for hematological and biochemical estimations, respectively. The vital organs such as liver, kidney and heart were also separated for further studies. These organs were then preserved in 10% buffered formalin for histopathological examination.

### 2.5 Biochemical Estimations

The quantification of the serum protein, albumin, globulin, urea, creatinine, cholesterol, triglycerides and assay of serum AST, ALT, and LDH activities were done using Agappe diagnostics kit as per the manufacturer's instructions. The haematology parameters counts including haemoglobin, RBC, WBC, platelets, monocytes, lymphocytes and neutrophils were determined by the haematology auto-analyzer.

### 2.6 Histopathological Findings on the Liver Sections in Experimental Group of Animals

After sacrifice, the heart, liver and kidney tissues were immediately removed and washed with ice-cold normal saline and then fixed in 10% formalin for a period of at least 24 h. Thin paraffin sections of 5µm thickness were made using a rotary microtome. Further to this, the sections were stained with Haematoxylin and Eosin[13]. The histopathological slides were examined and photographs were taken with a Zeiss AxioSkop 40 photomicroscope at 40X magnification.

## 3. Statistical Analysis

The obtained data were analyzed through Tukey's test. Results were expressed as mean ± standard deviaion of six rats in each group. *P* values less than 0.05 were considered statistically significant.

## 4. Results

### 4.1 Acute Oral Toxicity Results of the Etha nolic Extract of *A. microphylla*

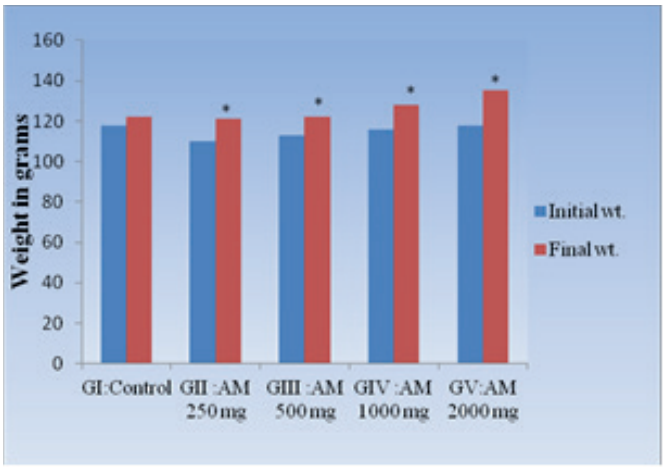
In the present acute toxicity study, after the administration of 250 mg and 2000 mg/kg b.w. of the extract, the rats were observed for behavioral changes, tremor, convulsion, food and water intake, and mortality until 72 h. Indeed, the rats did not reveal any signs of toxicity or mortality or abnormal behavioral pattern during the complete observational period for either of the dosages used.

### 4.2 Effect of Ethanolic Extract of *A. micro phylla* on the Body Weights of Rats in Sub-acute Toxicity Study

The sub-acute toxicity of the ethanolic extract of *A. microphylla* was evaluated by administering daily one dose of the following concentrations of the extract 250, 500, 1000 & 2000 mg/kg b.w. for 14 days. The rats displayed no indications of toxicity or mortality. Additionally, the rats did not show any significant alteration in water or food consumption. The results from the entire groups of rats (group-II to group-V) indicate a significant increase in the body weight except group-I (Figure 1).

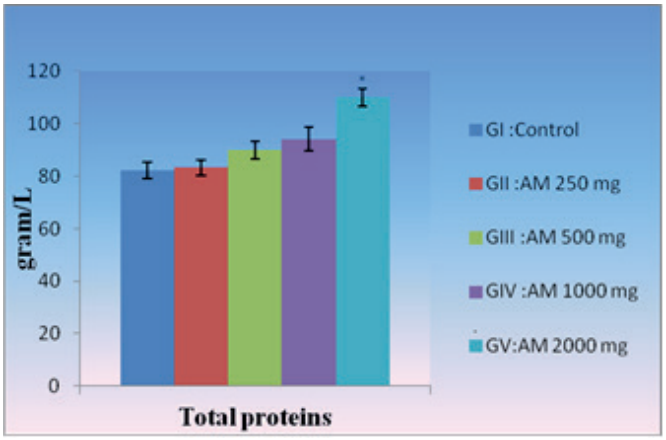
### 4.3 Effect of Ethanolic Extract of *A. micro phylla* on Total Serum Proteins in Sub-acute Toxicity Study

The current results indicate an insignificant change in serum concentration of total proteins in group-II to group-IV rats (Figure 2). However, there observed a slight increase (statistically insignificant) in the serum protein levels among all the groups except group-V, wherein, a statistically significant increase in serum proteins were observed when compared to the group-I rats.




Values are expressed as Mean±SD for six animals; *= Significant (p<0.05) when compared to initial weight

**Figure 1.** Effect of the ethanolic extract of *A. microphylla* on rat body weight in sub- acute toxicity study.

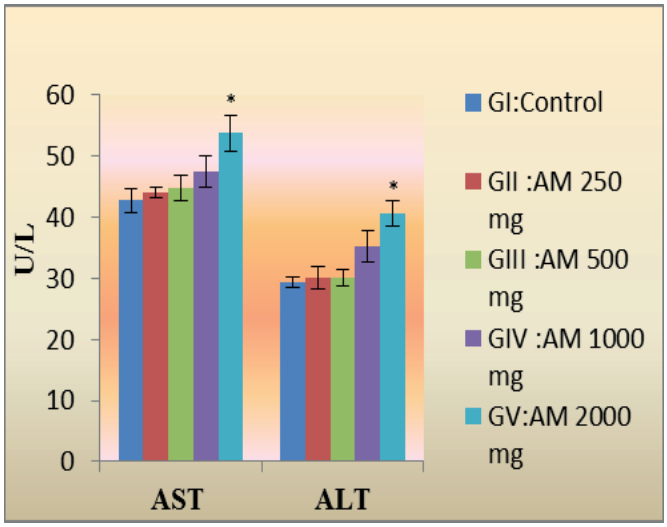



Values are expressed as Mean±SD for six animals; *= Significant (p<0.05) when compared to group

**Figure 2.** Effect of the ethanolic extract of *A. microphylla* on serum total protein in sub-acute toxicity study.

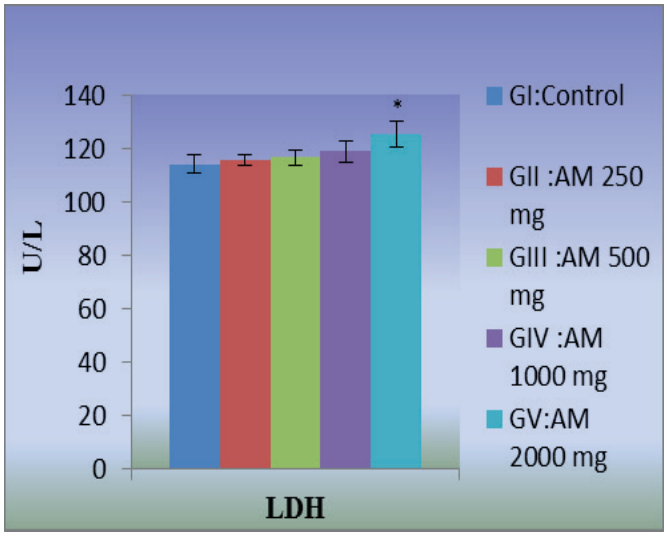
### 4.4 Effect of Ethanolic Extract of *A. micro phylla* on Serum Transaminases (AST & ALT) and LDH Enzymes Activities in Sub-acute Toxicity Study

The results of the present study indicate no significant changes (p>0.05) in the activities of serum ALT, AST, and LDH among the entire groups (II-IV), when compared to the group-I rats. Only in the 2000 mg treated rats (group-V), a significant (p<0.05) elevation in the activities of ALT, AST, and LDH enzymes were noted (Figures 3 & 4).




Values are expressed as Mean±SD for six animals; *= Significant (p<0.05) when compared to group I

**Figure 3.** Effect of ethanolic extract of *A. microphylla* on serum transaminases activities in sub-acute toxicity study.

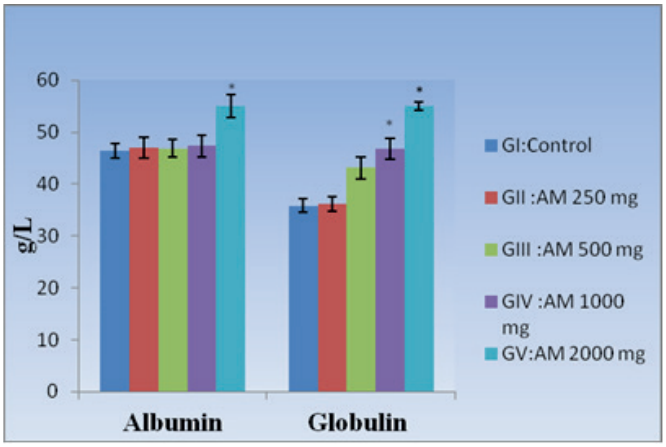



Values are expressed as Mean±SD for six animals; *= Significant (p<0.05) when compared to group I

**Figure 4.** Effect of ethanolic extract of *A. microphylla* LDH enzyme activities in sub-acute toxicity study.

## 4.5 Effect of Ethanolic Extract of *A. Microphylla* on Serum Albumin and Globulin Levels in Sub-acute Toxicity Study

The present sub-acute toxicity study shows no significant variation in the serum globulin levels from group-II to group-IV. However, albumin in group-V rats and globulin in group-IV & group-V rats, displayed a statistically significant increase while compared to control rats (Figure 5).

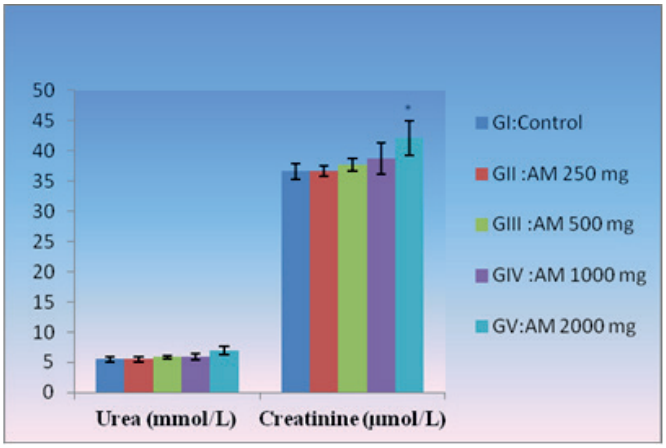



Values are expressed as Mean±SD for six animals; *= Significant (p<0.05) when compared to group I

**Figure 5.** Effect of the ethanolic extract of *A. microphylla* on serum albumin and globulin levels in sub-acute toxicity study.

## 4.6 Effect of Ethanolic Extract of *A. microphylla* on Serum Creatinine and Urea Levels in Sub- acute Toxicity Study

The toxicological analysis results under the present study indicate no evident rise in the concentration of creatinine (group-II to group-IV) and urea (group-II to group-V), when compared to the control rats (Figure 6). This is suggestive of the extract not disposing of any damage to rat kidneys between 250-1000 mg/kg b.w. concentrations. However, a statistically significant elevation of serum creatinine was evidenced in 2000 mg treated group of rats.

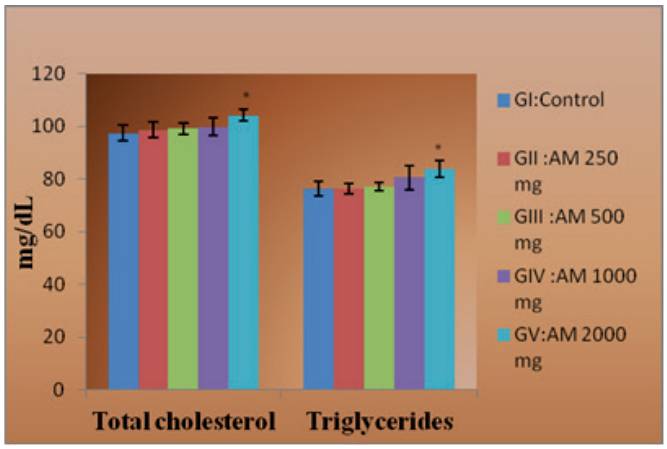



Values are expressed as Mean±SD for six animals; *= Significant (p<0.05) when compared to group I

**Figure 6.** Effect of the ethanolic extract of *A. microphylla* on serum urea and creatinine levels in sub-acute toxicity study.

### 4.7 Effect of Ethanolic Extract of *A. micro phylla* on Serum Cholesterol and Triglyc erides Levels in Sub-acute Toxicity Study

In the present study, the concentrations of the serum cholesterol and triglycerides were not altered significantly in the entire group of rats except group-V, in which a statistically significant but toxicologically insignificant change was observed (Figure 7).




Values are expressed as Mean±SD for six animals; *= Significant ($p<0.05$) when compared to group

**Figure 7.** Effect of the ethanolic extract of *A. microphylla* on serum cholesterol and triglycerides levels in sub-acute toxicity study.

### 4.8 Effect of Ethanolic Extract of *A. micro phylla* on Hematological Parameters in Sub-acute Toxicity Study

In the present sub-acute toxicity results, the hematological parameters were not altered significantly in the plant extract treated group (group-II to group-IV) when compared to group-I rats (Table 1). Nevertheless, a slight elevation in all the hematological parameters in group-V rats suggests that the extract possesses slight reactivity towards blood cells at 2000 mg/kg b.w. concentration.

### 4.9 Effect of Ethanolic Extract of *A. micro phylla* on Heart Histomorphology in Sub-acute Toxicity Study

The histopathological observations revealed no adverse effects on Azolla microphylla treated group myocardium (group-II to group-V) and appeared almost same as the control group (Figure 8). This is evidenced by the absence of necrosis and edema, lack of inflammatory cells infiltration and normal myofibrils.

**Table 1.** Effect of ethanolic extract of A. microphylla on hematological parameters in sub-acute toxicity study

| Parameters | Group I : Control | Group II : AM-250 mg | Group III : AM-500 mg | Group IV : AM-1000 mg | Group V : AM-2000 mg |
|---|---|---|---|---|---|
| Hemoglobin (g/dl) | 14.77 ± 0.62 | 14.35 ± 1.13 | 14.42 ± 0.51 | 14.76 ± 1.90 | 15.48 ± 1.96* |
| Total RBCs ($10^6$ /µL) | 6.75 ± 0.20 | 6.86 ± 0.5 | 7.19 ± 0.35 | 6.91 ± 0.33 | 7.25 ± 0.34* |
| Total WBCs ($10^3$/µl) | 8.17 ± 0.18 | 8.53 ± 0.43 | 8.64 ± 0.17 | 8.82 ± 0.20 | 9.09 ± 0.37* |
| Platelets count ($10^3$/µl) | 750.3 ± 6.29 | 750.35 ± 8.5 | 754.08 ± 8.4 | 760.28 ± 5.61 | 764.7 ± 4.12* |
| Monocytes (%) | 5.53 ± 0.35 | 5.73 ± 0.22 | 6.00 ± 0.34 | 6.60 ± 0.41 | 7.33 ± 0.56* |
| Lymphocytes (%) | 68.02 ± 1.02 | 68.68 ± 1.52 | 70.17 ± 1.16 | 69.53 ± 2.21 | 74.11 ± 1.81* |
| Neutrophils (%) | 16.95 ± 0.86 | 17.29 ± 0.31 | 17.52 ± 0.69 | 18.31 ± 1.03 | 21.78 ± 1.18* |

Values are expressed as Mean±SD for six animals; *= Significant when compared to group I

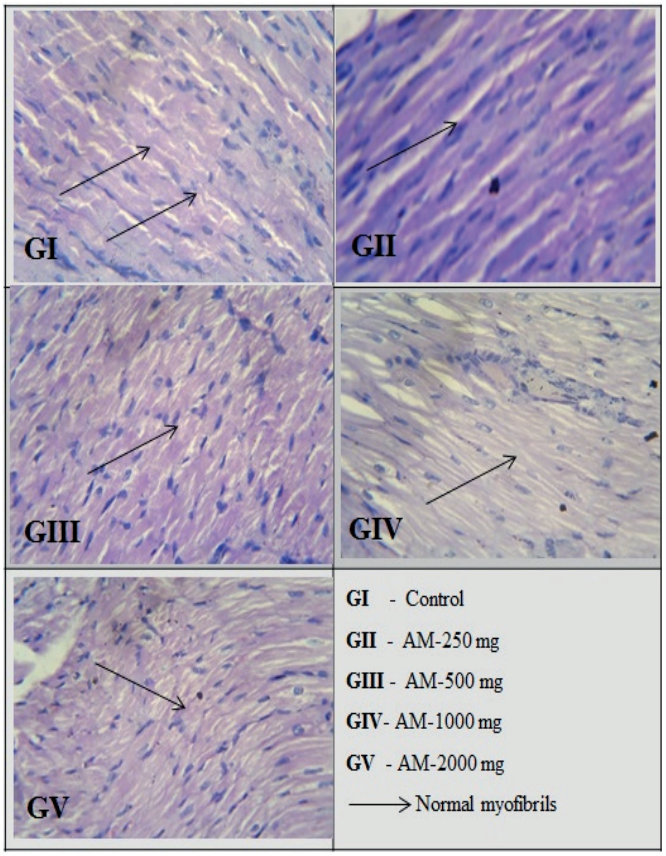



**Figure 8.** Histomorphological observations on heart tissues in sub-acute toxicity study.

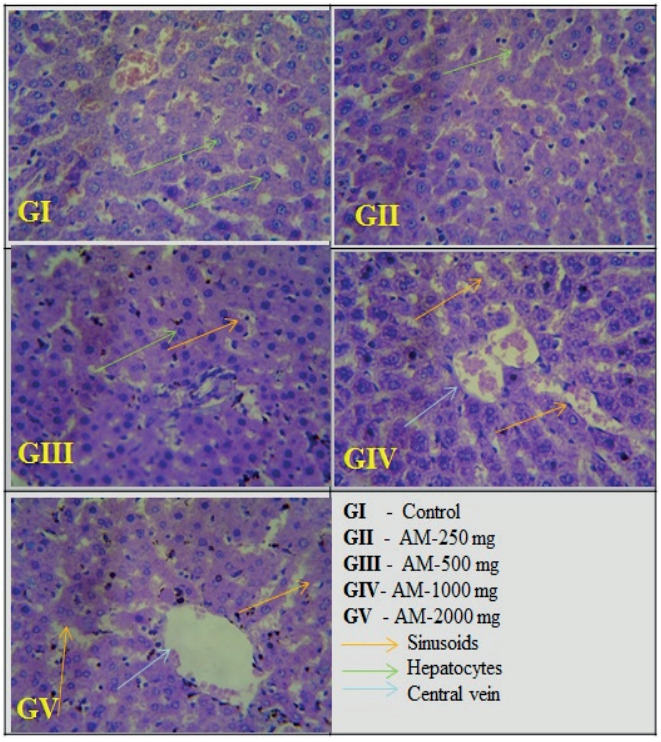



**Figure 9.** Histomorphological observations on liver tissues in sub-acute toxicity study.

## 4.10 Effect of Ethanolic Extract of *A. micro phylla* on Liver Histomorphology in Sub-acute Toxicity Study

The histopathological observations on the hepatic tissues in the present study indicate no adverse effects in the plant extract treated groups except group-IV & group-V, wherein, mild sinusoidal dilation was observed (Figure 9). Interestingly, the hepatocytes appeared normal with distinct cellular boundaries and absence of gross lesions or inflammatory reaction amongst all the groups when compared to the control rats.

## 4.11 Effect of Ethanolic Extract of *A. micro phylla* on Kidney Histomorphology in Sub-acute Toxicity Study

In the present investigation the Bowman's capsule, glomeruli, tubular epithelial cells, urinary space appeared normal from group-II to group-IV rats (Figure 10). The group-V rats also evidenced a similar anatomy but differed from the previous groups with an indication of mild blood vessel congestion.

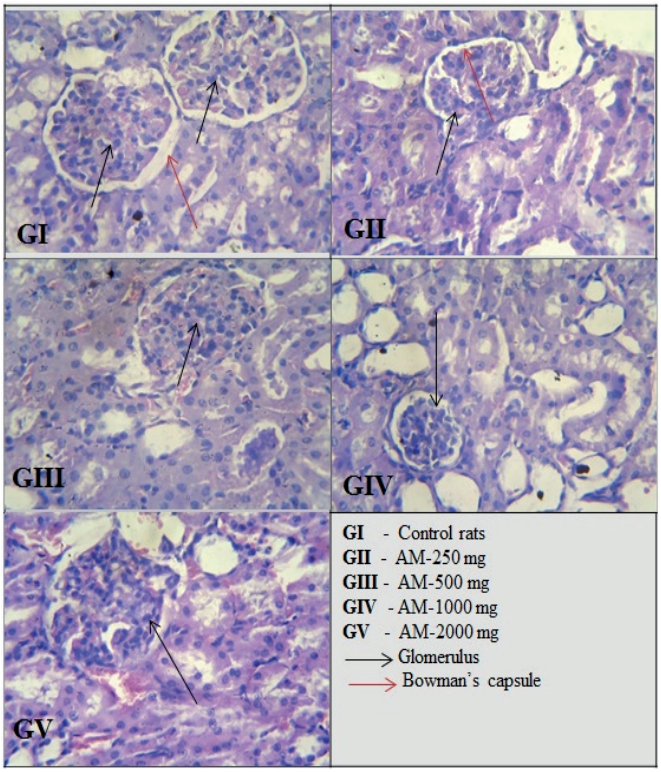



**Figure 10.** Histomorphological observations on kidney tissues in sub-acute toxicity study.

## 5. Discussion

In general, the acute toxic effects are observed soon after the single exposure of the drug, whereas, the sub-acute toxic effects are monitored over an extended period with/without repeated exposure of the same drug (at different concentrations) to relatively more number of animals. The acute toxic results suggest that the $LD_{50}$ of extract may be greater than 2000 mg/kg body weight. As per the Global Harmonized System (GHS) criterion, the herbal extracts with $LD_{50}$ greater than 2000 mg/kg b.w. are considered to be practically safe on acute exposure[14]. Since the plant extracts seldom shown acute toxic effects till 72 h, they are considered to be safe until 2000 mg/kg body weight.

Ideally, a significant reduction in food and water intake is suggested as a possible reason for intensive weight loss during the experimental period. The results of the present study are suggestive of the well-accepted diet and water by the rats treated with the extracts and had not imposed any serious metabolic alterations. The body weight change serves as a critical health indicator of animals[15]. The weight gain observed in group-II to group-V rats may be due to the unobstructed feed intake and optimal nutrient absorption from the extract. The trend in weight gain is directly proportional to the dosage administered.

A decrease in total protein is generally considered as a sign of the reduced synthetic function of the liver or a weakened hepatocellular function. The current findings are suggestive that the extract did not impose any damage to the hepatocellular/secretory functions of the liver at any of the doses tested. Prior work has shown that an increase in serum total protein levels may be due to conditions such as acute dehydration or increased protein synthesis in the liver[16]. As food and water were available throughout the experimental period, there was less chances for dehydration. Hence, the elevated protein concentration in group-V rats may be due to accelerated protein synthesis. Earlier works on *Eclipta alba* states the presence of coumestans, polypeptides, flavonoids, steroids, and terpenes caused a significant increase in the serum albumin levels of the rats[17]. Since, *A. microphylla* extract contains some of the aforesaid phytochemicals as well as a higher protein content, there is a better tendency to cause an elevation in the serum protein levels.

In general, the analysis of serum transaminases serves as a key segment whilst the toxicity evaluation of drugs and plant extracts. They are one of the prime indicators for liver and myocardial damage[18]. Increased activities of ALT and AST were reported in myocardial damage as well as during hepatotoxicity. An increase in serum LDH activity is an equally important biomarker to assess myocardial injuries. A statistically significant elevation but not a multiple fold increase of the serum transaminases are not directly linked to tissue injury, rather, it could be a non-specific reaction caused by inflammatory reactions and/or lysis of RBC during the processing of the blood[19]. The increase of enzyme activity in group-V rats might be due to aforesaid reason or due to some phytochemicals present in the extract that could have potential effect on the liver or heart at 2000 mg/kg concentration. Nevertheless, group-V rats neither shown weakened activity nor displayed any toxic effects until 14$^{th}$ day. In a previous attempt to identify the toxic effect of *Euphorbia hirta*, the authors evidenced no significant variations in the serum biochemical parameters and reported that the extract to be non-toxic until 90 days of oral administration[20]. The present study results are in harmony with their findings and can be considered to be non-toxic to rats till 2000 mg/kg body weight.

Many of the plant-based drugs are bio-transformed in the liver which might become toxic to the same and/or different organs. The hepatic protein synthesis function is best assayed by monitoring the levels of albumin and globulin. Albumin participates in the transportation of proteins while globulins take part in defining the immunological status of the animals. The ratio of albumin to globulin is very important to determine the health condition of animals. The ratio of albumin to globulin in the present study was in the range of 1-1.3 (data not shown). Generally, the A/G ratio decreases proportionally with the increase in age[21]. No significant variation in the ratio was observed among the entire groups. The increase in globulin levels observed in group-V rats may be due to the immunomodulatory effect of the plant extract. Endogenous biological molecules such as corticosteroids and thyroid hormones are known to stimulate albumin synthesis in hepatocytes[22].

Basically, any dysfunctions in the kidneys are explored by monitoring the serum levels of urea, creatinine and uric acid in addition to other biomarkers. Generally, elevated levels of serum creatinine and urea indicate damage caused to the kidney. The cortex and glomerulus are the major regions susceptible to toxic insults, as they assist in maintaining the excretion of creatinine and urea[23]. Physiologically synthesized creatinine directly passes into the circulation towards kidneys for excretion[24]. Their retention in the blood is a representation of kidney impairment. Urea is the breakdown product of protein metabolism. Previous reports suggest excess consumption of protein rich diet causes elevation of urea in the serum[25]. The increase in the serum creatinine level in group-V rats may be probably due to the reduced excretion from blood. Thus the findings of the study establish the non-toxic nature of the extract to the functioning of the kidneys in a dose-dependent manner.

The compounds capable of inducing an abnormal increase in the levels of total cholesterol and triglycerides are one of the most prominent risk factors of myocardial infarction. The herbal extracts triggering elevation of the serum lipid levels are considered to be potentially harmful. Thus any considerable elevation in the serum levels of cholesterol and triglycerides provides vital information on the modification of lipid metabolic pathway and the susceptibility towards atherosclerosis[26]. An abnormal increase in cholesterol would lead to hepatic cholesterol accumulation which is not observed in the present experimental rats. However, it appears that the 2000 mg/kg b.w. of the extract may cause an elevation in the lipid levels upon chronic treatment, which is presumably due to decreased cholesterol utilization or increased synthesis. The increase in the serum triglycerides concentration in group-V rats might be due to accelerated lipolysis or decreased fatty acid utilization. The results obtained from group-II to IV are in concordance with previous findings, wherein, the serum triglycerides and cholesterol levels remained unchanged during sub-acute toxicity study of *Hippobromus pauciflorus* extract in rats[27]. Thus it is inferred that *A. microphylla* extract at higher dosage (2000 mg/kg b.w.) has mild hyperlipidemic effect in rats.

It is to be emphasized here that herbal extracts affecting the erythropoiesis are capable of RBC lysis, immunosuppression, promotes allergic response, wherein, the hematological parameters varies considerably[28]. Hence, the following hematological parameters like RBC/WBC, platelets, monocytes, lymphocytes and neutrophils are the key factors to be tested while assessing the toxicity potential of the herbal extracts. Moreover, in certain situations like an unusual high protein diet, the body produces an excess number of RBCs and WBCs to meet the requirement of mature cells to stabilize the compounds in the extract, which could also be a reason for the observed slight rise on hematological parameters in group-V rats. Since blood cells are originated through erythropoiesis, the unresponsiveness to the extract on hematological parameters shows that the erythropoiesis is not adversely affected during the testing period, apparently reinstates the safe nature of the plant extract for further experimental studies.

In general most of the cardiotoxic agents cause coagulative necrosis, myofibrillar damage, edematous space, inflammatory cells infiltration, loss of membrane integrity and destabilization on the myocardium[29]. These changes can be monitored while doing the histopathological assessment. However, the present histopathological investigation didn't report any significant structural alterations. Generally, damage to myocyte results in the leakage of myocyte marker enzymes, consequently, an elevated level in the serum. Thus the mild elevation of serum transaminases and LDH enzymes observed in 2000 mg treated group may not be associated with the damaged myocytes. The insignificant changes of the biochemical parameters among the test groups indicate the non-toxic nature of the plant extract to the rat myocardium until 2000 mg/kg body weight, which is further supported by the undisturbed myocardial architecture.

The toxicology studies must be always coupled with suitable histopathological evaluation as they provide anatomical localization of the action of the extract[30]. Liver is one of the predominant organs susceptible to intensive stress during the toxicity analysis as they are involved in the biotransformation of various metabolites. The obstruction in the venous blood flow causes the sinusoids to dilate and represent a pathological state in two-thirds of the hepatic complications[31]. The mild sinusoidal dilation reported in the present study can be attributed to the biotransformation and processing of biological constituents of the extract. Thus the histopathological findings in the liver tissue suggest that at higher doses (>1000 mg/kg) the extract has some minor disturbing effects on the rat hepatocytes. However, until 1000 mg/kg b.w. concentration, the extract is safe for oral administration to rats which corroborates with the biochemical findings.

Normally the damage to the kidney is associated with degeneration of tubular epithelial cells, glomeruli congestion, infiltration of inflammatory cells, necrosis, edema and blood vessel congestion[32]. The observed vascular congestion in the 2000 mg extract treated rats (group-V) may be due to the formation of renal stones or due to the excess concentration of therapeutically active compound at this particular dosage eliciting prooxidant activity. Indeed the dosage and the length of time have clear correlation with the prooxidant activity of compounds present in the herbal extract. Earlier studies indicate that potential drugs like catechins, epicatechin and gallic acid induce oxidative stress at higher concentration[33]. The presence of excess quantities of similar phytochemicals in the extract at higher doses may be the reason for the observed blood vessel congestion in group-V rats. Interestingly, all the other concentrations of the extract didn't stimulate kidney damage and hence, suggested to be safe for pharmacological applications.

## 6. Conclusion

In acute toxicity study, the oral administration of a single dose of 250 and 2000 mg/kg b.w. of the ethanolic extract of *A. microphylla* exhibited no mortality/toxic signs upto 72 h and thus reflected the absence of acute toxic effects in rats. Furthermore, the sub-acute toxicity studies testing

four different concentrations (250, 500, 1000 & 2000 mg) for 14 days indicated no major changes until 1000 mg/kg body weight. However, a slight increase in the biochemical, hematological and histomorphological changes were observed in group-V rats treated with 2000 mg/kg b.w. of the extract. This observed rise in the tested parameters may be due to the hyper-concentration administration of biologically active substances in a stipulated short period of time, subsequently culminating in minor damages to the liver and kidneys. However, no adverse effects were noted in the histomorphology of the heart among the entire groups of rats. Thus, the sub-acute toxicity results suggest that the extract is non-toxic and safe to rats between 250-1000 mg/kg b.w. concentration under 14 days observational period. In addition, as there was no mortality upto 2000 mg/kg b.w., 50% lethal dose ($LD_{50}$) could not be determined, and hence it is presumed to be greater than 2000 mg/kg b.w./day. Further to the above findings, it is concluded that ethanolic extract of *Azolla microphylla* whole plant is non-toxic to rats in all the tested doses herein. Thus the present study results on this plant are suggestive of its non-toxic nature and can be used safely used in the intervention of human diseases.

## 7. Acknowledgements

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## 8. References

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