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Study of the acute oral toxicity of methanol extract of aerial parts from *Marsilea quadrifolia* Linn

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ABSTRACT

Marsilea quadrifolia Linn is a pteridophyte belonging to Family Marsileaceae commonly known as European water clover. *M. quadrifolia* is used to treat snake bite, cough, bronchitis, diabetes, psychiatric diseases, eye diseases, diarrhoea and skin diseases. Objective of the study is to identify a dose causing major adverse effects and an estimation of the minimum dose causing lethality, according to regulatory guidelines (OECD 425). Female Swiss Albino mice weighing 25-30 gm, aged 56 to 70 days were chosen for the study. Mice were divided into two groups (n=10/group). Group I served as control and treated with Saline. The Group II received methanol extract of *M. quadrifolia* orally ranging from 175 to 2000 mg/kg body weight by using oral feeding needle sleeved on to disposable syringe. They were kept in individual polypropylene cages provided with clean bedding of rice husk.. There was no mortality or behavioural changes observed in treated animals. All the animals belonging to the treated group survived throughout the 14 days observation period after dosing. The data revealed that LD_{50} of the extract was greater than 2000 mg/kg b.w. There was no significant variation found in body weight and organ to body mass index. In comparison with control group, there was significant increase in levels of ALT, AST,Bilirubin, total proteins, globulin levels, urea, cholesterol, triglycerides, LDL, platelet count, MCV, MCH, WBC count and lymphocytes whereas ALP and MCHC levels were reduced significantly. No significant changes were observed in HB, total RBC, total bilirubin, albumin. The methanol extract of aerial parts from *Marsilea quadrifolia* Linn had nontoxic effect on the biochemical and haematological parameters studied up to a dose of 2000 mg/kg. The lethal dose is therefore over 2000 mg/kg.

Keywords: *Marsilea quadrifolia,* Methanol, Albino mice, Snake bite

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INTRODUCTION

Toxicology is like science and an art like medicine. It includes observational data assembly & data utilization to expect outcome of exposure in human and animals. Several studies are resolute on toxicity analysis so as to determine the safeness of medicinal plants and their products. Toxicity analysis is essential, as some herbs consumed might have some toxic effects and many reports have been available for toxicity caused due to long term consumption of herbs. Herbs are alternative medicines for treatment of various diseases due to their unspecified adequacy, effectiveness, affordability, wellbeing and low cost[1]. There is also an rising increase in the consumption of herbal formulations by the public because of the strong confidence that these products are natural; hence, they are harmless for the treatment of ailments[2].M. quadrifolia Linn is an aquatic fern belongs to the family (Marsileaceae) commonly named has Niraaaikkirai, Aaraikeerai in Tamil, European water clover and Four leaf clover in English, Neeraral in Malayalam and Cauptiya, Sunsuniya in Hindi. It is widely distributed in tropical and temperate regions of world and found throughout India, in marshy places and along the banks of canals and rivers. M. quadrifolia is also eaten by various tribal communities such as Kadar's, Pulaiyars, Malasars, Malaimalasars, Mudhuvars of Anamailais hills, Western Ghats, Coimbatore district Tamil Nadu, India as per seasonal availability. Juice made from the leaves is diuretic and febrifuge and also used to treat snake bite and applied to abscesses etc.The plant is anti-inflammatory, diuretic, depurative, and febrifuge and refrigerant. Plants pacifies vitiated pitta, cough, bronchitis, diabetes, psychiatric diseases, eye diseases, and diarrhoea and skin disease[3].

MATERIALS AND METHODS

Collection of plant material

Fresh aerial parts of *M. quadrifolia* Linn were collected from field of anthiyur and athani region of erode district of Tamilnadu, India and authenticated by DR.P. Jayaraman PhD,

Director, Plant anatomy research, Chennai, Tamilnadu, India and a voucher specimen no PARC/2014/2119. Voucher specimen(No:JKKMCP/0102/14) has been deposited in the Department of Pharmacognosy, J.K.K Sampoorai Ammal College of Pharmacy, Komarapalayam, Tamilnadu, India.

Extraction

Coarsely powdered shade dried aerial parts of the plant *M. quadrifolia* Linn Soxhlet extractor was used for the preparation of the extracts. Successive solvent system was used for the extraction. The dried powdered material of aerial parts of M. *quadrifolia* Linn were loaded in thimble of soxhlet extractor and extracted with petroleum ether, ethyl acetate, acetone, methanol with increasing order of their polarity. A minimum of 60 cycles of siphoning was done for successive solvent, and the process was continuous for 72 hrs until the solvent in the extractor siphon tube became colourless. Extracts were concentrated at reduced pressure in a rotary vacuum evaporator and refrigerated until further use.

Acute toxicity studies

Objective of the study is to identify a dose causing major adverse effects and an estimation of the minimum dose causing lethality, according to regulatory guidelines (OECD 425)[4]. Female Swiss Albino mice weighing 25-30 gm, aged 56 to 70 days were chosen for the study. Mice were divided into two groups (n=10/group). Group I served as control and treated with Saline. The Group II received methanol extract of *M. quadrifolia* Linn (MEMQ) orally ranging from 175 to 2000 mg/kg body weight by using oral feeding needle sleeved on to disposable syringe. They were kept in individual polypropylene cages provided with clean bedding of rice husk. They were acclimatized for five days prior to dosing under standard housing conditions (temperature: 25 ± 2°C, relative humidity: between 30 and 70% with optimal air changes per hour and 12 h each of dark and light cycle) and provided with standard pelleted feed and U.V. treated water *ad libitum*. The experimental protocol was approved by Institutional animal ethical committee (IAEC), JKKMRFCP/IAEC/2015/01.

Biochemical & Haematological analysis

Urea, cholesterol, creatinine, triglyceride, high density lipoprotein (HDL), low density lipoprotein (LDL), very low density lipoprotein (VLDL), bilirubin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphate[ALP],total protein, albumin, globulins were measured. The blood samples from animals (both treated and vehicle control groups) were quiet in EDTA containing spouts for hematological study. hemoglobin (Hb), total RBC, packed cell volume (PVC), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelet count, white blood cells (WBC) count, neutrophils (N), lymphocytes (L), monocytes (M), and eosinophils (E) were determined with humalyzer.

Histopathological study

The vivacious organs Liver, Lungs, Spleen, Kidney, Pancreas and Brain isolated from sacrificed mice were fixed in 10% formalin, then after processing embedded in paraffin wax. Paraffin sections were made at 5 mm and stained with hematoxylin and eosin. The slides were studied under a light microscope and captured the magnified images of tissues structure for further study.

Statistical analysis

The data are presented as mean ± SEM using SPSS (Version 16.0) software. The obtained data was subjected to compare means of paired sample student's t test.

RESULT

Acute toxicity test

There were no toxic symptoms or mortality observed in treated animals. All the animals belonging to the treated group survived throughout the 14 days observation period after dosing. The data revealed that LD_{50} of the extract was greater than 2000 mg/kg b.w.

Behaviour parameter

No behavioural changes of fur & skin, Eyes [Exophthalmus & Lacrimation], salivation, urination, Faeces consistency was observed of the control and methanol extract treated animals. Respiration rate was elevated for first 30 mins and also an increase in motor activity was observed frequently in first 24 hrs and then up to 72 hrs. Grip strength was decreased first 4 hrs and then increased interval between 24 hrs then normalized throughout the study period Table-1.

Table 1: Effect of methanol extract of *M. Quadrifolia* on Behavioural test in mice

S. No.	Parameters	Observations											
		30 mins		4hr		24hr		48 hr		7th day		14th day	
		C	T	C	T	C	T	C	T	C	T	C	T
1.	Fur & skin	N	N	N	N	N	N	N	N	N	N	N	N
2.	Eyes (Exophthalmos, Lacrimation)	N	N	N	N	N	N	N	N	N	N	N	N
3.	Salivation	N	N	N	N	N	N	N	N	N	N	N	N
4.	Respiration	N	Increased	N	N	N	N	N	N	N	N	N	N
5.	Urination	N	N	N	N	N	N	N	N	N	N	N	N
6.	Faeces consistency	N	N	N	N	N	N	N	N	N	N	N	N
7.	Diarrhoea	A	A	A	A	A	A	A	A	A	A	A	A
8.	Sedation	A	A	A	Little Drowsy	A	A	A	A	A	A	A	A
9.	Sleep	A	A	A	A	A	A	A	A	A	A	A	A
10.	Loss of righting reflex	A	A	A	A	A	A	A	A	A	A	A	A
11.	Writhing	A	A	A	A	A	A	A	A	A	A	A	A
12.	Itching	A	P	A	A	A	A	A	A	A	A	A	A
13.	Straub phenomena	A	A	A	A	A	A	A	A	A	A	A	A
14.	Piloerection	A	A	A	A	A	A	A	A	A	A	A	A
15.	Motor activity	N	Increased	N	Increased	N	Increased	N	Increased	N	Increased	N	N

16.	Grip strength	N	Decreased	N	Decreased	N	Increased	N	N	N	N	N	N
17.	Convulsion	A	A	A	A	A	A	A	A	A	A	A	A
18.	Tremor	A	A	A	A	A	A	A	A	A	A	A	A
19.	Coma	A	A	A	A	A	A	A	A	A	A	A	A
20.	Mortality	A	A	A	A	A	A	A	A	A	A	A	A

C- control, T- Extract treatment, N = Normal, P = Present, A = Absent

Body weight and organ to body weight

The body weight and organ to body weight index of the both control as well methanol extract treated group was no significant changes throughout the study was summarized in Table-2, 3.

Table 2. Effect of methanol extract of *M. Quadrifolia* on body weight of mice at different days.

Groups	1st day Body weight (gm)	7th day Body weight (gm)	14thday Body weight(gm)
Normal control	29.66 ± 0.21	30.83± 0.30	30.16± 0.30
2000mg/kg MEMQ	30.16 ± 0.30	31.16± 0.47	30.66± 0.42

Values are expressed as Mean ± SEM; n=10.

Table 3. Effect of methanol extract of *M. Quadrifolia* on organ to body weight in mice

Groups	Liver	Heart	Kidneys	Brain	Spleen
Normal control	3.21±0.00	0.25±.006	1.06±0.006	2.54±.007	0.12±0.006
2000mg/kg MEMQ	3.12±0.00	0.33±.006	1.08±0.004	2.42±.005	0.12±0.006

Values are expressed as Mean ± SEM; n=10.

Biochemical & Haematological analysis

In comparison with control group, there was significant increase in levels of ALT, AST, total proteins, globulin levels, urea, cholesterol, triglycerides, LDL, platelet count, MCV, MCH, WBC count and lymphocytes whereas ALP and MCHC levels were reduced significantly. No significant changes were observed in HB, total RBC, total bilirubin, albumin was calculated and summarized in Table-3-7.

Table 4. Effect of methanol extract of *M. Quadrifolia* on renal functions in mice

Groups	Creatinine(serum)mg/dl	Urea(serum) mg/dl
Normal control	0.45±0.042	11.16±0.307
2000mg/kg MEMQ	0.46±0.025	12.16±0.307

Values are expressed as Mean ± SEM; n=10.

Table 5. Effect of methanol extract of *M. Quadrifolia* on liver functions test in mice

Groups	ALT U/L	AST U/L	ALP U/L	Bilirubin mg\dl	Protein g/dl	Albumin g/dl	Globulins g/dl
Normal control	205±0.307	310±0.365	154±1.085	0.85±0.003	5.68±0.600	4.3±0.030	2.0±0.080
2000mg/kg MEMQ	211±0.477	320±0.421	111±0.577	0.86±0.004	6.10±0.036	4.3±0.031	2.1±0.071

Values are expressed as Mean ± SEM; n=10.

Table 6. Effect of methanol extract of *M. Quadrifolia* on lipid profile test in mice

Groups	Cholesterol mg/dl	Triglycerides mg/dl	HDL mg/dl	LDL mg/dl	VLDL mg/dl
Normal control	160 ±0.981	121±0.851	29±0.501	105±1.101	22±0.301
2000mg/kg MEMQ	171±1.100	135±0.981	32±0.307	125±0.925	27±0.542

Values are expressed as Mean ± SEM; n=10.

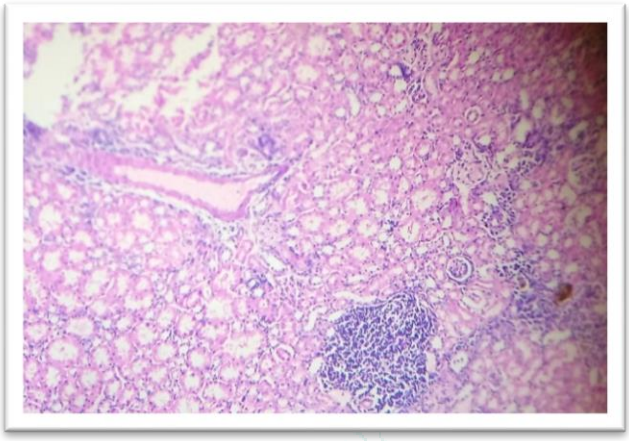
Table 7. Effect of methanol extract of *M. Quadrifolia* on Haematological test in mice

Groups	Hb g/dl	RBC 10^6/µl	Platelet Count 10^{12} /L	MCV [Fl]	MCHC [g/dl]	MCH [Pg]	WBC [10^9/L]	NP %	EP %	LC %	MC %
Normal control	11.8± 0.051	7.8± 0.271	237± 1.210	48.2± 0.041	32.1± 0.210	15.2± 0.541	3.5± 0.124	11± 0.031	1± 0.002	84± 0.974	3± 0.151
2000mg/kg MEMQ	11.9± 0.023	7.9± 0.081	250± 1.920	52.4± 0.982	27.2± 0.810	15.6± 0.021	5.9± 0.189	11.3± 0.052	2± 0.001	89± 1.10	4± 0.500

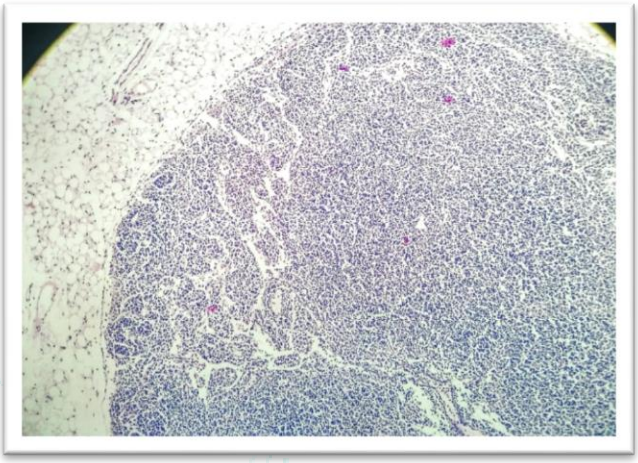
Values are expressed as Mean ± SEM; n=10.

Histopathological examination

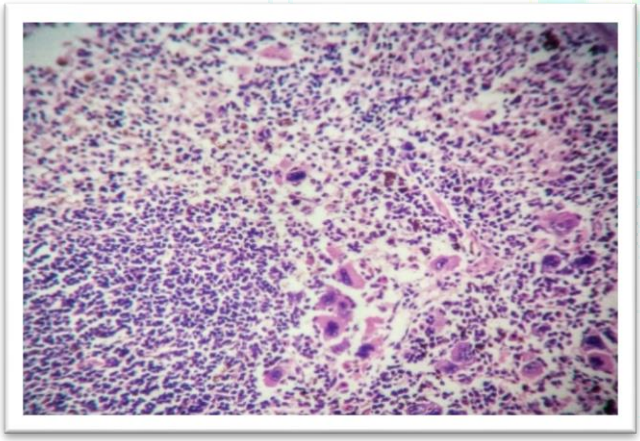
Section A: Microscopic examination of Kidney of normal control mice showing clear. Section A_1: Methanol extract (2000mg/kg) section show kidney with focal interstitial dense lymphocytic infiltration. Tubules and glomeruli are normal. Section B: Microscopic examination of Lymphnode of normal control mice showing clear. Section B_1: Methanol extract (2000mg/kg) section show lymphnode with surrounding adipose tissue. Lymphnode show no pathology. Section C: Microscopic examination of Liver of normal control mice showing clear. Section C_1: Methanol extract (2000mg/kg) section show liver tissue with mild increase in reticuloendothelial cells. Section D: Microscopic examination of Lung of normal control mice showing clear. Section D_1: Methanol extract (2000mg/kg) section shows many respiratory and terminal bronchioles have dense lymphocytic infiltration in the surrounding stroma. Section E: Microscopic examination of Spleen of normal control mice showing clear. Section D_1: Methanol extract (2000mg/kg) section show spleen with marked increase in hematopoietic cells including megakaryocytes (Fig-1).



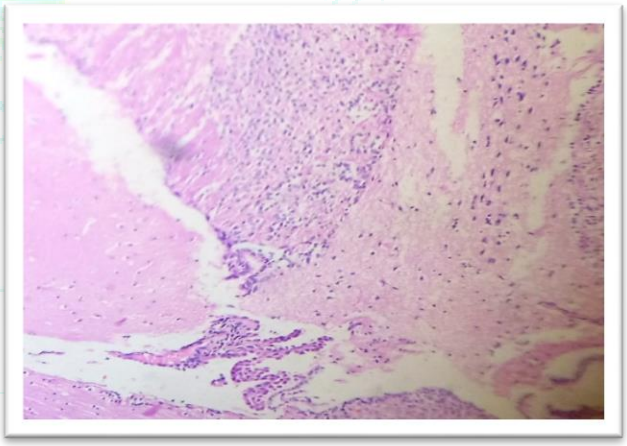
A. Normal Kidney



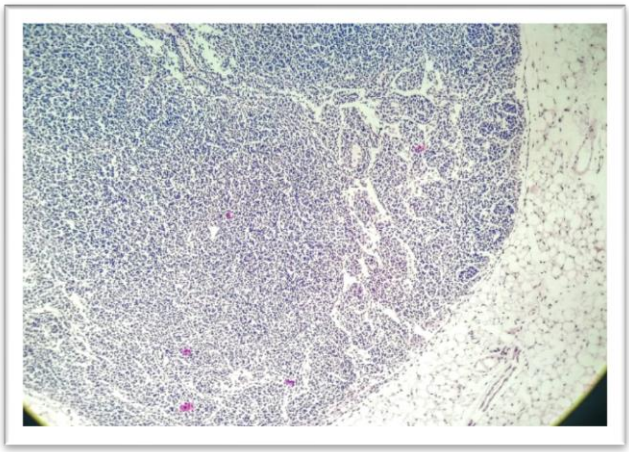
B_1. Treated Lymphnode



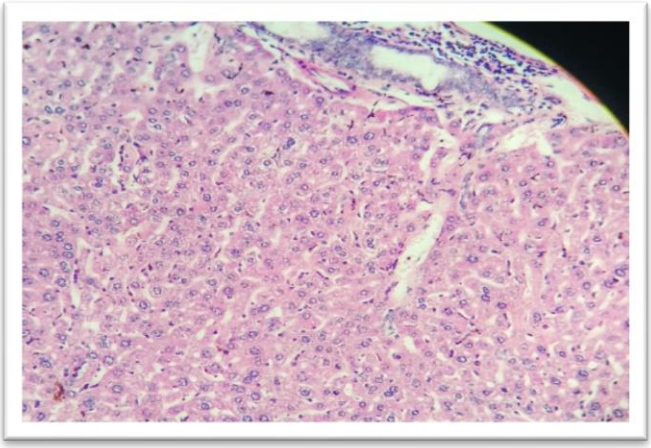
A_1. Treated Kidney



C. Normal Liver

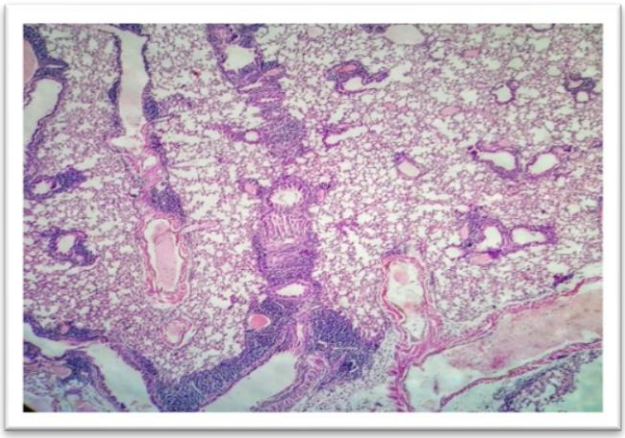


B. Normal Lymphode

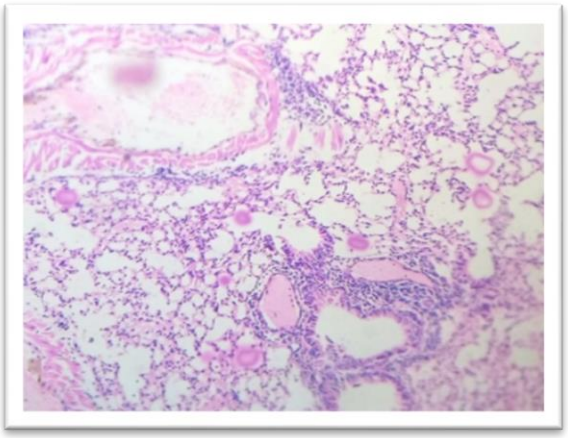


C_1. Treated Liver

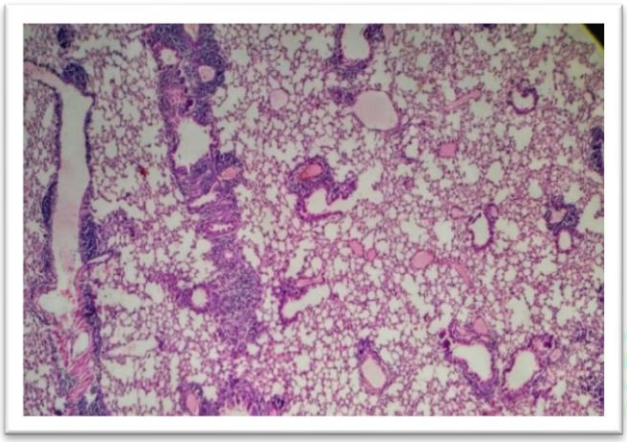
Figure 1(A): Histopathological examination of control and methanol extract treated groups.



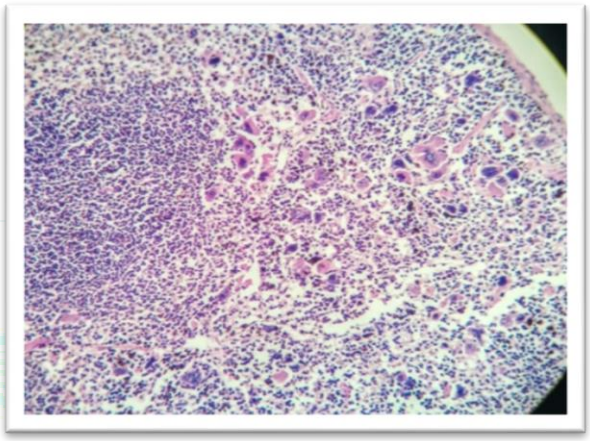
D. Normal Lung



E. Normal Spleen



D_1. Treated Lung



E_1. Treated Spleen

Figure 1(B): Histopathological examination of control and methanol extract treated groups.

DISCUSSION

Natural compounds take an eminence role in therapeutic relevance because of seldom adverse effects. Yet these bioactive compounds from medicinal plants are traditional to be safe without any prejudicial health effect, and thus extensively used as self-medication[5]. Don't have of adverse effects of these natural compounds and scientific validation on the toxicity. Therefore, in direction of acute oral toxicity study is to a great extent needed, will not only help to identify the choice and concentration of dose that could be used subsequently, but also to reveal the possible clinical signs elicited by the substances under investigation. Therefore in addition it is also a very able parameter to do research the therapeutic index of drugs and xenobiotics[6]. In this oral acute toxicity study, the Swiss albino mice were utilized to perceive the toxicity effects of the methanol extract of aerial parts from *M.Quadrifolia* Linn In suspect on historical research, the oral route administration is the greatest convenient and generally used one when studying acute toxicity. When compare to rats, the mice shown that better prediction for human acute lethal dose[7]. In this study, the mice in the control and treated groups were administrated with vehicles and methanol extract, respectively. The mice were monitored daily as far as 14 days for any toxic signs and mortality. The scientific symptom is one of the major important observations to indicate the toxicity effects on organs in the treated groups[8]. It was observed that the period of 14 days & evaluation of acute toxicity, it intake of food and water were normal with non- significant body weight variations. No animal was found dead were observed in treatment group in first 24 h , but some changes found in behavioural pattern respiration rate was elevated for first 30 mins and also an increase in motor activity was observed frequently in first 24 hrs and then upto 72 hrs. Grip strength was decreased first 4 hrs and then increased interval between 24 hrs then normalized throughout the study period. To it, the physical appearance was found to be normal such as fur, skin and eyes were found to be normal. Commonly, the alterations of body weight gain and internal organ weights of mice would reflect the toxicity after exposure to the toxic substances[9].Organ weight also is an important index of physiological and pathological status in animals and whilst the body weight and organ of body weight index of mice showed as normal. So it indicates that the administration of the methanol extract has negligible level of toxicity on the growth of the animals. The food intake and water consumption is important role in the study of safety of a product with therapeutic purpose, as regular intake of nutrients is essential to the physiological status of the animal and to the accomplishment of the proper response to the drugs tested[10,11]. In this study, the food intake and water consumption also was good, didn't induce appetite suppression and other effects, this indicates there was no disturbance in metabolism. Liver injury caused by hepatotoxic drugs can result in eminent AST, ALT[12]. ALP is most often measured to indicate bile duct obstruction[13].In this study, significant increase of AST,ALT, total proteins and globulin as well as significant decreases in the ALP and also

no changes in albumin attention might be an proposal that the methanol extract of *M. Quadrifolia* have hepatoprotective. In this study lipid profiles also observed increased levels of cholesterol, triglycerides, LDL, VLDL and HDL were found in the methanol extract group could suggest a hyperlipedemic effects. Serum levels of urea and Creatinine were shown to be a clinical value that denotes renal impairment[14]. Urea is formed by deamination of amino acids in the liver and then it is transported by blood to the kidneys, where it is excreted with urine. Creatinine is a waste product that is normally filtered from the blood and excreted with urine. Increased in creatinine level in response, it indicates renal diseases and may be a result if impaired glomerular function[15,16]. The results from this study, serum urea and creatinine, only urea levels were found mild elevated, but histopathological evaluation shows tubules and glomeruli are normal. This may perhaps be a suggestion that the methanol extract did not affect with the capacity of kidney to excrete these metabolites. So above manifest that the methanol extracts 2000mg/kg did not cause renal impairment or kidney damage. The haematopoietic system is one of the utmost sensitive targets for noxious compounds and an important index of physiological and pathological status in man and animal[17]. In this study, significant increase in levels of platelet count, MCV, MCH, WBC count and lymphocytes. Whereas MCHC levels were reduced significantly. No significant changes were observed in HB, total RBC. The severity of the causative stress condition may be attributed to an increase in leukocyte mobilization[18] .Some of these compounds such as alkaloids and flavonoids are most common plant metabolites that widely affect immune system in various ways. According to[19] Flavonoids are able to activate immune cells such as mast cells, Basophils, neutrophils, eosinophils, Total globulin lymphocytes, macrophages. We alleged that methanol extract caused a significant increase in the level of WBC. The WBC value in present study showed an immune system to protect the mice against infection that might have been affected by chemical and also secondary infections.

CONCLUSION

The results of this study suggested that the aerial parts of methanol extract from *M.Quadrifolia* Linn up to the dose of 2000mg/kg b.w is safe, when administered orally to female mice.The histology examination enactment no changes in the structure of internal organs in both control and methanol extract treated group. This could be an affirmation for the medicinal use of *M.Quadrifolia* Linn in folk medicine. However, the preliminary results suggested that it should be further expected for long term use and repeated dose effects to ensure safety of this plant.

Conflict of Interest

The authors have no conflict of interest

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