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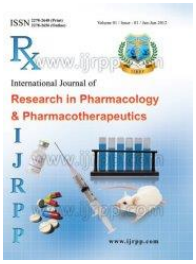

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Acute and subchronic toxicity studies of Limnophila heterophylla and Michelia champaca

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ABSTRACT

In an attempt to mobilize indigenous healthcare knowledge, acute and sub chronic toxicity studies were performed on the methanol extracts of leaves of *Limnophila heterophylla* and *Michelia champaca*. In the acute toxicity study, a single oral dose (2000 mg/kg) of the methanol extracts of leaves of *Limnophila heterophylla* (MELH) and *Michelia champaca* (MEMC) was administered independently to rats and observed for 14 days meant for signs of acute toxicity. In sub chronic toxicity study, rats were administered with MELH & MEMC separately (250 and 500 mg/kg) daily for three months. Biochemical and haematological analysis were carried out on blood samples at the end of the treatment. The results showed that administration of 2000 mg/kg of MELH and MEMC to animals did not produce any mortality. In biochemical and haematological analysis**,** no significant differences between control and test animals was observed. The findings suggested that methanol extracts of *Limnophila heterophylla* and *Michelia champaca* were well tolerated both in acute and sub chronic toxicity studies.

Keywords: *Limnophila heterophylla*, *Michelia champaca*, Biochemical, Haematological, Sub chronic toxicity.

INTRODUCTION

Natural products including herbs, animals and minerals function the lead compounds for the event of recent medicines and conjointly for the treatment and prevention of varied human ailments. This accepted trendy drugs has bit by bit developed within the recent years by varied efforts done by the researchers. However, ancient drugs still remain as basis within the development of recent medication [1]. In recent years, herbs and herbal medicines have continued to receive interest and a focus from the individuals as these products are safe and free from side effects [2]. The growing range of herbal drug users round the globe and lack of scientific information on the protection profile of herbal drug build it necessary to conduct toxicity study of herbal drug [3]. Toxicity related to herbal products has alerted several national and international restrictive authorities to develop and implement varied set of pointers for assessing, observance and preventing the toxicity related to the herbal products. For instance, Uppsala observance committee (UMC) of the World Health Organization (WHO) collates and communicates data relating to herbal adverse drug

reactions whereas Organization for Economic Cooperation and Development (OECD) sets pointers for conducting varied toxicity studies.

Toxicity tests are most generally wont to examine specific adverse effects or specific endpoints like cancer, cardiotoxicity and skin/eye irritation. Toxicity testing conjointly useful in crucial the No Observed Adverse Effect Level (NOAEL) dose and is useful for additional clinical trials [4]. Acute and sub chronic toxicity tests square measure routine toxicity tests dispensed by the pharmaceutical companies within the development of new medicines. So as to assess the virulent nature of a compound, acute oral toxicity is that the opening to be dispensed [5]. Acute toxicity testing involves the determination of dose, the dose that kills fifty % of the tested cluster of animals, whereas sub chronic toxicity checking involves the determination of long run effects of the test compound upon perennial administration. In view of developing improved ancient medicines (ITM's) that square measure cheap, safe, economical and user- friendly or the identification of plant based mostly drug targets, it's projected that diagnosing testing methods of botanicals ought to begin with the *in vivo* examination of extracts in relevant animal models to substantiate the ethno pharmacological/ ethnopharmaceutical use [6].

Exhaustive analysis relating to isolation of additional phytochemicals and medicine study on this healthful plants continues to be necessary therefore on explore the plant relating to its healthful importance.

Limnophila heterophylla is an aquatic herb, mainly submerged, but with shoots that often emerge above the water surface, rooting at nodes. The plant finds lot of applications in the traditional system of medicine to treat wounds [7]. Different parts of *Limnophila heterophylla* possess varied pharmacological activities like COX inhibitor [8], antimicrobial [9] and wound healing [10]. The plant encloses terpene, flavanoids, terpinoids and oils [11]. *Michelia*, known by the scientific name *Michelia champaca*, is a very tall tree that grows up to 30m tall. *Michelia champaca* is used ethnomedically for the handling of astringent, constipation, dyspepsia, dysmenorrhea, fever, febrifuge, nausea, stomachic, skin disease, tonic, ulcers and wounds [12]. Earlier pharmacological reports of *Michelia champaca* had demonstrated its cytotoxic activity [13], anti-inflammatory [14], antihyperglycemic [15], leishmanicidal [16], antibacterial [17], wound healing [18], diuretic [19], antiulcer [20], antifertility [21], antihelmintic [22] and cardio protective [23] activities. Several phytoconstituents like alkaloids, flavonoids, triterpenoids, saponins, tannins, sterols and steroids have been isolated from different parts *Michelia champaca*.

This study was undertaken to judge the acute and sub chronic oral toxicity of the methanol extracts of leaves of *Limnophila heterophylla* and *Michelia champaca* on some biological and metabolic parameters in rats.

MATERIALS

Plant materials

The plants were collected from Tirupati (Andhra Pradesh), India and further, plants were distinguished, affirmed and validated by Dr. Madavchetty, Professor, Botany office, Sri Venkateswara University, Tirupati. Voucher specimen of these plants (*Limnophila heterophylla* - GIP006/2013-2014 & *Michelia champaca*- GIP005/2013-2014) have been kept in the GITAM Institute of Pharmacy, GITAM University, Visakhapatnam, Andhra Pradesh, India.

Extraction

Leaves of *Limnophila heterophylla* and *Michelia champaca* were dried under shade and then powdered with a mechanical grinder to obtain a coarse powder. Powder was passed through 40 mesh sieve and extracted with methanol separately in Soxhlet apparatus at 60°C. The solvent was completely removed by rotary vacuum evaporator and concentrated. The extracts were freeze dried and stored in a vacuum desiccator for further toxicity studies.

Experimental animals

Albino Wistar rats of either sex, weighing 200-250 gm were procured and maintained in standard laboratory conditions. The animals were fed with standard pellet diet and water ad libitum. They were allowed to acclimatize for one week before experimentation. All experiments were carried out according to the guidelines for care and use of experimental animals and approved by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) [Registration No: IAEC/GIP/-1287/SR-UGC/approved/3/15].

Instruments

SYSMAX semiautomatic haematology analyser was used in this experiment.

METHODS

Acute toxicity study

Acute toxicity study of methanol extracts of *Limnophila heterophylla* (MELH) and *Michelia champaca* (MEMC) were carried out in rats by using Organization for Economic Co-operation and Development (OECD) guideline 425. Before oral administration of a single doses of the MELH and MEMC, the rats were deprived of food for 3 hrs. A single oral doses of 2000 mg/kg of the individual methanol extract was given using oral gavage for short term (i.e., 48 hr) and long term (i.e., 14 days) to the rats. Surviving animals were observed for a further period of 14 days for toxic symptoms of piloerection, as well as lachrymatory, locomotor and respiratory activities.

Sub chronic toxicity study

Five groups of six rats each were kept in four separate metal cages. Group I was kept as control and received normal clean drinking water ad libitum for three months. Group II and III received 250 and 500 mg/kg of methanol extract of *Limnophila heterophylla* daily for three months. Likewise, Group IV and V received 250 and 500 mg/kg of methanol extract of *Michelia champaca* daily for three months. The animals in each group were weighed on day zero (baseline) and weekly thereafter. Blood samples of rats in each treatment group was obtained by tail bleeding, at baseline and termination of treatment, into eppendorf tubes without anticoagulant, centrifuged at 4000×g for 5 min and the serum obtained stored at -40°C for biochemical analysis. Other blood samples were collected into separate tubes already coated with trisodium citrate for haematological analysis. All rats were sacrificed after the blood collection. The internal organs and some tissues were weighed to determine relative organs.

Serum biochemical analysis

Serum glutamic oxaloacetic transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), alkaline phosphatase (ALP), bilirubin, urea and creatinine samples were determined using a semi-automated blood chemistry analyser.

Serum transaminases (GOT and GPT)

Serum transaminases (GOT and GPT) were determined by the method of Reitman and Frankel [24]. Each substrate (0.5mL) [either α -L-alanine (200mM) or L-aspartate (200mM) with 2mM α -ketoglutarate] was incubated for 5 min at 37°C. A 0.1mL of serum was added and the volume shall be adjusted to 1.0mL with sodium phosphate buffer (pH 7.4; 0.1M). The reaction mixture was incubated for 30 and 60 min for GPT and GOT, respectively. A 0.5mL of 2, 4-dinitrophenyl hydrazine (1mM) was added to the reaction mixture and left for 30 min at room temperature. Finally, the color was developed by the addition of 5mL NaOH (0.4 N) and the product formed was read at 505nm. Data were expressed as IUL^{-1}.

Alkaline phosphatase

Alkaline phosphatase (ALP) was assayed by the method of Kind and King [25]. The reaction mixture of 3.0 ml containing 1.5 ml of buffer (carbonate-bicarbonate buffer, 0.1M, pH 10.0), 1 ml of substrate and requisite amount of the enzyme sources was incubated at 37^{o}C for 15minutes. The reaction was arrested by the addition of 1.0 ml of Folins phenol reagent. The control tubes were received the enzyme after arresting the reaction. The contents were centrifuged and to the supernatant, 1.0 ml of 15% sodium carbonate solution, 1.0ml of substrate and 0.1ml of magnesium chloride (0.1M), was added and mixture was incubated for 10 minutes at 37^{0}C. The colour was read out 640 nm against the blank.

Bilirubin

Bilirubin content was estimated by method of Malloy and Evelyn [26]. The two test tubes were taken and each into was added 0.2ml of serum sample and 1.8 ml of distilled water. To the unknown, 0.5 ml of diazo reagent and to the blank, 0.5 ml of 1.5% HCl was added. Finally, to each tube, 2.5 ml of methanol was added and then allowed to stand for 30 minutes in ice and absorbance was read at 540nm. For a standard curve, the above standard was diluted 1 in 5ml methanol. The amount of direct reacting bilirubin was determined similarly by substituting 2.5ml of water for 2.5ml of methanol. Values were expressed as mg/dl.

Estimation of blood urea

Blood urea in test serum was determined by standard enzymatic method as described by Talke and Schubert (1965) and later modified by Tiffany et al. [27]. Add 0.1 ml of test serum sample to 1 ml of ready to use blood urea agent in test tubes kept in a series, mixed gently and aspirated into the Trace 30 semi auto analyser. After completion of assay, the result displayed on the screen were recorder and expressed in mg/dl.

Estimation of creatinine

The creatinine in test serum was estimated by standard enzymatic method (Picric acid method) by following the method described by Fabiny and Ertinghausen [28]. In to a series of test tubes equal volume of 1 ml ready to use picric acid and sodium hydroxide and 0.1 ml of test serum added and mixed gently and aspirated in Trace- 30 semi auto analyser as above. Result obtained on screen were recorded and expressed in mg/dl.

Haematological analysis

Red blood cells (RBC), mean cell volume (MCV), mean corpuscular haemoglobin (MCH), white blood cells (WBC), haemoglobin (HGB) and platelet counts (PLT) were determined with a semi-automated haematology analyser.

Statistical analysis

All experimental data were expressed as mean ± standard error of the mean (SEM). This Statistical analysis was carried out using one-way analysis of variance (ANOVA) followed by Dunnet-t-test with the SPSS statistical software for comparison to the control group. $p<0.05$ was considered as statistically significant.

RESULTS

Acute toxicity study

In the acute toxicity study, oral administration of the methanol extracts of *Limnophila heterophylla* and *Michelia champaca* at 2000 mg/kg did not produce any deaths and clinical signs of toxicity in 14 days experimental period in rats. Also, animals did not show signs of acute toxicity such as piloerection, lacrimation, changes in locomotion and respiration.

Sub chronic toxicity study

Body and organ weights

There were no significant differences in changes in calculated body weights of test animals compared to the control after administration of the methanol extracts both plants for 90 days. The change in body weight with period of treatment and organ weights at termination of treatment in control and methanol extracts of both plants treated animals were shown separately in Table 1 and 2. The results showed no significant difference in body weight changes between MELH & MEMC treated and control animals with time. Similarly, there were no significant changes in the organ weights, expressed as percentage of body weight, at termination between control and methanol extracts of *Limnophila heterophylla* and *Michelia champaca* treated animals.

Table 1: Effect of methanol extracts of *Limnophila heterophylla* and *Michelia champaca* on body weight

Weight (gm)	Group-I [Control]	Group-II 250 [mg/kg]	Group-III 500 [mg/kg]	Group-IV 250 [mg/kg]	Group-V 500 [mg/kg]
Initial	183.67	183.50	184.17	183.50	184.17
Final	281.83	265.17	268.33	262.95	266.18

Values are Mean ±SEM (n= 6).

Serum biochemical parameters

The effects of sub chronic administration of methanol extracts of *Limnophila heterophylla* and *Michelia champaca* to rats on serum biochemical parameters at termination of treatment were mentioned in Fig 1 and 2. The results of serum biochemical indices indicate that MELH and MEMC caused no significant changes in the levels of SGOT, SGPT, ALP and total bilirubin were observed. Likewise, when compared to the controls a significant decrease in the levels of urea and creatinine were found.

Table 2: Effect of methanol extract of *Limnophila heterophylla* and *Michelia champaca* on organs weight

Organ	Group-I [Control]	Group-II 250 [mg/kg]	Group-III 500 [mg/kg]	Group-IV 250 [mg/kg]	Group-V 500 [mg/kg]
Liver	3.22 ± 0.03	3.59 ± 0.31	3.69 ± 0.01	3.41 ± 0.11	3.59 ± 0.43
Lungs	0.56 ± 0.06	0.55 ± 0.11	0.57 ± 0.03	0.52 ± 0.03	0.49 ± 0.23
Heart	0.38 ± 0.05	0.36 ± 0.02	0.36 ± 0.03	0.31 ± 0.02	0.34 ± 0.01
Spleen	0.21 ± 0.01	0.20 ± 0.01	0.24 ± 0.00	0.16 ± 0.25	0.22 ± 0.02
Kidneys	0.60 ± 0.02	0.58 ± 0.01	0.62 ± 0.01	0.51 ± 0.37	0.61 ± 0.11

Values are Mean ± SEM (n= 6).

Haematological parameters

The effect of sub chronic administration of MELH & MEMC on certain haematological indices at termination of treatment was explained in Fig 3, 4 and 5. The results showed that there was no significant difference in all parameters like; red blood cells (RBC), mean cell volume (MCV), mean corpuscular haemoglobin (MCH), white blood cells (WBC), haemoglobin (HGB) and platelet counts (PLT) measured between control and methanol extracts of *Limnophila heterophylla* and *Michelia champaca* treated animals.

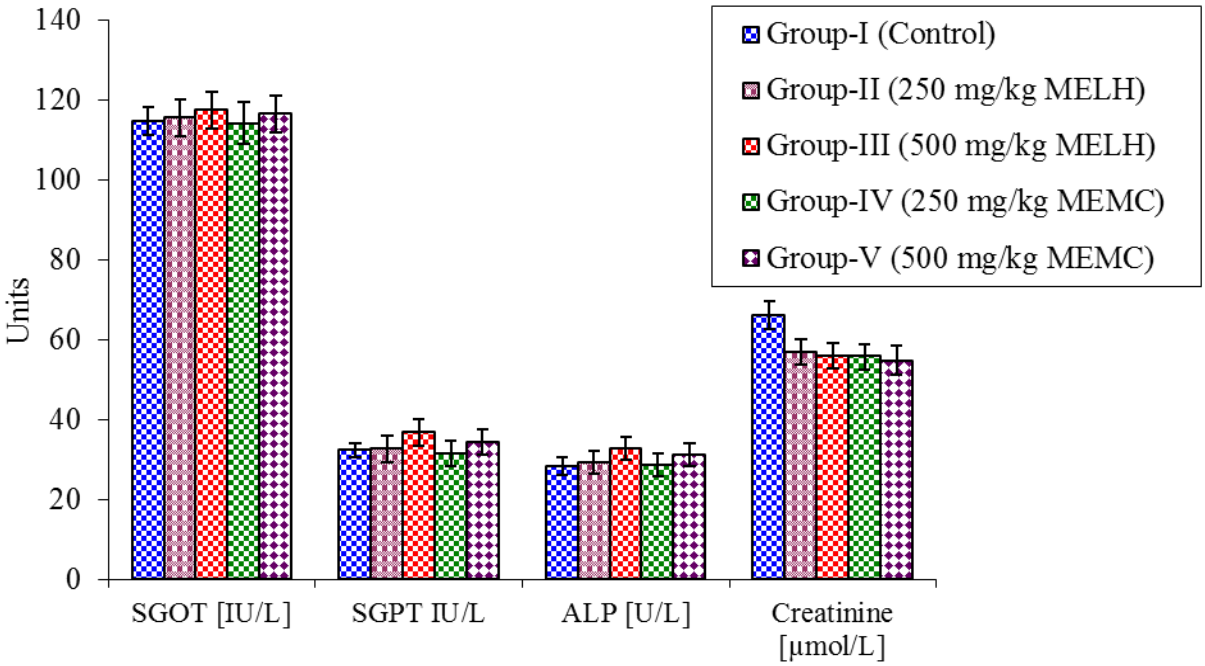

Fig 1: Biochemical parameters in experimental animals after 90 days of MELH and MEMC administration

Biochemical parameters: SGOT (Serum Glutamic Oxalacetic transaminase); SGPT ((Serum Glutamic Pyruvate transaminase); ALP (Alkaline phosphatase); CRE Creatinine. The data represents the Mean ± SD for each group of rats, n = 6 (number of animals per group).

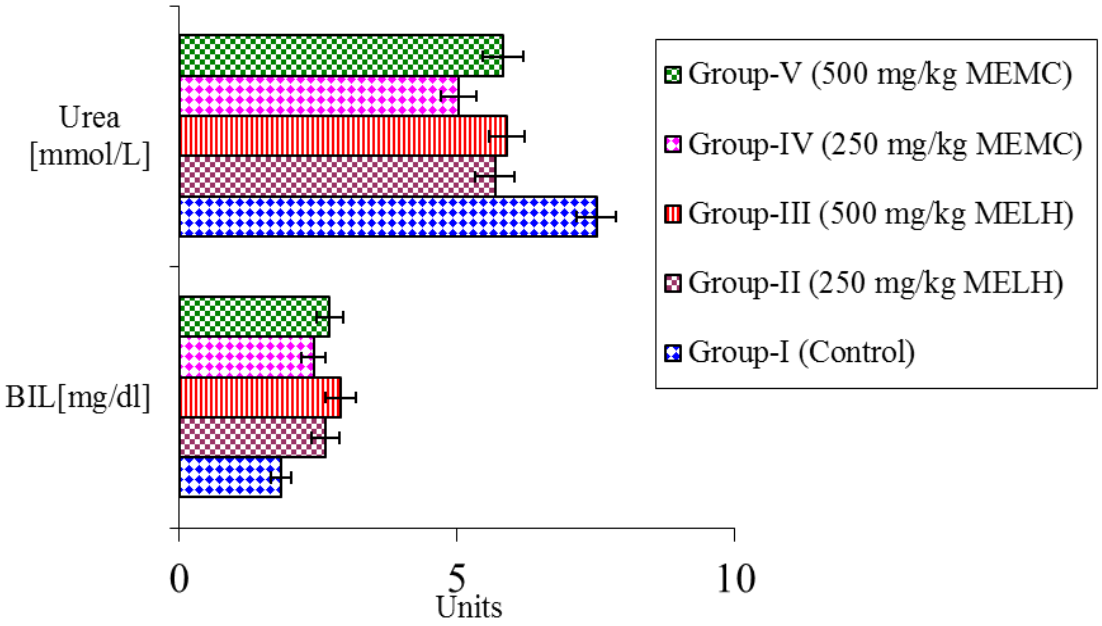

Fig 2: Biochemical parameters in experimental animals after 90 days of MELH and MEMC administration

Biochemical parameters: BIL: Bilirubin; URIC (Uric acid); CRE Creatinine. The data represents the Mean ± SD for each group of rats, n = 6 (number of animals per group).

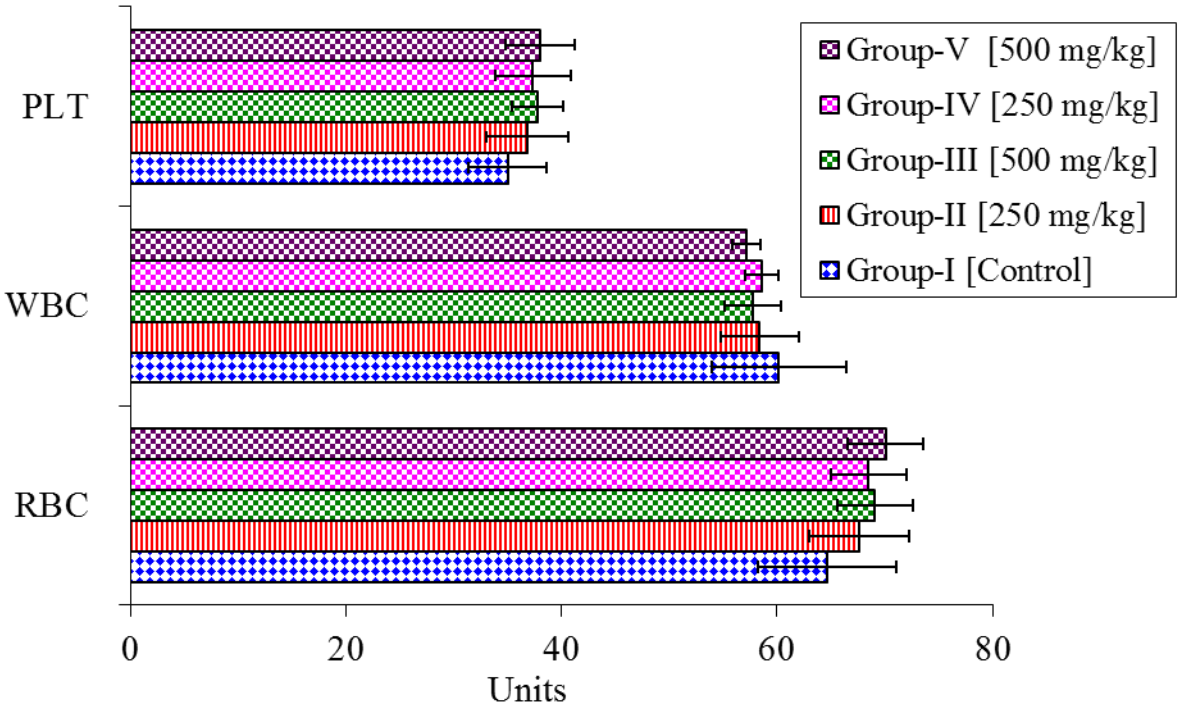

Fig 3: Haematological parameters in experimental animals after 90 days of MELH and MEMC administration

Hematological parameters: PLT (Platelet Count), WBC (White Blood Cell Count) and RBC (Red Blood Cell Count), Values are means ± S.E.M. of N = 6.

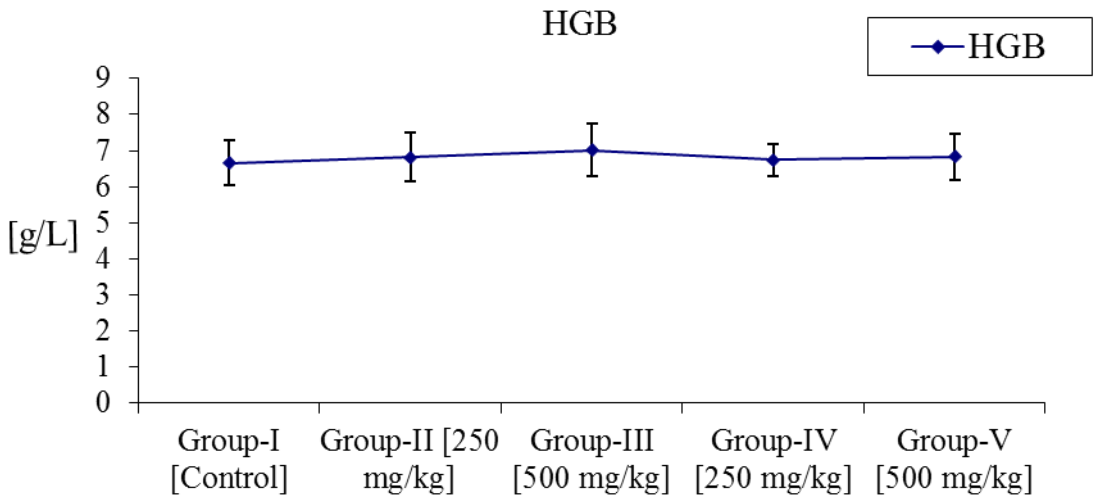

Fig 4: Haematological parameters in experimental animals after 90 days of MELH and MEMC administration

Hematological parameters: HGB (Haemoglobin), Values are means ± S.E.M. of N = 6.

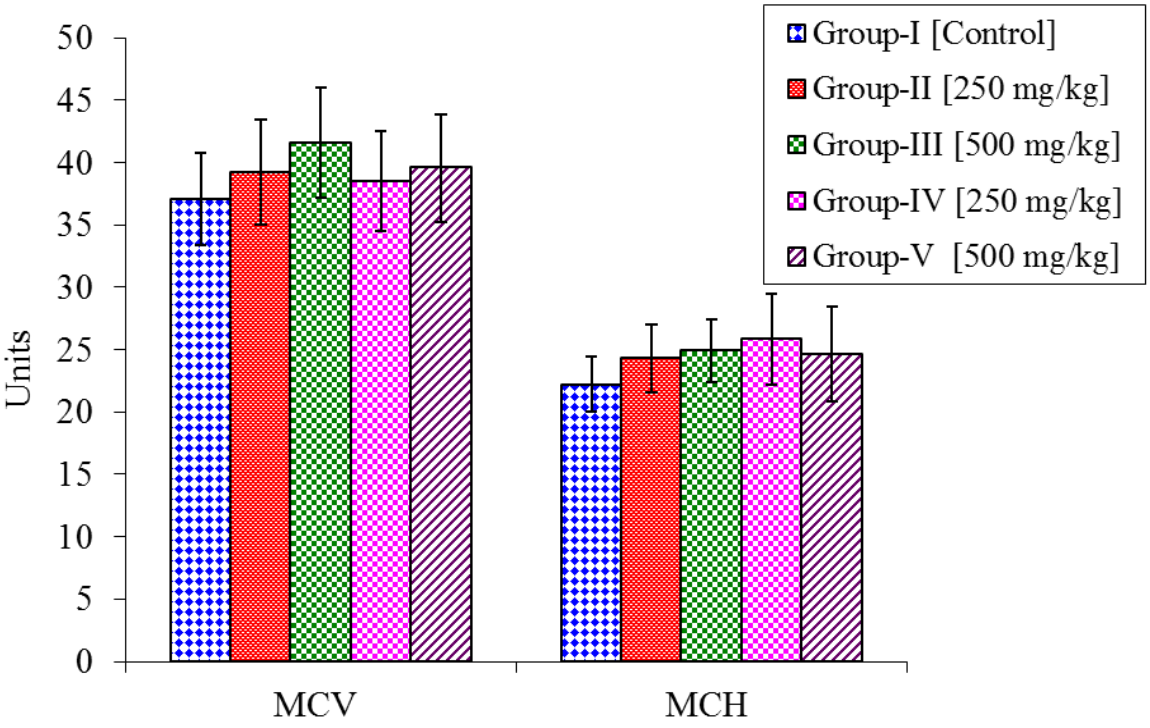

Fig 5: Haematological parameters in experimental animals after 90 days of MELH and MEMC administration

Hematological parameters: MCV (Mean Corpuscular Volume) and MCH (Mean Cell haemoglobin), Values are means ± S.E.M. of N = 6.

DISCUSSION

In developing countries, herbal products ready from medicative plants became notable in care and a few are incorrectly thought-about as safe as they're obtained from natural sources. However, these bioactive compounds from ancient medicative plants square measure complete to be safe while not understanding the attainable health effects and so usually used as self-medication [29]. However, there's an absence of information on the pharmacology profile and adverse effects of those active compounds isolated from the herbal plants. Therefore, acute toxicity study is needed not solely to spot the additional vary of doses in animal studies however conjointly to elucidate the probable clinical signs induced by the test compounds beneath investigation. It's conjointly a vital effective parameter for hard the therapeutic index of medication and chemicals [30]. Results obtained from toxicity studies on animals are going to be crucial for positive judgement on the security of medicative plants if they're found to possess adequate potential for development into pharmacologic compounds [31].

The limit dose of 2000 mg/kg didn't turn out in mortality or any clinical sign of acute toxicity in rats within the short term (i.e., 48 hr) and long run (i.e., fourteen days) observatory amount suggesting that the oral LD_{50} of the extract is more than 2000 mg/kg. The limit test is employed in things wherever the investigator has data indicating that the take a look at material is probably going to be non-toxic or of low toxicity. Within the sub chronic study, the extract didn't seem to have an effect on the conventional growth of take a look at animals as proven by comparable weight gain by each management and take a look at animals over the three-month treatment amount. Furthermore, there was no important amendment once excised liver, lungs, heart, spleen and kidney were expressed relative to weight up to speed and take a look at animals. Relative weight is additional indicative of toxicity than absolute weight [32].

The values of biochemical parameters, like SGOT, SGPT, ALP and bilirubin square measure usual markers of the conventional liver functions [33]. During this study, there have been no adverse effects on the standard markers of liver toxicity (serum levels of SGOT, SGPT, ALP and bilirubin). It's going to be complete that the methanol extracts of each the plants didn't induce important harm to those organs. The values of biochemical parameters, like creatinine and urea, square measure sensible indicators of the conventional urinary organ perform [34]. Urea is that the initial acute excretory organ marker upon excretory organ (kidney) injury and creatinine is that the most trustable excretory organ marker and will increase only the bulk of excretory organ perform is lost [35]. In present study, no important variations were discovered in MELH and MEMC-treated teams compared with the control group. Therefore, this result reveals a reduction, though not statistically important, within the levels of urea and creatinine at all the dose levels compared to controls and has no influence on the perform of kidney rats.

Blood cells are produced within the bone marrow. Glorious haemotoxicants like paracetamol cause reduction in RBCs resulting in anaemia [36] and a few bioactive phytochemicals have an effect on Hematocrit (HCT) levels [37]. In the haematological studies, there was no important distinction between take a look at and management animals. Hence, the extracts might not have injurious effects on bone marrow perform. Analysis of blood parameters in animal studies has relevancy to guage the danger of alterations of the haemopoietic system in toxicity studies, for necessary application to humans [38]. During this study, administration of the methanol extracts of each plants once ninety days up to a dose of five hundred mg/kg weight induces no significant increase in all haematological parameters. The white blood cells defend the body from infection by foreign organisms, the erythrocyte boost the system and therefore the platelets defend blood vessels from epithelial tissue harm furthermore as initiate repair of those vessels. This, thus suggests a powerful immuno-modulatory, antioxidant, endothelial protection and repair activity of *Limnophila heterophylla* and *Michelia champaca* extracts. The mean corpuscular haemoglobin (MCV) and mean corpuscular haemoglobin (MCH) that square measure erythrocyte indices utilized in classifying styles of anaemia didn't show any important changes in experimental animals when put next to the controls, once more validating the erythrocyte protection by the extracts from oxidative damage.

CONCLUSION

Treatment with single oral doses of 2000 mg/kg did not produce result in any toxic signs or mortality in the acute toxicity study. The administration of the methanol extracts of *Limnophila heterophylla* and *Michelia champaca* separately did not show any signs of sub chronic toxicity on the body weights, organ weights, biochemical parameters and haematological parameters at a doses of 250 and 500 mg/kg body weight. Despite the fact that the acute toxicity of the methanol extracts was not significantly different from the sub chronic toxicity apart from significant changes in biochemical and haematological parameters. Overall, it can be concluded that the MELH and MEMC were well tolerated in both in acute and sub chronic toxicity studies.

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