

Research

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Sub-Acute Toxicity Study of *Calotropis gigantea* Latex Extracts in Male Swiss Albino Mice

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ABSTRACT

Background: *Calotropis gigantea* of family Asclepiadaceae, is traditionally used in ayurveda for its anti-helminthic, anti-pyretic, and anti-malarial activities. The present study was designed to investigate the sub-acute toxic effects of ethanol and water extract of *Calotropis gigantea* latex in mouse model using haematology, serum biochemistry and histopathological changes as toxicity indices.

Methods: In the sub-acute 28-day toxicity study, ethanol and water extract of *Calotropis gigantea* latex were administered intraperitoneally at the dose levels of 50, 100, 200, 500, 1000 and 2000 mg/kg body wt./day.

Results: Significant ($p < 0.05$) difference were not observed in relative organ weights and haematological, hepatic and renal biomarkers up to the dose level of 500 mg/kg body wt./day for 28 days except blood glucose and serum glutamate pyruvate transaminase (SGPT) in comparison to the control group. No significant toxicity was seen up to the dose level of 1000 mg/kg body wt./day for 28 days in case of blood glucose and SGPT.

Conclusion: The findings suggest that *Calotropis gigantea* latex extracts do not cause sub-acute toxicity up to the level of 1000 mg/kg body wt./day for 28 days and may be considered as phytomedicinal therapeutic agents.

KEYWORDS: *Calotropis gigantea*; Sub-acute toxicity; Relative organ weight; Haematological hepatic and renal biomarkers; Histopathological changes.

ABBREVIATIONS: EECGL: Ethanol extract of *Calotropis gigantea* latex; WECGL: Water extract of *Calotropis gigantea* latex; EDTA: Ethylenediaminetetraacetic acid; CPCSEA: Committee for the Purpose of Control and Supervision of Experiments on Animals; RBC: Red Blood Cell; WBC: White Blood Cell; SGOT: Serum Glutamate Oxaloacetate Transaminase; SGPT: Serum Glutamate Pyruvate Transaminase; ALP: Alkaline phosphatase.

INTRODUCTION

Presently, 25% of prescribed drugs worldwide are derived from plant sources in spite of the great progress and advancement of organic synthesis.¹ Medicinal plants offer unlimited opportunities for the discovery of new drugs in biomedical field. Most of the natural products used in folk remedy have scientific evidences with regard to their biological activities. However, there is little evidence available concerning the possible toxicity those drugs or products from medicinal plants may cause to the consumers.² The traditional use of any plant for medicinal purposes requires guarantees for the safety of such plant. Herbal medicines are usually complex mixtures of many bioactive compounds. Herbal medicines may differ from single-agent pharmaceuticals, phytomedicines due to the different mechanisms of action of their bioactive constituents.³ This is also reflected in their dose-response relationships and in synergistic/combinatorial effects. Pharmacological investigations have established their rising relevance in search of more reliable herbal drugs free of any side effects.⁴ Different types of interactions can arise

whenever a chemical substance or drug administered to a biological system and a series of dose-related responses can occur. Thus, to identify potential health hazards before the drugs are administered to man, toxicity testing in animals is usually done on new drugs with doses well above the expected therapeutic range. Toxicity studies include a wide range of tests in different species with regular monitoring for biochemical or physiological anomalies seen in long-term administration of the drug.⁵

The types of toxicity studies which are routinely executed by pharmaceutical manufacturers in the search of a new drug include acute, sub-acute and chronic toxicity. Administration of a single dose or multiple doses in a period not beyond 24 hours, up to a limit of 2000 mg/kg produces acute toxicity. Objective of acute toxicity tests is to detect a dose producing major adverse effects and an assessment of the minimum dose causing lethality.⁶

Calotropis gigantea (Linn.) R. Br. of family Asclepiadaceae is a perennial shrub and widely distributed in tropical and subtropical region and most abundant in Bangladesh, India, Burma and Pakistan.⁷⁻¹⁰ The root, stem, leaves, flower and latex of *C. gigantea* are reported to be utilized in traditional medicine for the treatment of toothache, ear ache, eczema, syphilis, elephantiasis, injury, pain, ulceration, epilepsy, anxiety and mental disorders.^{11,12}

Traditionally, the root of *Calotropis gigantea* is used in treatment of leprosy, asthma, bronchitis, and as an expectorant.^{13,14} *Calotropis gigantea* reported to exhibit free radical scavenging^{15,16} and pro-coagulation activity,¹⁷ pregnancy interceptive,¹⁸ anticancer,¹⁹ immunomodulatory,²⁰ wound healing,²¹ anti-inflammatory²²⁻²⁵ and hepatoprotective^{16,26} activity.

The major phytochemicals of *Calotropis gigantea* are flavonol, glycoside, uscharidin, calotropin, frugoside, calotroposides A to G. Other constituents are α -amyrin, β -amyrin, taraxasterol, β -sitosterol, α - and β -amyrinmethylbutazone, gigantursenylacetate A and B. It's latex rich in lupeol, calotropin, calotoxin and uscharchin.²⁷ In current times there is a growing awareness and interest in medicinal plants and their preparations.²⁸ Lack of scientific and clinical data in support of better understanding of the efficacy and safety of the drugs, is the major difficulty to the use of traditional herbal preparations.

The sub-acute toxicity data may be required to envisage the safety and effects of long term exposure to a specific medicinal plant. This study therefore was carried out to evaluate the sub-acute toxic effects of the ethanol and water extract of *Calotropis gigantea* latex in mice.

MATERIALS AND METHODS

Collection and Extraction of Plant Material

C. gigantea plant was collected from the out fields of Vidyasagar

University, Midnapore, West Bengal, India and authenticated from Botanical Survey of India (BSI), Ministry of Environment and Forest (MoEF), Government of India, Howrah (Identification No. CNH/2014/Tech.II/55).

The fresh latex of *C. gigantea* was collected from the aerial parts of mature plants and was then spread into petri dishes for air dry in room temperature under shade up to 4 to 5 days. Then 250 g of dried latex was subjected for size reduction to coarse powder. Then in 450 ml of ethanol and water, powdered latex was dissolved separately and was incubated for 48 hours in room temperature and was filtered using Whatmann filter paper. The filtrates were dried in EYELA CCA 1110 rotary evaporator to produce ethanol extracts of *Calotropis gigantea* latex (EECGL) and water extracts of *Calotropis gigantea* latex (WECGL) extracts (yields 4.5% and 2.8% respectively).²⁹ Dried extracts were stored in air tight containers at 4°C till further use.

Animals

Healthy male Swiss albino mice (20-25 g) were selected for toxicity test. The mice were grouped and housed in poly acrylic cage (38×23×10 cm) with 6 animals per cage. The animals were kept on a 12 h light: 12 h dark regime at 25°C for 7 days before commencement of the experiment. The animals had free access to standard diet and water.³⁰ Mice were deprived of food but not water prior to administration of the test extracts. The study was approved by the Institutional Animal Ethical Committee (IAEC), registered under Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment, Forests and Climate Change (MoEFCC), Government of India and performed in compliance with the relevant laws and guidelines of the CPCSEA.

Treatment Schedule

The sub-acute toxicity study on the above mentioned plant extracts was performed as per the Organisation for Economic Co-operation and Development (OECD) guidelines 407³¹ with slight modifications, where 2000 mg/kg was used as the limit test dose. The ethanol and water extracts of *Calotropis gigantea* latex were administered intraperitoneally prior up to the bladder³² at the dose levels of 50, 100, 200, 500, 1000 and 2000 mg/kg body weight to the group II to VI animals respectively and distilled water to the control group (Group I) animals by sterile syringe daily for 28 days.^{33,34} Toxicity studies were conducted as per internationally accepted protocol in Swiss albino mice. The animals were divided into 6 groups containing 6 animals each. The experimental design was given below:

Group I : Control

Group II : EECGL /WECGL (50 mg/kg body weight)

Group III : EECGL/WECGL (100 mg/kg body weight)

Group IV : EECGL/WECGL (200 mg/kg body weight)

Group V : EECGL/WECGL (500 mg/kg body weight)

Group VI : EECGL/WECGL (1000 mg/kg body weight)

Group VII : EECGL/WECGL (2000 mg/kg body weight)

The feed and volume of water consumed by mice in each group were monitored daily. Weights of the mice in all the groups were recorded daily during the treatment schedule and on the last day of study. Doses of the extract administered were adjusted accordingly. On the 29th day of the experiment, the mice were anesthetized with pentobarbital sodium (50 mg/kg, i/p) and sacrificed by cervical dislocation. The test organs of the animals were opened up surgically and blood samples were collected by cardiac puncture.

Blood Sample Collection

On the last day of the experiment, 2 sets of blood samples were collected from all the animals *via* cardiac puncture using a 5 ml sterile syringe. Two ml of blood sample was collected into sterile container containing ethylenediaminetetraacetic acid (EDTA) as anticoagulant for the determination of haematological parameters. For serum analysis blood samples were collected into another anticoagulant free sterile container, allowed to stand at room temperature and centrifuged at 2000 rpm for 10 minutes. The supernatants were then collected and stored at -20°C for biochemical analysis.

Organ Weight

On 29th day, all the animals were sacrificed. Heart, liver, lungs, spleen, and kidneys were carefully dissected out and weighed.

Haematological Analysis

Red blood cell (RBC) count: Blood was diluted with RBC dilution fluid (1:200). Total erythrocytes were counted in the Neubaur haemocytometer chamber.³⁵

White blood cell (WBC) count: Blood was diluted with white blood cell (WBC) dilution fluid (1:20) and loaded in Neubaur haemocytometer chamber. Four large (1 sq mm) corner squares of the haemocytometer chamber were counted under the microscope.³⁵

Determination of haemoglobin: At first 20 µl of blood was transferred into a test tube containing 5 ml of Drabkin's solution. After adjusting the photoelectric colorimeter at 540 nm with a blank the OD of sample was read.³⁶

Estimation of Urea

Urea was determined by the modified method of Natelson et al.^{37,38} To 0.1 ml of serum, 3.3 ml of water, 0.3 ml each of 10% sodium tungstate and 0.67 N sulphuric acid were added. The suspensions were centrifuged and to 1.0 ml of the supernatant, 1.0 ml of water, 0.4 ml of diacetylmonoxime and 2.6 ml of 0.67 N sulphuric acid-phosphoric acid reagents were added. Standard urea (20 to 50 µg/ml) were also treated in a similar manner and

all the tubes were heated in a boiling water bath for 30 minutes, cooled and the color developed was measured at 480 nm in a Shimadzu spectrophotometer (UV-Shimadzu-245, Japan). The values were expressed as mg of urea/dl of blood.

Estimation of Creatinine

Creatinine was described according to the modified method of Brod and Sirota.^{39,40} At first, protein free filtrate was prepared. One ml of serum was precipitated with 8.0 ml of water, 0.5 ml of 2/3 N sulphuric acid and 0.5 ml of 40% sodium tungstate. After that, 5.0 ml clear filtrate was taken and 1.5 ml of saturated picric acid and 1.5 ml of 0.75 N sodium hydroxide were added to it. Standard and blank were also prepared similarly. The color intensity was measured at 530 nm in a Shimadzu spectrophotometer (UV-Shimadzu-245, Japan). The values were expressed as mg of creatinine/dl of blood.

Measurement of Serum Alkaline Phosphate (ALP)

Activity of serum alkaline phosphatase were estimated by taking 0.25 mL of serum in a centrifuge tube containing 1 ml buffer (1 mM of p-nitrophenol phosphate in 1 M Tris buffer, pH 8.0); the mixture was then subjected to be incubated at 37°C for 30 minutes in a water bath. The activity was measured spectrophotometrically at 420 nm.⁴¹

Measurement of Serum Glutamate Oxaloacetate Transaminase (SGOT) and Serum Glutamate Pyruvate Transaminase (SGPT)

For the estimation of SGOT, to 1.0 ml of the buffered substrate (200 mM/L of DL-aspartate and 2 mM/L of α-ketoglutarate, pH=7.4), 0.1 ml of serum was added and incubated at 37°C for one hour. Then 1 ml of dinitrophenyl hydrazine (DNPH) was added and kept for 20 minutes at room temperature. After 20 minutes, 10 ml of 0.4 N sodium hydroxide was added and the color intensity was measured at 505-540 nm in a Shimadzu spectrophotometer (UV-Shimadzu-245, Japan) after 10 minutes against the reagent blank. The standard and blank were also prepared by the same process. The enzyme activity in serum was expressed as IU/lit.

For the estimation of SGPT, to 1.0 ml of the buffered substrate (200 mM/L of DL-alanine and 2 mM/L of α-ketoglutarate pH=7.4), 0.1 ml of serum was added and incubated at 37°C for on hour. The reaction was arrested by the addition of 1.0 ml of dinitrophenyl hydrazine and left aside for 20 minutes at room temperature. The color developed by the addition of 10 ml of 0.4 N sodium hydroxide was read at 505-540 nm in a Shimadzu spectrophotometer (UV-Shimadzu-245, Japan) against the reagent blank. The enzyme activity in serum was expressed as IU/lit.⁴²

Estimation of Blood Glucose

Blood glucose was measured according to the method of Nelson-Somogyi.^{43,44} Somogyi's copper reagent was prepared by

dissolving 24 g anhydrous sodium carbonate and 12 g of sodium potassium tartrate in about 250 ml of distilled water. To this 4 g copper sulphate as a 10% (w/v) solution was added and mixed followed by the addition of 16 g of sodium bicarbonate. Then 180 g of sodium sulphate was dissolved in about 500 ml of distilled water and boiled to expel air. After cooling, the two solutions were mixed and the volume was made up to 1000 ml. Nelson's arsenomolybdate reagent was prepared by dissolving 25 g ammonium heptamolybdate in 450 ml of water. Then 21 ml of sulphuric acid was added and mixed well. To the mixture 3.0 g disodium hydrogen arsenate dissolved in 25 ml of distilled water was added. The solution was mixed well and incubated for 24 hours at 37 °C. From the sample, one ml of aliquot was pipetted out. To this 1.0 ml of Somogyi's copper reagent was added. The mixture was then placed in a bath of boiling water and heated for 20 minutes. After cooling under tap water 1.0 ml of Nelson's arsenomolybdate reagent was added with immediate mixing till the effervescence ceased. The intensity of color was measured spectrophotometrically at 540 nm.

Measurement of Serum Cholesterol

At first 0.1 ml of serum was mixed with 6 ml of glacial acetic acid. After addition of 4 ml of color reagent (1 ml of 10% FeCl₃, 6 H₂O, 15 ml of conc. H₂SO₄) it was mixed vigorously and allowed to stand for 20 min. The reading was taken at 570 nm.⁴⁵ The amount of cholesterol present is calculated by plotting the standard curve.

Measurement of Serum Total Protein

Different dilutions of BSA solutions are prepared by mixing stock BSA solution (1 mg/ml) and water. The final volume in

each of the test tubes is 6 ml. The BSA range is 0.05 to 1 mg/ml. From these different dilutions, protein solutions were pipetted out to different test tubes and 5 ml of alkaline copper sulphate reagent were added. The solutions were mixed well. Then 0.5 ml of reagent Folin-Ciocalteu solution (reagent solutions) was added to each tube and incubated at 37°C for 30 min. The optical density was measured at 660 nm.⁴⁶ The absorbance was plotted against protein concentration to get a standard calibration curve.

Histopathology Analysis

All the animals from each group were sacrificed for histopathological examinations of major internal organs. Organs such as liver, kidney were collected from all the animals. The collected organs were weighed and preserved in 10% neutral buffered formalin, then dehydrated in alcohols and embedded in paraffin. Five micron thickness of tissue sections were stained with haematoxylin and eosin (H and E) for histopathological study.

Statistical Analysis

All the parameters were performed in triplicate manner. The data was expressed as Mean±SEM, comparisons between control and treated groups were analyzed by using the one-way analysis of variance (ANOVA) considering p<0.05 as a limit of significance.

RESULTS

Relative Organ Weights (ROW) of Male Mice

Relative organ weights of 28-days treated mice are shown in Tables 1A and 1B. The relative organ weights recorded in the treatment groups did not show any significant difference (p<0.05)

Weight	Control	50 mg/kg body wt.	100 mg/kg body wt.	200 mg/kg body wt.	500 mg/kg body wt.	1000 mg/kg body wt.	2000 mg/kg body wt.
Initial body weight (gm)	27.66±0.33	27±0.57	27.7±0.33	27.88±0.57	28±0.57	28±0.57	28±0.57
Final body weight (gm)	31±0.57	31±0.57	31±0.57	31±0.57	32±0.57	32±0.57	35±0.57*
Weight of heart (gm)	0.22±.005	0.22±.005	0.22±.005	0.21±.005	0.21±.003	0.2±.003	0.19±.005*
Weight of spleen (gm)	0.24±.008	0.24±.008	0.26±.005	0.27±.003	0.29±.005	0.30±.008	0.32±.011*
Weight of liver (gm)	0.33±.008	0.33±.008	0.34±.008	0.36±.005	0.36±.008	0.41±.008	0.41±.011*
Weight of kidney(gm)	0.38±.003	0.36±.005	0.36±.005	0.35±.005	0.35±.005	0.32±.003	0.3±.005*

Results are expressed as Mean ± SEM. Analysis is done by one way ANOVA. Comparison was done between control group versus all other groups (*indicates p<0.05).
Table 1A: Effect of water extract of *Calotropis gigantea* latex (WECGL) administration for 28 days on body and different organ weight in Swiss albino mice.

Weight	Control	50 mg/kg body wt.	100 mg/kg body wt.	200 mg/kg body wt.	500 mg/kg body wt.	1000 mg/kg body wt.	2000 mg/kg body wt.
Initial body weight (gm)	27±0.57	27±0.57	27.33±0.33	28±0.57	28±0.57	28.66±0.88	29±0.57
Final body weight (gm)	31.33±0.66	31±0.57	31±.57	33±0.57	34±0.57	34.66±0.88	35±0.57
Weight of heart (gm)	0.23±.005	0.22±.005	0.22±.005	0.22±.008	0.2±.005	0.2±.008	0.19±.005*
Weight of spleen (gm)	0.27±.005	0.27±.003	0.27±.005	0.28±.005	0.29±.005	0.3±.005	0.32±.008*
Weight of liver (gm)	0.31±.005	0.32±.005	0.33±.005	0.32±.005	0.32±.011	0.33±.005	0.35±.015*
Weight of kidney(gm)	0.39±.005	0.36±.008	0.36±.005	0.35±.005	0.35±.005	0.33±.003	0.31±.005*

Results are expressed as Mean±SEM; n=6. Analysis is done by one way ANOVA. Comparison was done between control group versus all other groups (*indicates p<0.05).
Table 1B: Effect of ethanolic extract of *Calotropis gigantea* latex (EECGL) administration for 28 days on body and different organ weight in Swiss albino mice.

Haematological parameters	Control	50 mg/kg body wt.	100 mg/kg body wt.	200 mg/kg body wt.	500 mg/kg body wt.	1000 mg/kg body wt.	2000 mg/kg body wt.
Haemoglobin (%)	15±0.577	15±0.55	14.8±0.25	14.5±0.4	14.5±0.54	14±0.55	13.5±0.54*
Total RBC count (cu.mm)	6±0.145	6±0.012	5.8±0.104	5.8±0.05	5.5±0.057	5.1±0.04	5.1±0.02
Total WBC count (cu.mm)	5100±58	5100±50	5200±62	5300±60	5300±78	5400±65	5560±70*

Results are expressed as Mean ± SEM; n=6. Analysis is done by one way ANOVA. Comparison was done between control group versus all other groups (*indicates p<0.05).

Table 2A: Haematological parameters after WECGL administration for 28 days in Swiss albino mice.

Haematological parameters	Control	50 mg/kg body wt.	100 mg/kg body wt.	200 mg/kg body wt.	500 mg/kg body wt.	1000 mg/kg body wt.	2000 mg/kg body wt.
Haemoglobin (%)	15±0.577	15±0.52	14.54±0.25	14.5±0.43	14.5±0.56	14±0.55	13.8±0.64*
Total RBC count (cu.mm)	6±0.145	6±0.109	5.5±0.124	5.5±0.057	5.6±0.057	5.2±0.042	5.2±0.022
Total WBC count (cu.mm)	5100±58	5100±52	5300±82	5345±80	5400±88	5545±85	5550±80*

Results are expressed as Mean±SEM; n=6. Analysis is done by one way ANOVA. Comparison was done between control group versus all other groups (*indicates p<0.05).

Table 2B: Haematological parameters after EECGL administration for 28 days in Swiss albino mice.

up to the dose level of 1000 mg/kg body wt. compared to the control.

Haematological Parameters Analysis

The effects of *C. gigantea* latex extracts at sub-acute doses on haematological parameters are presented in Tables 2A and 2B. Most haematological parameters (haemoglobin, total RBC count, total WBC count) in treated mice were not significantly different from the control group animals, with the exception of marginal variations in the parameters. No significant toxicity seen up to the dose level of 1000 mg/kg body wt. in haemoglobin percentage and total WBC count in latex extracts treated mice than that of the control animals. There was no trace of toxicity in total RBC counts in latex extracts treated mice up to the dose level of 2000 mg/kg body wt.

Effect of EECGL and WECGL on Hepatic and Renal Biomarkers of Mice

The results of hepatic and renal biomarkers following the treatment of *C. gigantea* latex extracts in mice are shown on Figures 1 to 8.

No significant sub-acute toxicity was seen in the level of urea (Figure 1), creatinine (Figure 2), alkaline phosphatase (Figure 3), and serum cholesterol (Figure 7) in mice treated with EECGL and WECGL up to the dose level of 500 mg/kg body wt./day for 28 days when compared with the control animals.

In case of blood glucose (Figure 6), SGPT (Figure 5) and serum total protein (Figure 8), significant sub-acute toxicity was not observed up to the dose level of 1000 mg/kg body wt./

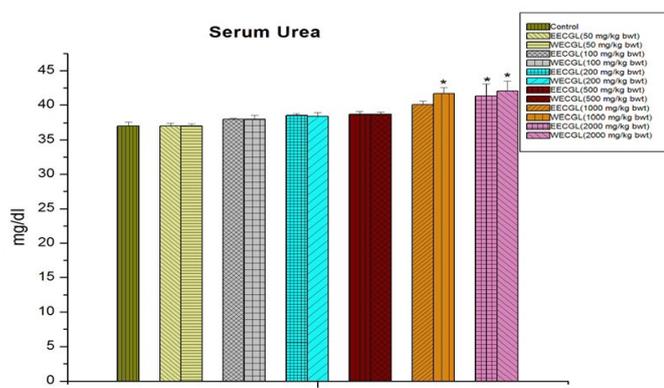


Figure 1: Shows serum urea level after treatment of EECGL and WECGL for 28 days in Swiss albino mice. Results are expressed as Mean±SEM; n=6. Analysis is done by one way ANOVA. Comparison was done between control group versus all other groups (*indicates p<0.05).

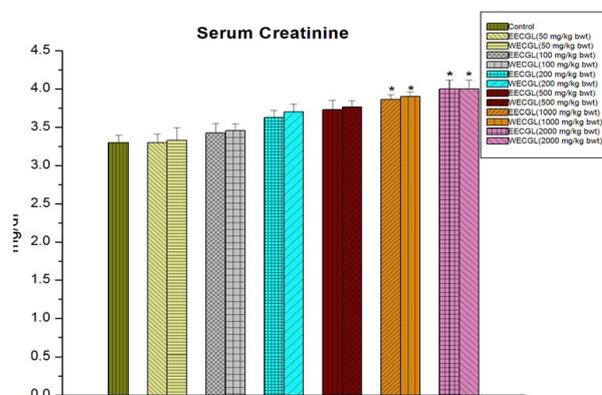


Figure 2: Serum creatinine after treatment of EECGL and WECGL for 28 days in Swiss albino mice. Results are expressed as Mean±SEM; n=6. Analysis is done by one way ANOVA. Comparison was done between control group versus all other groups (*indicates p<0.05).

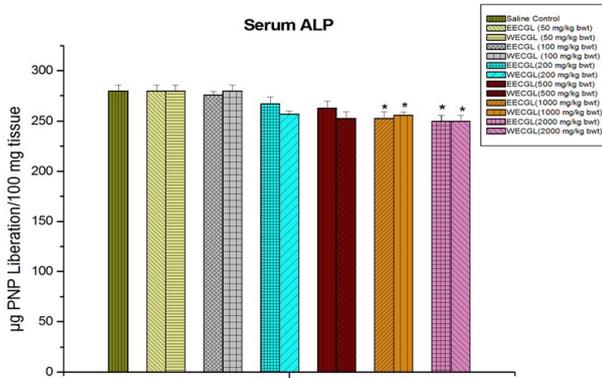


Figure 3: Shows serum ALP after treatment of EECGL and WECGL for 28 days in Swiss albino mice. Results are expressed as Mean±SEM; n=6. Analysis is done by one way ANOVA. Comparison was done between control group versus all other groups (*indicates p<0.05).

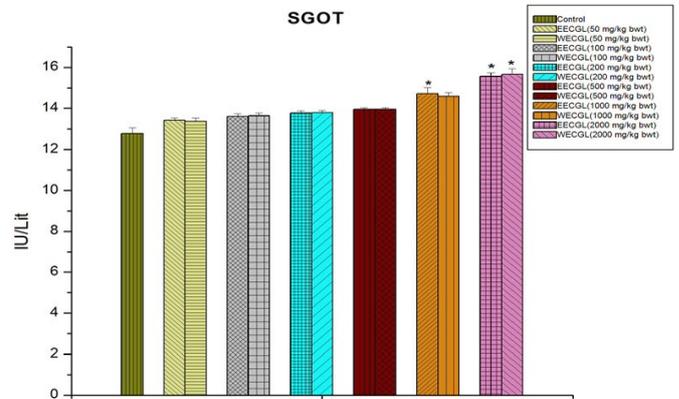


Figure 4: The effect of SGOT after treatment of EECGL and WECGL for 28 days in Swiss albino mice. Results are expressed as Mean±SEM; n=6. Analysis is done by one way ANOVA. Comparison was done between control group versus all other groups (*indicates p<0.05).

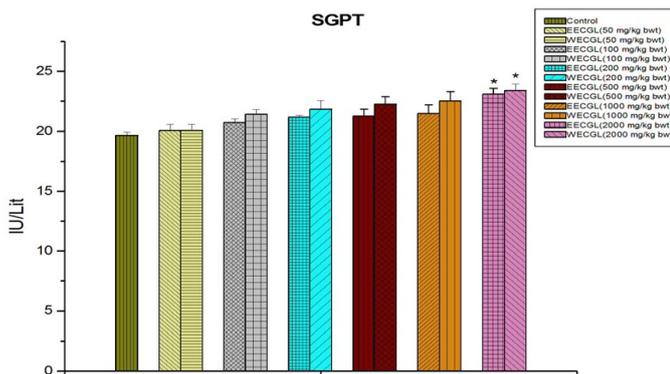


Figure 5: Level of SGPT after treatment of EECGL and WECGL for 28 days in Swiss albino mice. Results are expressed as Mean±SEM; n=6. Analysis is done by one way ANOVA. Comparison was done between control group versus all other groups (*indicates p<0.05).

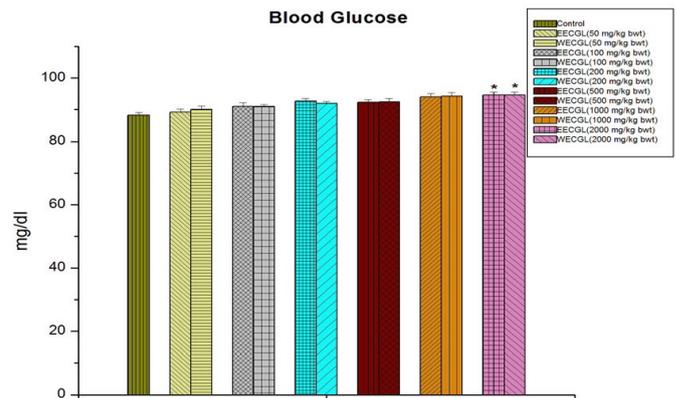


Figure 6: Shows the blood glucose level after treatment of EECGL and WECGL for 28 days in Swiss albino mice. Results are expressed as Mean±SEM; n=6. Analysis is done by one way ANOVA. Comparison was done between control group versus all other groups (*indicates p<0.05).

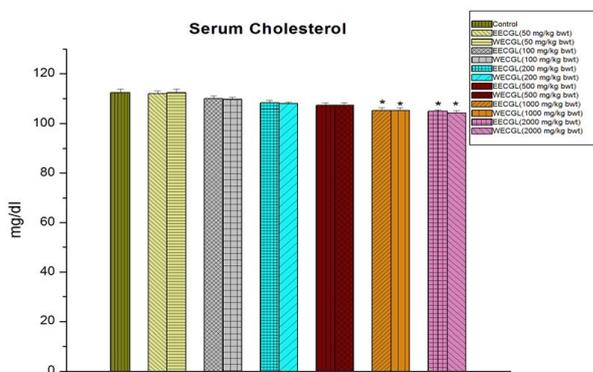


Figure 7: The diagram shows the measurement of serum cholesterol after treatment of EECGL and WECGL for 28 days in Swiss albino mice. Results are expressed as Mean±SEM; n=6. Analysis is done by one way ANOVA. Comparison was done between control group versus all other groups (*indicates p<0.05).

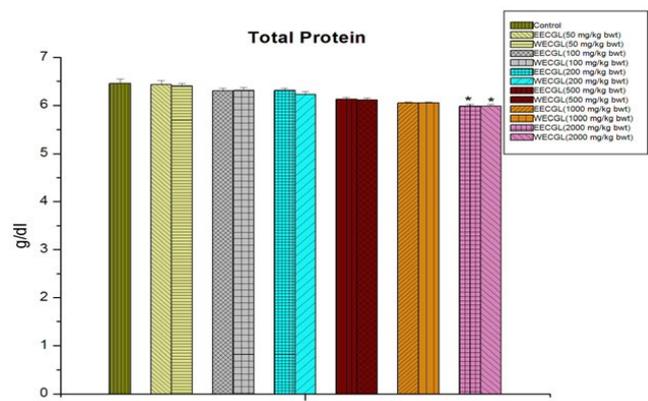


Figure 8: Shows serum total protein after treatment of EECGL and WECGL for 28 days in Swiss albino mice. Results are expressed as Mean±SEM; n=6. Analysis is done by one way ANOVA. Comparison was done between control group versus all other groups (*indicates p<0.05).

day.

WECGL produces no toxicity in case of SGOT (Figure 4) up to 1000 mg/kg body wt./day but EECGL causes significant ($p < 0.05$) toxicity at this dose level.

Histopathological Study

Histopathological studies of the liver and kidney tissues in the

control and the *C. gigantea* extract treated groups showed no differences, indicating that treatment of EECGL and WECGL did not result in any adverse or abnormal toxicological effect on these vital organs (Figures 9, 10, 11 and 12).

DISCUSSION

For centuries, natural products especially medicinal plants are used for the treatment of different diseases.⁴⁷ In the screening of

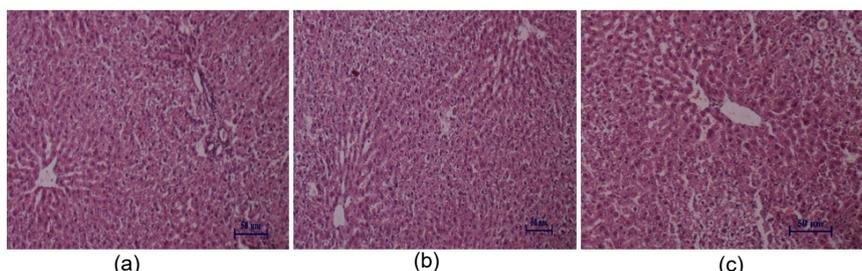


Figure 9: Histopathological studies of liver tissue: (a) Control mice, (b) EECGL treated (50 mg/kg body wt.), (c) EECGL treated (2000 mg/kg body wt.).

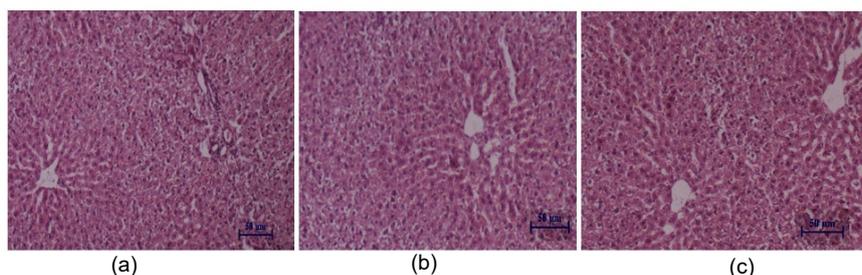


Figure 10: Histopathological studies of liver tissue: (a) Control mice, (b) WECGL treated (50 mg/kg body wt.), (c) WECGL treated (2000 mg/kg body wt.).

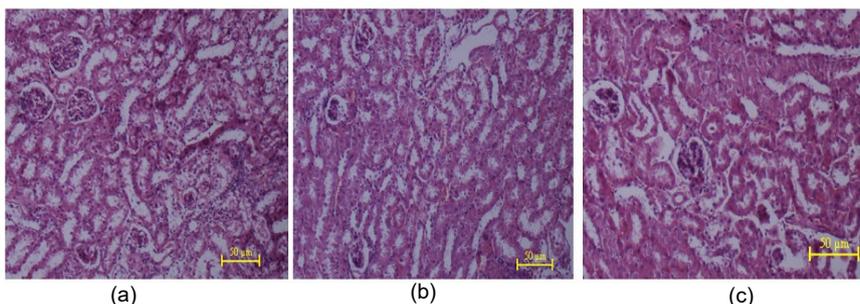


Figure 11: Histopathological studies of kidney tissue: (a) Control mice, (b) EECGL treated (50 mg/kg body wt.), (c) EECGL treated (2000 mg/kg body wt.).

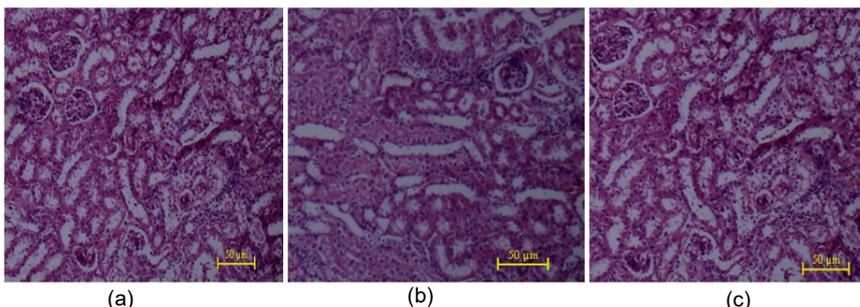


Figure 12: Histopathological studies of kidney tissue: (a) Control mice, (b) WECGL treated (50 mg/kg body wt.), (c) WECGL treated (2000 mg/kg body wt.).

pharmacological efficacy of a medicinal plant, the initial step is the assessment and evaluation of its toxic characteristics and the present study was undertaken to investigate the sub-acute toxicity of *C. gigantea* in a mammalian model.

No significant changes was observed in the weights of the heart, liver, spleen, kidneys suggesting that the administration of *C. gigantea* extract at the sub-acute doses had no effect on the normal growth. In toxicity studies, the worth of weighing organs takes account of their sensitivity to envisage toxicity, enzyme stimulation, physiologic perturbations, and acute injury.⁴⁸ The relative organs weights are also relatively sensitive indicators for particular organs in toxicity studies.⁴⁹ The findings of this study exposed that the vital organs, such as liver, kidneys, were not adversely affected for toxicity throughout the treatment. Since there was no reduction in body and relative organ weights of the treated animals at any of the tested dose level, we concluded that the extracts are nontoxic to the analyzed organs.

To evaluate the probable changes in hepatic and renal functions influenced by the extracts the serum haematology and clinical biochemistry studies were done liver and kidney function analysis is very important in the toxicity evaluation of plant extracts as they are both necessary for the survival of an organism.⁵⁰ The assessment of activities of serum marker enzymes plays important role in the evaluation of plant extract for its toxicity risk. The enzymes considered in this study, are valuable marker enzymes of liver cytolysis and liver cell membrane damage. SGOT and SGPT, the transaminases are well known good indicators of liver function and used as biomarkers to conclude the probable toxicity of drugs. Normally, destruction to the liver parenchymal cells will result in an increase of both these enzymes in the blood.⁵¹ Alkaline phosphatase is a marker enzyme for the plasma membrane and endoplasmic reticulum of the tissue studied. High levels of alkaline phosphatase are reported in liver diseases or hepatotoxicity.⁵² The insignificant changes in alkaline phosphatase in male mice at all dose level suggest that acute administration of *C. gigantea* extract does not affect the hepatocyte function in mice. Renal dysfunction can be measured by simultaneous measurements of urea, creatinine and their normal levels observed at reduced renal problems.⁵³ Higher than normal levels of serum creatinine and urea are good indicators of renal function abnormality.⁵⁴ Thus, the decrease in serum creatinine concentrations with concomitant decrease in the serum urea concentration in the treated rats suggests that functioning of the kidney is normal. In the present study, changes in serum urea, creatinine levels in *C. gigantea* extract treated groups showed non-significant differences indicating a normal renal function up to the dose level of 500 mg/kg body wt.

Evaluation of haematological parameters can be used to estimate the extent of the harmful effect of *C. gigantea* extract on the blood of an animal. It can also be used to explain blood related functions of a plant extract or its products.⁵⁵ Moreover, for risk evaluation such analysis is important as haematological

changes have higher prognostic value for human toxicity when the data are obtained from mammalian studies.⁵⁶ A haemogram was undertaken for all the *C. gigantea* extract treated and control groups and the results show no significant effects up to the dose level of 1000 mg/kg bw. The non-significant effect of the extract on total red blood cells, Hb percentage indicates that the *C. gigantea* extract does not affect the RBC morphology or formation, or its osmotic fragility.⁵⁷ Leukocytes are the first line of cellular defence that counter tissue injury, infectious agents or inflammatory process. Furthermore, *C. gigantea* extracts produced no significant changes in WBC count, which further confirmed the above findings. A normal haematological profile of *C. gigantea* extract treated groups also further justified the non-toxic nature of *C. gigantea* extract. In histopathological studies, the liver of treated animals showed normal histological feature at 50, 2000 mg/kg. No degeneration of hepatocyte, focal steatosis, congestion of central vein and inflammation of portal tract when compared with control animals. The kidney of treated rats showed normal glomeruli and there is no necrosis of tubular epithelium in the kidney. Gross histological examination of liver and kidney on did not reveal any abnormalities. Thus, it was concluded that *C. gigantea* did not produce any toxic effect in male albino mice.

From these findings, we may conclude that EECGL and WECGL are not toxic up to the dose level of 500 mg/kg body wt./day for 28 days and did not produce any noteworthy significant ($p < 0.05$) alterations in relative organ weights, haematological, hepatic and renal biomarkers except blood glucose, serum protein and serum glutamate pyruvate transaminase (SGPT). No significant toxicity was seen up to the dose level of 1000 mg/kg body wt./day for 28 days in case of blood glucose, serum protein and SGPT, which are important toxicity biomarkers. As there were no significant adverse effects on the liver and kidney histology, haematological and serum biomarkers up to the dose level of 500 mg/kg body wt., it may be concluded that the ethanolic and water extract of *C. gigantea* latex did not induce any damage to the vital organs and may be quite safe for mammals.

CONCLUSION

In conclusion, the present investigation establishes that the ethanolic extract and water extract of *C. gigantea* latex may be considered as absolutely safe up to 500 mg/kg body wt. dose level, as it did not create any toxicological effects on selected body organs, haematological and serum biomarkers of mice during the sub-acute toxicity study. So, the findings suggest that ethanolic and water extracts of *Calotropis gigantea* latex do not produce any effective sub-acute toxicity in mice and may be considered as phytomedicinal therapeutic agents.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

REFERENCES

- Habib MR, Karim MR. Effect of anhydrosophoradiol-3-acetate of *Calotropis gigantea* (Linn.) flower as antitumor agent against Ehrlich's ascites carcinoma in mice. *Pharmacol Rep.* 2013; 65: 761-767. Web site. http://www.if-pan.krakow.pl/pjp/pdf/2013/3_761.pdf. Accessed November 2, 2016.
- Dias FDL, Takahashi CS. Cytogenetic evaluation of aqueous extracts of the medicinal plants *Alpinia mutans* rose (Zingiberaceae) and *Pogostemon hynceanus* benth (Labiateae) on Wistar rats and *Allium cepa* (Liliaceae) root tip cells. *Braz J Genet.* 1994; 17(2): 175-180. Web site. <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3872372/>. Accessed November 2, 2016.
- Olejniczak K, Günzel P, Bass R. Preclinical testing strategies. *Drug Int J.* 2001; 35(2): 321-336. doi: [10.1177/009286150103500202](https://doi.org/10.1177/009286150103500202)
- Sengupta M, Sharma GD, Chakroborty B. Hepatoprotective and immunomodulatory properties of aqueous extract of *Curcuma longa* in carbon tetrachloride intoxicated Swiss albino mice. *Asian Pac J Trop Biomed.* 2011; 1: 193-199. doi: [10.1016/S2221-1691\(11\)60026-9](https://doi.org/10.1016/S2221-1691(11)60026-9)
- Ukwuani AN, Abubakar MG, Hassan SW, Agaie BM. Toxicological studies of hydromethanolic leaves extract of *Grewia crenata*. *Int J Pharma Sci Drug Res.* 2012; 4: 245-249. Web site. <http://ijpsdr.com/pdf/vol4-issue4/5.pdf>. Accessed November 2, 2016.
- Robinson S, Ockert D, Stei P, Dreher D. Challenging the regulatory requirement for conventional acute toxicity studies in pharmaceutical drug development. *Toxicol.* 2007; 231(2-3): 96.
- Ghani A. *Medicinal Plants of Bangladesh*. 2nd ed. Dhaka, Bangladesh: Asiatic Society of Bangladesh; 2003.
- Kumar S. Hepatoprotective activity of Hepax-A polyherbal formulation. *Asian Pac J Trop Biomed.* 2011; 1(2): 142-146. doi: [10.1016/S2221-1691\(11\)60013-0](https://doi.org/10.1016/S2221-1691(11)60013-0)
- Wills PJ, Asha VV. Acute and subacute toxicity studies of *Lygodium flexuosum* extracts in rats. *Asian Pac J Trop Biomed.* 2012; S200-S202. doi: [10.1016/S2221-1691\(12\)60159-2](https://doi.org/10.1016/S2221-1691(12)60159-2)
- Wills PJ, Asha VV, Pour BM, Sasidharan S. *In vivo* toxicity study of *Lantana camara*. *Asian Pac J Trop Biomed.* 2011; 1(3): 230-232. doi: [10.1016/S2221-1691\(11\)60033-6](https://doi.org/10.1016/S2221-1691(11)60033-6)
- Dash VB. *Materia Medica of Ayurveda: Based on: Madanapala's Nighantu*. New Delhi, India: B. Jain Publishers; 2002.
- Pal DC, Jain SK. *Tribal Medicine*. Calcutta, India: Naya Prokash; 1998.
- Varier PS. *Indian Medicinal Plants*. Vol.1. New Delhi, India: Orient Longman Pvt. Ltd; 2003: 341-343.
- Kirtikar KR, Basu BD. *Indian Medicinal Plants*. Vol.3. Dehradun, India: International Book Distributors; 2005: 1607-1609.
- Mueen Ahmed KK, Rana AC, Dixit VK. Free radical scavenging activity of *Calotropis species*. *Ind Drugs.* 2003; 40: 654-655.
- Kshirsagar A, Purnima A, Ingawale D, Vyawahare N, Ingale K, Hadambar A. Antioxidant and hepatoprotective activity of ethanolic extract of *Calotropis gigantea* against paracetamol induced liver damage in mice. *J Cell Tissue Res.* 2009; 9: 1859-1864. Web site. <http://search.proquest.com/openview/49e893182b2b288a4f552ef2c325a268/1?pq-origsite=gscholar>. Accessed November 2, 2016.
- Rajesh R, Raghavendra Gowda CD, Nataraju A, Dhananjaya BL, Kemparaju K. Procoagulant activity of *Calotropis gigantea* latex associated with fibrin (ogen)olytic activity. *Toxicol.* 2005; 46: 84-92.
- Srivastava SR, Keshri G, Bhargavan B, Singh C, Singh MM. Pregnancy interceptive activity of the roots of *Calotropis gigantea* Linn. in rats. *Contraception.* 2007; 75: 318-322
- Pardesi GS, Gadgoli C, Vaidya MD, Hasni HY, More BH, Bhuskat P. Preliminary studies on antimetabolic and anticancer activity of *Calotropis gigantea*. *Pharmacologyonline.* 2008a; 1: 38-47.
- Pardesi GS, Gadgoli C, Vaidya MD, Hasni HY, More BH, Bhuskat P. Immunomodulatory activity of *Calotropis gigantea* by cyclophosphamide induced myelosuppression. *Pharmacologyonline.* 2008b; 2: 164-167. Web site. <http://pharmacologyonline.silae.it/files/newsletter/2008/vol2/18.Pardesi.pdf>. Accessed November 2, 2016.
- Deshmukh PT, Fernandes J, Akarte A. Wound healing activity of *Calotropis gigantea* root bark in rats. *J Ethnopharmacol.* 2009; 125(1): 178-181. doi: [10.1016/j.jep.2009.06.007](https://doi.org/10.1016/j.jep.2009.06.007)
- Adak M, Gupta JK. Evaluation of anti-inflammatory activity of *Calotropis gigantea* (AKANDA) in various biological systems. *Nepal Med Coll J.* 2006; 8: 156-161. Web site. <http://europepmc.org/abstract/med/17203820>. Accessed November 2, 2016.
- Chitme HR, Chandra R, Kaushik S. Studies on anti-inflammatory activity of *Calotropis gigantea* in experimental animals. *Asia Pac J Pharmacol.* 2006; 16: 163-168.

24. Saumya D, Sanjita D, Manas KD, Saumya PB. Evaluation of anti-inflammatory effect of *Calotropis gigantea* and Tridax procumbens on Wistar albino rats. *J Pharm Sci Res.* 2009; 4: 123-126. Web site. <http://www.jpsr.pharmainfo.in/Documents/Volumes/Vol1Issue4/jpsr01040916.pdf>. Accessed November 2, 2016.
25. Awasthi S, Irshad M, Das MK, Ganti SS, Rizvi MA. Anti-inflammatory activity of *Calotropis gigantea* and Tridax procumbens on carrageenin-induced paw edema in rats. *Ethnobotanical Leaflets.* 2009; 13: 568-577. Web site. <http://opensiuc.lib.siu.edu/ebl/vol2009/iss5/2/>. Accessed November 2, 2016.
26. Singh HK, Pant KK, Hussain Z. Hepatoprotective effects of *Calotropis gigantea* extract against carbon tetrachloride induced liver injury in rats. *Acta Pharm.* 2009; 59(1): 89-96. doi: [10.2478/v10007-009-0002-2](https://doi.org/10.2478/v10007-009-0002-2)
27. Mishra M, Gautam RK, Mathur R. Evaluation of antifertility potential of *Calotropis gigantea* linn. in female albino rats. *J Pharmaceutical Res Opinion.* 2011; 1(3): 92-93. Web site. <http://innovativejournal.in/index.php/jpro/article/view/660>. Accessed November 2, 2016.
28. Ogbonnia SO, Nkemhule FE, Anyika EN. Evaluation of acute and subchronic toxicity of *Stachytarpheta angustifolia* extract in animals. *African J Biotech.* 2009; 8(9): 1793-1799.
29. Maity P, Bepari M, Maiti Choudhury S. Evaluation of antimutagenic and cytotoxic potential of *Anacardium occidentale* leaf extracts in *Allium cepa* root tip cells and against sarcoma-180 cells. *Int J Sci. Res.* 2015; 4(4): 290-293. Web site. <http://www.ijsr.net/archive/v4i4/SUB152818.pdf>. Accessed November 2, 2016.
30. Biswas M, Karan TK, Bhattacharya S, Kumar RBS, Ghosh AK, Haldar PK. Acute and sub-chronic toxicity study of *Terminalia arjuna* leaf in swiss albino mice. *Pharmacologyonline.* 2011; 1: 366-371. Web site. http://www.ayurvedjournal.com/JAHM_201513_06.pdf. Accessed November 2, 2016.
31. OECD. OECD Guidelines for the Testing of Chemicals. Acute oral toxicity: Up-and-down-procedure (UDP). 2008: 27. Web site. https://ntp.niehs.nih.gov/iccvam/docs/acutetox_docs/udpproc/udpfn01/append/apph.pdf. Accessed November 2, 2016.
32. Coria-Avila GA, Gavrilá AM, Ménard S, Ismail N, Pfaus JG. Cecum location in rats and the implications for intraperitoneal injections. *Lab Anim (NY).* 2007; 36(7): 25-30. Web site. <http://search.proquest.com/openview/9b54a67f3ae9c6f31ebb57d7199690f3/1?pq-origsite=gscholar&cbl=2041949>. Accessed November 2, 2016.
33. Sunday R, Ilesanmi O, Obuotor E. Acute and subacute toxicity of aqueous extract of *Abrus Precatorius* seed in wister rats. *Internet J Pharmacol.* 2013; 11(1).
34. Hosseinzadeh H, Shakib SS, Sameni AK, Taghiabadi E. Acute and subacute toxicity of safranal, a constituent of Saffron, in mice and rats. *Iran J Pharm Res.* 2013; 12(1): 93-99.
35. Wintrobe MM. *Clinical Hematology.* 6th ed. Lea and Febiger, PA, USA: Library of Congress, Print USA; 1967.
36. Dacie JV, Lewis SM. *Practical Haematology.* 5th ed. London, England, UK: Churchill Livingstone; 1975: 628.
37. Natelson S, Scott ML, Beffa C. A rapid method for the estimation of urea in biological fluids. *Am J Clin Pathol.* 1951; 21: 275-281.
38. Aseervatham J, Palanivelu S, Sachdanandam P. Cytoprotective effect of *Semecarpus anacardium* against toxicity induced by Streptozotocin in rats. *J Exp Pharmacol.* 2010; 2: 135-143. doi: [10.2147/JEP.S11466](https://doi.org/10.2147/JEP.S11466)
39. Brod J, Sirota JH. The renal clearance of endogenous "creatinine" in man. *J Clin Investigation.* 1948; 27: 645-654. doi: [10.1172/JCI102012](https://doi.org/10.1172/JCI102012)
40. Maheswari C, Venkatnarayanan R. Protective effect of Orthosiphonstamineus leaves against lead acetate and cadmium chloride induced dysfunction in rats. *Int Res J Pharmacy.* 2013; 4(4).
41. Malamy M, Horecker BL. Alkaline phosphatase (crystalline). In: Willis AW, ed. *Methods in Enzymology.* Vol 9. Massachusetts, USA: Academic Press; 1966: 639-642.
42. Goel BK. Routine biochemical test. In: Mukherjee KL, ed. *Medical Laboratory Technology.* Vol.3. New Delhi, India: Tata McGraw-Hill Publishing Company Ltd; 1988: 985-1079.
43. Nelson N. A photometric adaptation of the somogyi method for the determination of glucose. *J Biol Chem.* 1944; 153: 375-380. Web site. <http://www.jbc.org/content/153/2/375.full.pdf>. Accessed November 2, 2016.
44. Somogyi M. Notes on sugar determination. *JBC.* 1952; 195: 19-23. Web site. <http://www.jbc.org/content/195/1/19.long>. Accessed November 2, 2016.
45. Zlatkis A, Zak B, Boyle AJ. A new method for the direct determination of serum cholesterol. *J Lab Clin Med.* 1953; 41(3): 486-492.
46. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem.* 1951; 193: 265-275. Web site. <http://www.jbc.org/content/193/1/265>.

[full.pdf](#). Accessed November 2, 2016.

47. Ridditid W, Sae-Wong C, Reanmongkol W, Wongnawa M. Antinociceptive activity of the methanolic extract of *Kaempferia galanga* linn. in experimental animals. *J Ethnopharmacol*. 2008; 118(2): 225-230. doi: [10.1016/j.jep.2008.04.002](https://doi.org/10.1016/j.jep.2008.04.002)

48. Michael B, Yano B, Sellers RS. Evaluation of organ weights for rodent and non-rodent toxicity studies: A review of regulatory guidelines and a survey of current practices. *Toxicol Pathol*. 2007; 35(5): 742-750. doi: [10.1080/01926230701595292](https://doi.org/10.1080/01926230701595292)

49. Kluwe WM. Renal function tests as indicators of kidney injury in subacute toxicity studies. *Toxicol Appl Pharmacol*. 1981; 57(3): 414-424. doi: [10.1016/0041-008X\(81\)90239-8](https://doi.org/10.1016/0041-008X(81)90239-8)

50. Olorunnisola OS, Bradley G, Afolayan AJ. Acute and sub-chronic toxicity studies of methanolic extract of *Tulbaghia violacea* rhizomes in Wistar rats. *Afr J Biotechnol*. 2012; 11: 14934-14940.

51. Wolf PL, Williams D, Coplon N, Coulson AS. Low aspartate trans-aminase activity in serum of patients undergoing chronic hemodialysis. *Clin Chem*. 1972; 18(6): 567. Web site. <http://clinchem.aaccjnls.org/content/18/6/567.long>. Accessed November 2, 2016.

52. Brautbar N, Williams J II. Industrial solvents and liver toxic-

ity: Risk assessment, risk factors and mechanisms. *Int J Hygiene Environ Health*. 2002; 205(6): 479-491. doi: [10.1078/1438-4639-00175](https://doi.org/10.1078/1438-4639-00175)

53. Davis ME, Bredt ND. *Principles and Methods of Toxicology*. New York, NY, USA: Raven Press; 1994.

54. Whelton A, Watson AJ, Rock RC. Nitrogen metabolites and renal function, in Tietz. In: Burtis CA, Ashwood ER, eds. *Textbook of Clinical Chemistry*. Philadelphia, PA, USA: W. B. Saunders; 1994.

55. Yakubu MT, Akanji MA, Oladiji AT. Haematological evaluation in male albino rats following chronic administration of aqueous extract of *Fadogia agrestis* stem. *Pharmacognosy Magazine*. 2007; 3(9): 34-38. Web site. <http://www.phcog.com/article.asp?issn=0973-1296;year=2007;volume=3;issue=9;spage=34;epage=38;aulast=Yakubu;type=0>. Accessed November 2, 2016.

56. Olson H, Betton G, Robinson D. Concordance of the toxicity of pharmaceuticals in humans and in animals. *Regul Toxicol Pharmacol*. 2000; 32(1): 56-67. doi: [10.1006/rtp.2000.1399](https://doi.org/10.1006/rtp.2000.1399)

57. Guyton AC, Hall JE. *Textbook of Medical Physiology*. Philadelphia, PA, USA: W. B. Saunders; 2000.