Chapter 3: Toxicity study of *Calotropis gigantea Linn* latex extracts

Abstract

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Abstract

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 acute and sub-acute toxic effects of the ethanol (EECGL) and water (WECGL) extract of *Calotropis gigantea* latex in brine shrimp and zebra fish embryo, human lymphocytesas well as in Swiss albino mice. The results showed that EECGL and WECGL do not show any significant change in ROS generation, DNA fragmentation in lymphocytes using comet assay.The LC50 value of EECGL and WECGL for brine shrimp (*A. salina)* was 1024 and 1280 µg/ml respectively. The two extracts at 2000 µg/ml concentration exhibited 100 % mortality in *A. salina*. Slight edema was observed on abdominal area and thoracic cavity of zebra fish embryos at 48 hpf after the treatment of EECGL and WECGL at the concentration of 2000 µg/ml. EECGL and WECGL do not show any significant change in ROS generation, DNA fragmentation in human lymphocytes. In the sub-acute repeated dose 28-day toxicity study, ethanol and methanol latex extract of *Calotropis gigantea* were administered intraperitoneally at the dose levels of 50, 100, 200, 500, 1000 and 2000 mg/kg body wt. /day. No significant ($p<0.05$) difference were observed in relative organ weights and haematological, hepatic and renal biomarkers up to the dose level of 500mg/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 in mice up to the dose level of 1000 mg/kg body wt. /day for 28 days in case of blood glucose and SGPT.

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3.1 Introduction

Presently 25% of prescribed drugs worldwide are derived from plant sources in spite of the great progress and advancement of organic synthesis (Habib and Karim, 2013). 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 of those drugs or products from medicinal plants may cause to the consumers (Dias and Takahashi, 1994). 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 (Olejniczak et al.,2001). 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 (Sengupta et al.,2011). 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 (Ukwuani et al.,2012).

The vast majority of toxicity testing is carried out in the framework of regulatory necessities governing particular types of chemical in different parts of the world. Regulatory bodies frequently give importance to the necessity of toxicity tests to preserve existing levels of human health and environmental protection (European Commission, 2004).

Over the past three decades present testing regimes have developed significantly. After the change of existing practices new methods have been added. Organisation for Economic Cooperation and Development (OECD) has a major influence on the developments of test guidelines programme. OECD has developed standardised methods of testing and all 30 OECD member countries have accepted these through an agreement on the mutual acceptance of data (OECD,2001). The OECD approach has mostly removed the necessity for testing in accordance to different protocols to satisfy regulatory authorities in different countries, and has thus substantively reduced the total number of animals used for certain standard tests.

Toxicity tests

The types of toxicity studies which are routinely executed by pharmaceutical manufactures 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 (Robinson et al.,2007).

Acute toxicity test

Acute toxicity tests are generally the first tests conducted and they provide critical data on the relative toxicity likely to arise from a single or brief exposure. The aim of the acute toxicity test is to find out the therapeutic index, which is the ratio between the lethal dose and the pharmacologically effective dose, in the same species and strains (LD_{50}). The acute test may also provide initial information on the mode of toxic action of a substance and to determine the LD_{50} value that provide indices of potential types of drug activity.

Acute toxicity has been defined by the organization for economic corporation and development (OECD). The purpose of toxicity testing is to provide evidence concerning the toxicological properties of plant extracts, products, chemicals and commercial products, so as to decide whether they are safe for use and to establish the safe limits in conditions of use.The modification to the classical LD50 test includes the fixed-dose procedure, OECD TG 420 (OECD,2001), the acute-toxic-class method, OECD TG 423 (OECD,2001), and the up-and-down procedure, OECD TG 425 (OECD,2001). The test is of value in minimizing the number of animals required to estimate the acute oral toxicity of a chemical. The method permits estimation of an LD50 with a confidence interval and the results allow a substance to be ranked and classified according to the Globally Harmonised System (GHS) (Bhardwaj and Gupta.,2012).

Sub-acute toxicity test

In animal model, sub-acute test has been conducted from 14 days to 28-days. The data from these studies provide valuable information on the cumulative exposure of target organs, and on general health hazards likely to occur as a consequence of repeated low-dose exposure to a chemical. There are OECD Organization for Economic Cooperation and Development) Test Guidelines describing short-term repeat-dose toxicity testing are Repeated Dose 28-day Oral Toxicity Study in Rodents (OECD TG407).Repeated-dose toxicity studies have three main objectives (i) to identify toxicity that develops only after a certain length of continuous exposure to the chemical, (ii) to identify the organs most affected and (iii) to determine the doses at which each effect occurs.

The toxicity data may be required to envisage the safety and effects of long term exposure to a specific medicinal plant. To the best of our knowledge, there is lack of report in the literature about toxicity profile of *Calotropis gigantea* latex. The objective of this study was

to test for the acute toxicity of *Calotropis gigantea* latex exudate so as to validate its use in an effective and safe way. Brine shrimp test (BST) tests and morphological toxicity in zebra fish embryos was performed to achieve this objective. *In-vitro* toxicity study on human lymphocyte were carried out to know the safety concern of test extracts.

This study was also carried out to evaluate the sub-acute toxic effects of the ethanol and methanol extract of *Calotropis giganteal* atex in mice model using haematology, serum biochemistry, and histopathological changes as important toxicity indices.

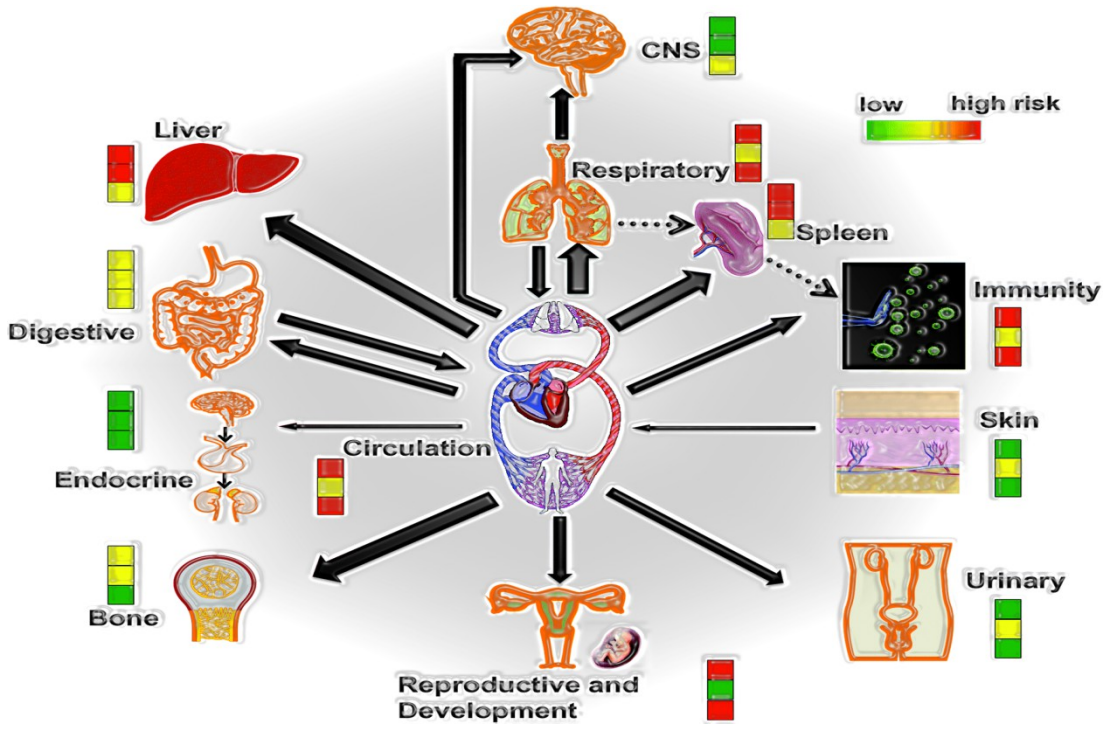


Figure 3.1 Effects of toxicants on the physiological system

3.2 Materials and methods

3.2.1 Chemicals

Sodium tungstate,sulphuric acid (H_2SO_4), diacetylmonoxime, urea, picric acid, sodium hydroxide (NaOH),p-nitrophenol phosphate (PNPP),dinitrophenyl hydrazine (DNPH),DL-aspartate, α ketoglutarate, DL-alanine, glacial acetic acid, cholesterol, ferric chloride(FeCl3), Bovine serum albumin (BSA), sodium carbonate ($NaHCO_3$), sodium potassium tartarate, copper sulphate ($CuSO_4$), sodium hydroxide (NaOH), Folin-Ciocalteau solution,haematoxylin, eosin ,5-Dithiobis-(2-nitrobenzoic acid) (DTNB), Tris–HCl, Tris buffer, Titron X- 100, phenol, chloroform, iso-amyl alcohol and other chemicals were purchased from Sigma-Aldrich, St. Louis, MO, USA; Himedia, India, Ltd., Mumbai, India; and Merck India, Ltd., Mumbai,India were purchased for the experimentation

3.2.2 Collection and extraction of plant material (Maity et al.,2015)

This section has been discussed in chapter-2.

3.2.3*In-vitro* acute toxicity study

3.2.3.1 Brine shrimp lethality test

A toxicity evaluation of medicinal plants, in this study brine shrimp lethality test (BSLT) with *Artemia salina* (*A.salina*) was designated. Artemias (Artemiidae) are type of salt-water shrimp invertebrate, which are fond in most 500 salty lakes (Mirzaei and Mirzaei, 2013). Most plant toxicity studies and research have been carried out by *A.salina*, along with it also seed for screening of chemical and natural products toxicity and isolation of active components in herbal extracts (Sadighara et al., 2010). BST is a simple tool to guide screening active plant extracts, where one of the simplest biological responses to monitor is lethality, since there is only one criterion: either dead or alive. BST reflects a good correlation

with cytotoxicity (McLaughlin, 1991) and bioactivity of a given compound or material (Sorgeloos, 1978).

Figure 3.2 Brine shrimp larvae

3.2.3.2Collection and hatching of brine shrimp

The brine shrimp egg was collected from Comprehensive Area Development Project(Tamluk) of West Bengal Government. The *Artemia*brine shrimp eggs were kept in a hatching chamber (1L) filled withartificial sea water.Regular air flow with average pressure and proper light was supplied for 48 hrs. and pH was adjusted to alkaline range 8.5. After hatching the active nauplii were collected with a plastic pipette for study (Amara and EL-Masry, 2008; Kumaret al., 2011).

3.2.3.3Procedure of brine shrimp lethality bioassay

About 4.5 ml of brine solution was taken into each test tube. Suitable dilution of the test substance (10-500µg/ml) was made as per the concentration. The 0.5 ml diluted solution was added to the test tubes. Ten active shrimps were added into each test tube by drawing them with glass capillary tube. Others were placed in brine water to serve a negative control. The solution was mixed thoroughly with the help of cyclomixer. After24 h the nauplii were

examined and the average number of survived larvae was determined. The toxicity rate of extracts was estimated using the following equation.

% of mortality or death rate = (d test-d control/A control) x 100

Where, d test = the average number of dead nauplii in the experimental groups, d control= the average number of dead nauplii in the control group, and a control= the average number of living nauplii in the control group.

3.2.3.4 *In-vitro* toxicity study on the morphology of zebrafish embryos

Zebrafish **(***Daniorerio***)** is a member ofcyprinidae family, originated in South Asia. Zebrafish prefers warm water, but they thrive in many environments.Zebrafish have been used as a model organism for biological research since 1930s and it has become a popular model for developmental biology, toxicology and recently for drug discovery (Kimmel et al.,1995; Peterson et al., 200; Parnget al., 2002).

3.2.3.5Collection and hatching of zebra fish embryo

Adult zebra fish were purchased and maintained at temperature (28±1 ˚C) with photoperiod was 12:12 h light/darkness. Fishes were fed daily with live *Artemiasalina*in morning and readymade spirulina flakes at evening time. Fish were placed 1:2 (male/female) in standard water in for different tanks of 3 litre capacity. A short period of darkness was made twice by switching of the lights of the room to stimulate spawning followed by a long dark period of 12 h; sufficient air was supplied through an external pump without any disturbance in flow of water. After 16 h eggs were collected from all the tanks, rinsed from debris and transferred to the petridish with same tank water 28±1 ˚C. Normally dividing and spherical at the 256 cell stage [2.5 hour posts fertilization (hpf) to oblong stage 3.7 hpf were selected and utilized for the present study.

3.2.3.6 Procedure of toxicity study on zebra fish embryo

Stock solutions of the test extract (EECGL and WECGL) were prepared and stored at -20˚C before use, serially diluted in beaker to take desired concentration of extracts (10-1000µg/ml). Selections of fertilized eggs were done with the help of binocular microscope (Olympus). Eggs as required for the test was exposed to the different test solution in glass Petridis. Selected eggs were placed in 250 µl of test solution in 96-well plates individually. EECGL and WECGL in different concentration was treated, then the plates were covered and observed at 24 and 48 hpf with inverted microscope (Carlessonet al.,2011)

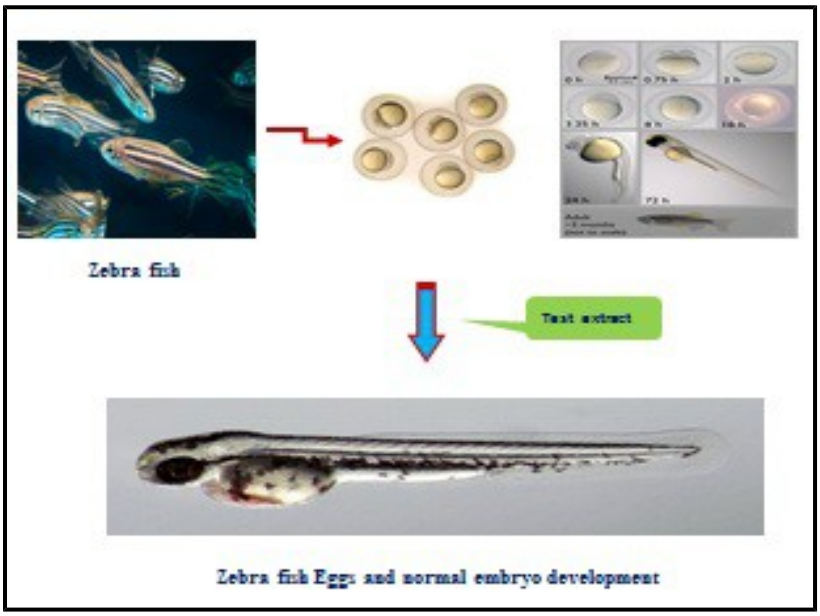


Figure 3.3 Experimental approach for zebra fish embryo toxicity screening

3.2.4Toxicity study on human lymphocytes

3.2.4.1 Collection and separation of humanlymphocytes

Blood samples were collected from the healthy young human subjects for the separation of lymphocytes. The lymphocytes were isolated from heparinized blood samples according to the method of Hudson and Hay (Hudson and Hay, 1989). Blood was taken and diluted with phosphate-buffered saline (pH 7.0) in equal ratio and then layered very carefully on Histopaque-1077, centrifuged at 2000 rpm for 30-40 min and lymphocytes were carefully removed. Then centrifuged at 1500 rpm for 10 min to get the required pellet of lymphocytes.

3.2.4.2 Culture of human lymphocytes

For *in vitro* toxicity experiments, normal human lymphocytes were cultured in RPMI 1640 medium, penicillin, and streptomycin, L-glutamine under 5% CO_2, and 95% humidified atmosphere at 37^0C.

3.2.4.5 Estimation of reduced glutathione (GSH)in human lymphocytes

At first sulfosalicylic acid was mixed with lymphocytesand the mixture was centrifuged for 10 min at 3000 rpm. Then 2 ml of 0.6 mM DTNB was added with the supernatant and was shaken well. Reading was taken at 412 nm(Griffith, 1981).

3.2.4.6 Estimation of Intracellular ROS measurement

Lymphocytes were treated with EECGL and WECGL for 24 h. After treatment, cells were washed with phosphate buffer followed by incubation with 1 mg ml^{-1} H_2DCFDA for 30 min at 37˚C(Roy et al., 2008). DCF fluorescence was determined at 485 nm excitation and 520 nm emission using a fluorescence spectrophotometer and was also observed by fluorescence microscopy (LEICA DFC295, Germany).

3.2.4.7 DNA fragmentation study by alkaline comet assay

Briefly, Lymphocytes were treated with EECGL and WECGL for 24 h. Then the cell pellet was mixed with 0.7% agarose for performing the comet assay(Alcˆantaraet al., 2011). The glass slide was smeared with 1% agarose and covered with cell pellet. After that slides were poured in lysis buffer and electrophorized. Then the slides were stained with fluorescence stain ethidium bromide and image was captured under fluorescence microscope (LEICA DFC295, Germany).

3.2.5 Sub-acute toxicity study in mice

3.2.5.1Animals

Healthy male Swiss albino mice weighing 20-25 g were selected for toxicity test. The mice were grouped and housed in poly acrylic cage (38x 23x10 cm) with 6 animal per cage and maintained under standard laboratory conditions (temperature 25 ± 2 ˚C and dark/light cycle 14/10) for 7 days before commencement of the experiment. They were allowed free access to diet and water (Biswas et al., 2011).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 & Climate Change, and Govt. of India and performed in compliance with the relevant laws and guidelines of the CPCSEA.

3.2.5.2Treatment schedule

The sub-acute toxicity study on the above mentioned plant extracts was performed as per the OECD guidelines 407 with slight modifications, where 2000 mg/kg was used as the limit test dose.The ethanol and methanol extracts of *Calotropis gigantea* latex were administered intraperitoneally prior up to the bladder (Coria-Avila et al.,2007) 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

(Sunday et al.,2013 and Hosseinzadeh et al.,2013). Toxicity studies were conducted as per internationally accepted protocol in Swiss albino mice. The animals were divided into six groups containing six 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)

3.2.5.3 Organ weight

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

3.2.5.4 Blood sample collection

On the last day of the experiment, two 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 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 -20^0 C for biochemical analysis.

3.2.5.5 Red blood cell (RBC) count

Blood was diluted 1:200 with RBC dilution fluid. Erythrocytes were counted in the haemocytometer chamber (Wintrobe, 1967).

3.2.5.6 White blood cell (WBC) count

Blood was diluted 1: 20 with white blood cell (WBC) dilution fluid and loaded in haemocytometer chamber. Four large (1sq mm) corner squares of the haemocytometer chamber were counted under the microscope (Wintrobe, 1967).

3.2.5.7 Determination of haemoglobin

At first20 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 (Dacie and Lewis, 1975).

3.2.5.8 Estimation of urea

Urea was determined by the modified method of Natelson ((Natelsonet al., 1951). 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 1.0 ml of water, 0.4 ml of diacetylmonoxime and 2.6 ml of 0.67 N sulphuric acid-phosphoric acid reagent were addedto 1.0 ml of the supernatant. Standard urea (20 to 50 µg/ml) were also prepared 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 spectrophotometer (UV-Shimadzu-245,Japan). The values were expressed as mg of urea/dl of blood.

3.2.5.9 Estimation of creatinine

Creatinine was measured according to the modified method of Brod and Sirota (Brod and Sirota, 1948). At first protein free filtrate was prepared by mixing 1ml of serum with 8.0 ml of water, 0.5 ml of 2/3 N sulphuric acid and 0.5 ml of 40% sodium tunsgstate. 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 colour intensity was measured at 530 nm in spectrophotometer (UV-Shimadzu-245, Japan). The values were expressed as mg of creatinine/dl of blood.

3.2.5.10 Measurement of serum alkaline phosphatase(ALP)

Activity of serum alkaline phosphatase were estimated by taking 0.25ml of serum in a centrifuge tube containing 1 ml buffer (1 mM of p-nitrophenol phosphate in 1M Tris buffer, pH 8.0); the mixture was then subjected to be incubated at 37ºC for 30 minutes in a water bath. The activity was measured spectrophtometrically at 420 nm (Malamy and Horecker, 1966).

3.2.5.11Measurement of serum glutamate oxaloacetate transaminase (SGOT) and serumgluamate 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 one ml of dinitrophenyl hydrazine (DNPH) was added and kept for 20 minutes at room temperature. After 20 minutes, 10 ml of 0.4N sodium hydroxide was added and the color intensity was measured at 505-540 nm in a 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 an 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 colour developed by the addition of 10 ml of 0.4N sodium hydroxide was read at 505-540 nm in spectrophotometer (UV-Shimadzu-245, Japan) against the reagent blank. The enzyme activity in serum was expressed as IU/lit (Goel, 1988).

3.2.5.12 Estimation of blood glucose

Blood glucose was measured according to the method of Nelson-Somogyi. 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 37^0C. 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 colour was measured spectrophtometrically at 540 nm (Nelson, 1944 and Somogyi,1952).

3.2.5.13 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 H_2O, 15 ml of conc. H_2SO_4) it was mixed vigorously and allowed to stand for 20 min. The reading was taken at 570nm. The amount of cholesterol present is calculated by plotting the standard curve (Zlatkis et al., 1953).

3.2.5.14Measurement of serum total protein

Different dilutions (10µl to 50µl) 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. From these different dilutions, protein reagents (98:1:1)consisting of sodium carbonate (Na_2CO_3)in 0.1 N sodium hydroxide (NaOH), sodium potassium tartarate in distilled water, copper sulphate (Cu_2SO_4) in distilled water were added to different test tubes and 10 µlof sample and 500 µl of normal saline (0.9 gm%) were also added. The solutions were mixed well. Then 500µl of reagent Folin-Ciocalteau solution was added to each tube and incubated at 37^0C for 30 min. The standards were prepared similarly. The optical density was measured at 660 nm (Lowry et al., 1951). The absorbance was plotted against protein concentration to get a standard calibration curve.

3.2.5.15 Histopathological study

All the animals from each group were sacrificed for histopathological examinations of major vital 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 (Standish et al.,2006).

3.2.6 Statistical analysis

The results were expressed as the Mean ± Standard error of mean (SEM). Statistical analysis of the collected data was performed by student's t-test. The difference was considered significant when $p<0.05$.

3.3 Results

3.3.1 *In-vitro* acute toxicity study

3.3.1.1 Effect of EECGL and WECGL on Brine shrimp lethality bioassay

In the present study the average of LC_{50} by *A.salina* in EECGL and WECGL was 1024 and 1280 µg/ml respectively. The bioactivity of two extracts against *A.Salina* exhibited 72 % and 65% mortality at 2000µg/ml concentration.

Table: 3.1 Lethal concentrations of EECGL and WECGL for brine shrimp bioassay

Samples	*Concentration(µg/ml)*	*% of Mortality*	*LC50 (µg/ml)*
Ethanolic extract of Calotropisgigantea (EECGL)	*10*	*5*	*1024*
	50	*11*	
	100	*16*	
	200	*32*	
	500	*46*	
	1000	*48*	
	2000	*72*	
Samples	*Concentration(µg/ml)*	*% of Mortality*	*LC50 (µg/ml)*
Water extract of Calotropisgigantea (WECGL)	*50*	*5*	*1280*
	100	*8*	
	200	*26*	
	500	*35*	
	1000	*40*	
	2000	*65*	

3.3.1.2 Effect of EECGL and WECGL on morphological toxicity in zebra fish embryos

Slight edema was observed on abdominal area and thoracic cavity of embryos at 24 hpf after treatment of EECGL and WECGL at optimum dose level.

Table: 3.2. Effect of EECGL and WECGL administration on Zebra fish embryos

Observation	*Control*	*EECGL (µg/ml)*						*WECGL (µg/ml)*					
		50	*100*	*200*	*500*	*1000*	*2000*	*50*	*100*	*200*	*500*	*1000*	*2000*
24 hpf													
Number of embryos	*5*	*5*	*5*	*5*	*5*	*5*	*5*	*5*	*5*	*5*	*5*	*5*	*5*
Tail Development	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
Eye Development	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
Head Development	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
No. of affected	*0*	*0*	*0*	*0*	*0*	*1*	*3*	*0*	*0*	*0*	*0*	*0*	*2*
Edema formation	*0*	*0*	*0*	*0*	*0*	*2*	*3*	*0*	*0*	*0*	*0*	*1*	*2*

3.3.1.3 Effect of EECGL and WECGL on human lymphocytes

3.3.1.3.1 Redox status (GSH levels)

The present study showed that increasing levels of GSH in normal lymphocyte by EECGL and WECGL. EECGL and WECGL were able to normalize the GSH in lymphocyte (Fig. 3.4).

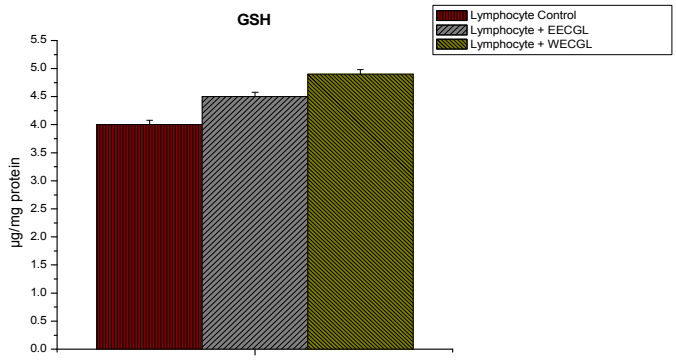


Figure 3.4Intracellular reduced glutathione (GSH) levels in EECGL and WECGL treated lymphocytes. The levels of GSH were expressed as µg of GSH mg^{-1} protein. Values are expressed as the mean ± SEM of three experiments.

3.3.1.3.2 Intracellular ROS level

In lymphocyte, ROS generation was not occurred, from the study it was confirmed that (Fig. 3.5) EECGL and WECGL did not produce intracellular ROS in human lymphocytes.

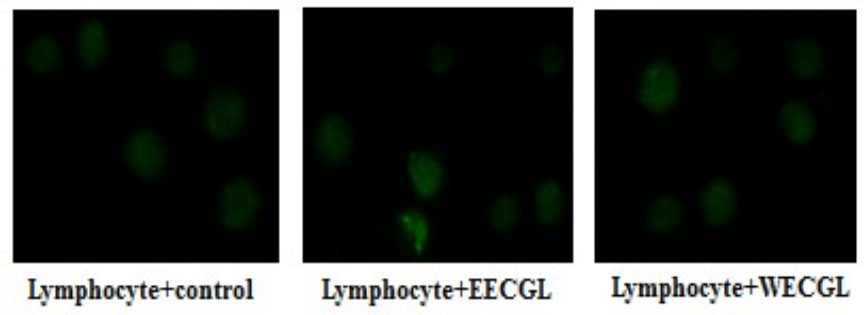


Figure 3.5Intracellular Reactive oxygen species (ROS) formation by H_2DCFDA staining. Qualitative characterization of ROS formation by $DCFH_2$-DA staining using fluorescence microscopy.

3.3.1.3.3 Genotoxicity of EECGL and WECGL on human lymphocyte cells

Genotoxicity of EECGL and WECGL was determined by the alkaline comet assay. After electrophoresis,treated cells were examined under a fluorescence microscope and images were captured (Fig. 3.6). No genotoxicity was seen in EECGL and WECGL treated human lymphocytes.

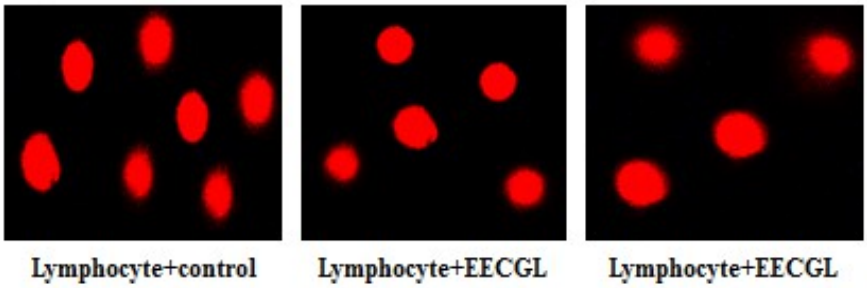


Figure 3.6Determination of the genotoxic effects of EECGL and WECGL in human lymphocytes by alkaline comet assay.

3.3.2*In-vivo* sub-acute toxicity study

3.3.2.1 Effect of ECGL and WECGL on organ weights

Relative organ weights of 28-days treated mice are shown in Table 3.3 and3.4. The relative organ weights recorded in the treatment groups did not show any significant difference ($p<0.05$) up to the dose level of 1000 mg/kg body wt. compared to the control.

Table 3.3: Effect of water extract of *Calotropisgigantea*latex(WECGL) administration for 28 days on Body and different organ weight in Swiss albino mice

Weight	*Control*	*50 mg/kg body wt.*	*100mg/kg body wt.*	*200mg/kg body wt.*	*500mg/kg body wt.*	*1000mg/kg body wt.*	*2000mg/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 groups versus all other groups. (*indicates $p<0.05$)

Table 3.4: Effect of ethanolic extract of *Calotropis gigantea* latex *(*EECGL) administration for 28 days on body and different organ weight in Swiss albino mice

Weight	*Control*	*50 mg/kg body wt.*	*100mg/kg body wt.*	*200mg/kg body wt.*	*500mg/kg body wt.*	*1000mg/kg body wt.*	*2000mg/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, Analysis is done by one way ANOVA. Comparison was done between control groups versus all other groups. (*indicates $p<0.05$)

3.3.2.2 Haematological parameters analysis

The effects of *C.gigantealatex* extractsat sub-acute doses on haematological parameters are presented in Table 3.5 and 3.6. Most haematological parameters (haemoglobin, total red blood cell count, total white blood cell 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 white blood cell counting latex extracts treated mice than that of the control animals. There was no trace of toxicity in total red blood cell counts in latex extracts treated mice up to the dose level of 2000 mg/kg body wt.

Table 3.5: Haematological parameters after WECGL administration for 28 daysin Swiss albino mice

Haematological Parameters	*Control*	*50 mg/kg body wt.*	*100mg/kg body wt.*	*200mg/kg body wt.*	*500mg/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, Analysis is done by one way ANOVA. Comparison was done between control groups versus all other groups. (*indicates $p<0.05$)

Table 3.6: Haematological parameters after EECGL administration for 28 daysin Swiss albino mice

Haematological Parameters	*Control*	*50 mg/kg body wt.*	*100mg/kg body wt.*	*200mg/kg body wt.*	*500mg/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, Analysis is done by one way ANOVA. Comparison was done between control groups versus all other groups. (*indicates $p<0.05$)

3.3.2.3 Effect of EECGL and WECGL onhepatic and renal biomarkers of mice

No significant sub-acute toxicity was seen in the level of urea (Figure 3.7), creatinine(Figure 3.8), alkaline phosphatase (Figure 3.9), and serum cholesterol (Figure 3.13) in mice treated with EECGL and WECGLup to the dose level of 500 mg/kg body wt. /dayfor 28 days when compared with the control animals.

In case of blood glucose (Figure 3.12), SGPT (Figure 3.11) and serum total protein (Figure 3.14), significantsub-acute toxicity was not observed up to the dose level of 1000 mg/kg body wt. /day.

WECGL produces no toxicity in case of SGOT(Figure 3.10) up to 1000 mg/kg body wt. /day but EECGL causes significant($p<0.05$) toxicity at this dose level.

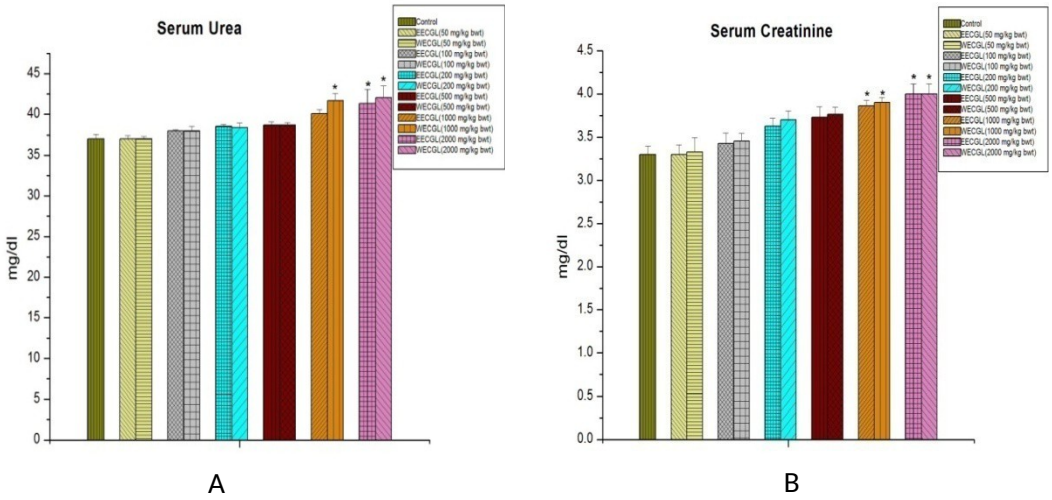


Figure 3.7 (A) shows serum urea level after treatment of EECGL and WECGL for 28 days in Swiss albino mice. (B) Serum creatinine level after treatment of EECGL and WECGL for 28 days in Swiss albino mice. Results are expressed as Mean ± SEM; n=6. Analysis is done by one way ANOVA. Comparison was done between control groups versus all other groups. (*indicates $p<0.05$).

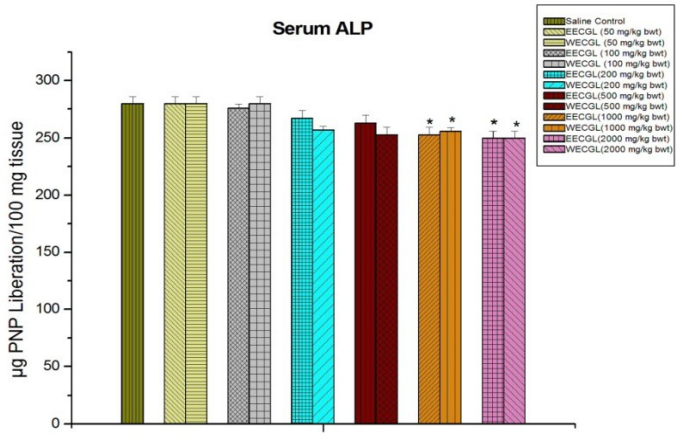


Figure 3.8shows serum ALP after treatment of EECGL and WECGL for 28 days in Swiss albino mice. Results are expressed as Mean ± SEM; n=6. Analysis is done by one way ANOVA. Comparison was done between control groups versus all other groups. (*indicates $p<0.05$).

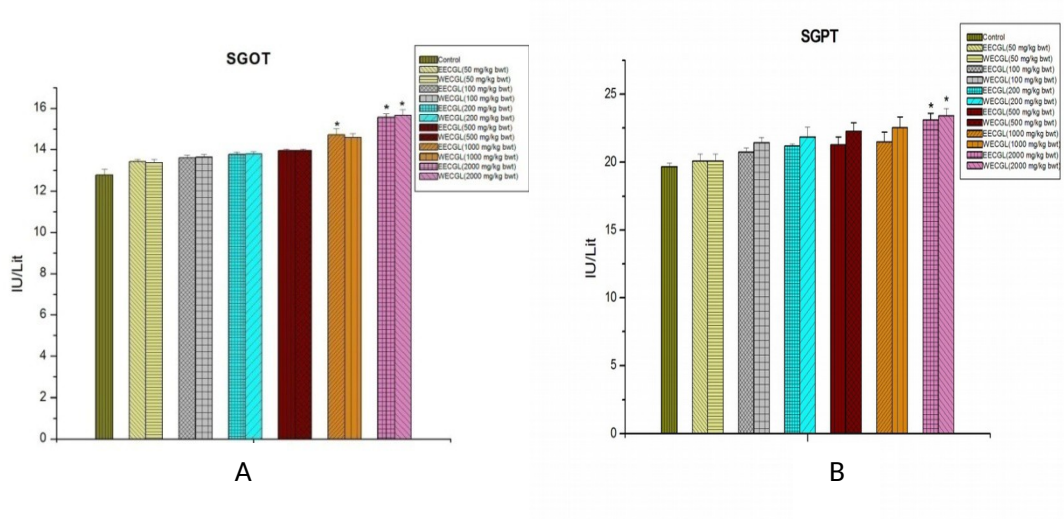


Figure 3.9. Showsthe effect of SGOT and SGPT after treatment of EECGL and WECGL for 28 days in Swiss albino mice. Results are expressed as Mean ± SEM; n=6. Analysis is done by one way ANOVA. Comparison was done between control groups versus all other groups. (*indicates $p<0.05$).

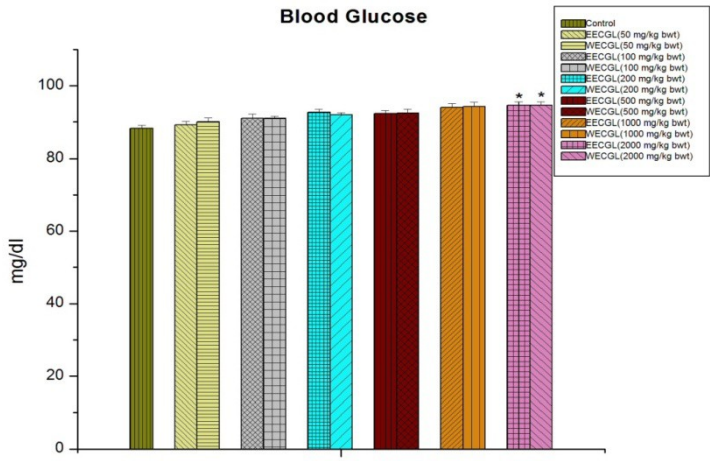


Figure 3.10. Shows the blood glucose level after treatment of EECGL and WECGL for 28 days in Swiss albino mice. Results are expressed as Mean ± SEM; n=6. Analysis is done by one way ANOVA. Comparison was done between control groups versus all other groups. (*indicates p<0.05).

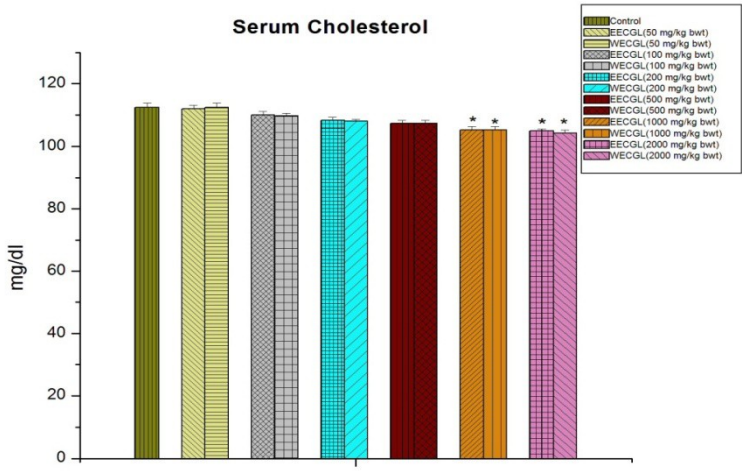


Figure 3.11. Shows the serum cholesterol level after treatment of EECGL and WECGL for 28 days in Swiss albino mice. Results are expressed as Mean ± SEM; n=6. Analysis is done by one way ANOVA. Comparison was done between control groups versus all other groups. (*indicates p<0.05).

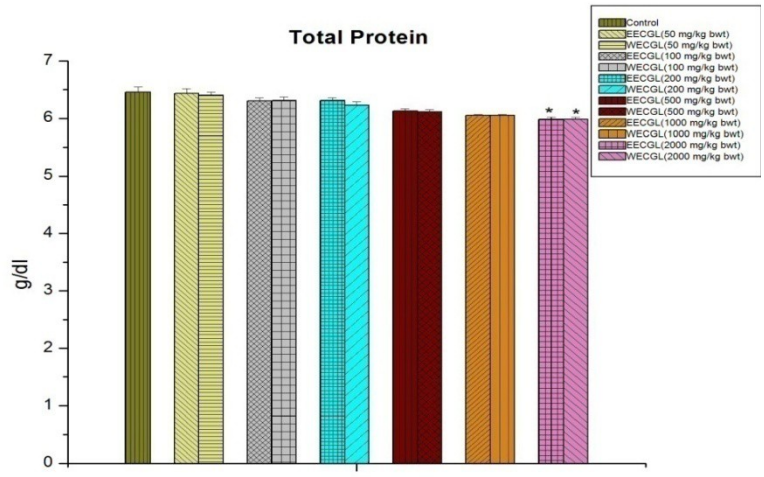
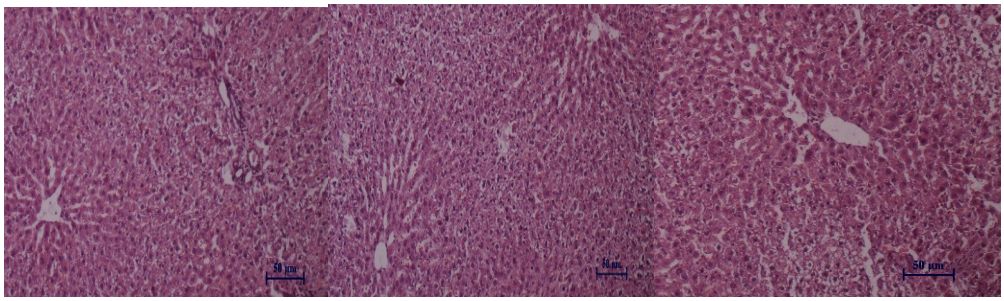
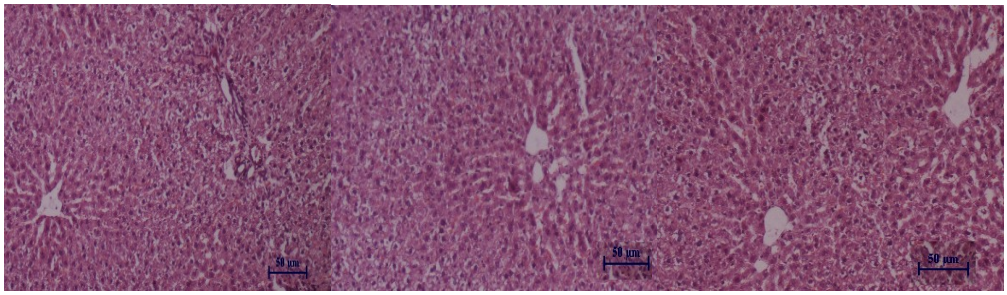


Figure 3.12. Shows serum total protein after treatment of EECGL and WECGL for 28 days in Swiss albino mice. Results are expressed as Mean ± SEM; n=6. Analysis is done by one way ANOVA. Comparison was done between control groups versus all other groups. (*indicates $p<0.05$)



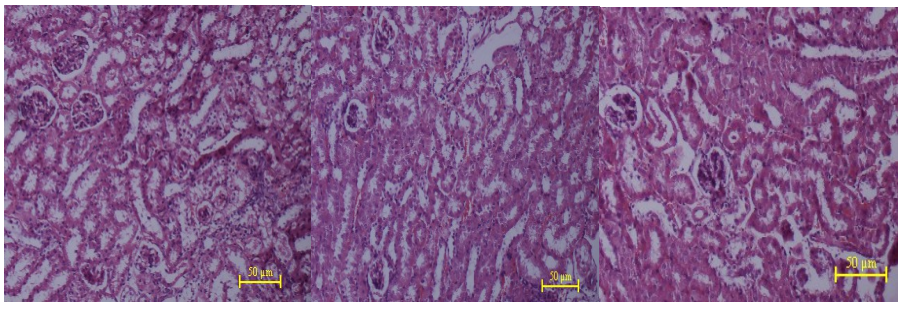
(a) (b) (c)

Figure 3.13. Shows the histopathological studies of liver tissue; (a) Control mice, (b) EECGL treated (50 mg/kg body wt.), (c) EECGL treated (2000 mg/kg body wt.)



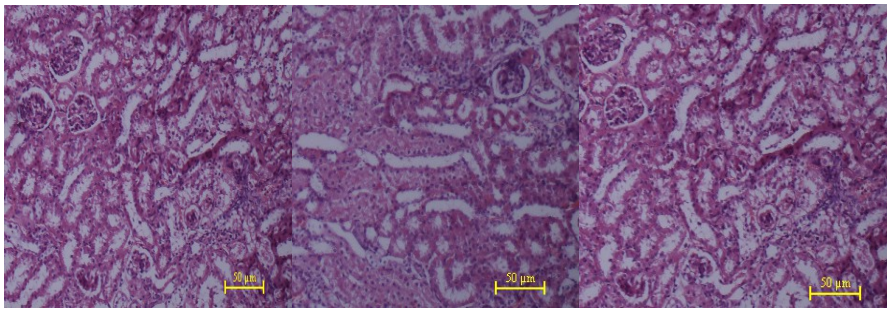
(a) (b) (c)

Figure 3.14.Showshistopathological studies of liver tissue; (a) Control mice, (b) WECGL treated (50 mg/kg body wt.), (c) WECGL treated (2000 mg/kg body wt.)



(a) (b) (c)

Figure 3.15. Shows thehistopathological studies of kidney tissue; (a) Control mice, (b) EECGL treated (50 mg/kg body wt.), (c) EECGL treated (2000 mg/kg body wt.)



(a) (b) (c)

Figure 3.16. Shows the histopathological studies of kidney tissue; (a) Control mice, (b) WECGL treated (50 mg/kg body wt.), (c) WECGL treated (2000 mg/kgbody wt.)

3.4 Discussion

This chapter illustrates a variety of general models and approaches that can be used in toxicological studies. The study covered both traditional and novel methods developed that have potential application to toxicology.

Brine shrimp and zebrafish toxicity study was included for more effective, efficientand sensitive refining of toxicants (extracts) in order to find out a minute change on biologicalproperty of organism.Brine shrimp and zebrafish toxicity differed from each other which may be due to differences in amount and type of cytotoxic compounds. In this study, there was a direct correlation between mortality rate and concentration level. The maximum and minimum mortality dose levels were 2000 and 10 µg/ml respectively

inbrine shrimp. Compounds and extracts with LC_{50} values less than 1000 µg/ml were considered toxic (Meyer et al., 1982). Therefore EECGL and WECGL in this study had biological potential as it showed nontoxic properties. Toxicity test with embryo is valuable for assessing potential impacts on growth, reproduction.From the result it was found that there was no significant morphological development like tail, head, embryonic development, pigmentation and hatching were observed in zebra fish at low and high concentration of EECGL and WECGL. But at high concentration of EECGL and WECGL on zebra fish eggs hour post fertilization reveals a minute toxic effect on hatch larvae, with a slight edema of thoracic cavity and abdominal area. This shows that EECGL and WECGL may not have any harmful on zebra fish embryos in an optimum dose level.

Glutathione is a potent inhibitor of neoplastic process. It plays an important role as an endogenous antioxidant system. Therefore in treated human lymphocytes, GSH level was slightly increased (not significant) compared to control lymphocytes. Similarly ROS formation was also not increased. The comet assay, which can detect DNA single- and double-strand breaks *in situ* has also been found to be relevant for the characterization of apoptotic cells. It is a simple, sensitive, reliable and reproducible method for the detection of DNA damage (Hughes et al., 1996). The ability of the comet assay to quantify DNA strand breaks and alkali labile sites has been well documented (McKelvey-Martin et al., 1993; Fairburn et al., 1995; Collins et al., 1997). In the present study, we have shown that the ethonolic and water extract of *Calotropisgigantea* latex exhibit no significant DNA damage in human lymphocytes.

In the screening of 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.Toxicity study on two extracts was performed and observed were made, acute

toxicity of EECGL and WECGL on mice was found to be safe up to the dose of 2000mg/kg body weight which indicates that EECGL and WECGL is safe for use.

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. The relative organs weights are also relatively sensitive indicators for particular organs in toxicity studies (Kluwe,1981).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 analysed 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 (Olorunnisolaet al., 2012). 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 (Wolf et al.,1972). 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 (Brautbar and Williams, 2002). 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 (Davis and Bredt, 1994).Higher than normal levels of serum creatinine and urea are good indicators of renal function abnormality (Whelton, 1994). 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 upto 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(Yakubu et al.,2007). 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(Olson et al., 2000). The haemogram showed no significant effects upto the dose level of 1000 mg/kg bwt. The non-significant effect of the extract on total red blood cells, haemoglobin percentage indicates that the *C.gigantea* extract does not affect the RBC morphology or formation, or its osmotic fragility (Guyton & Hall, 2000). 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. gigantean* extract. In histopathological studies, the liver of treated animals showed normal histological feature at both of 50 and 2000 mg/kg dose. 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 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 thatthe LC50 value of EECGL and WECGL for brine shrimp (A. salina) was 1024 and 1280 µg/ml respectively. The two extracts at 2000 µg/ml concentration exhibited 100 % mortality in *A. salina.* Slight edema was observed on abdominal area and thoracic cavity of zebra fish embryos at 48 hpf after the treatment of EECGL and WECGL at the concentration of 2000 µg/ml. EECGL and WECGL do not show any significant change in ROS generation, DNA fragmentation in human lymphocytes. EECGL and WECGLare 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) in mice. 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.

3.5Conclusion

From the above study it was shown thatethanolic extract and water extract of *C.gigantea latex*produces no toxic effect inbrine shrimp, zebrafish and *in vitro* human lymphocyte model at an optimum dose level. The present investigation establishes that EECGL and WECGL are absolutelysafe for mice up to 500 mg/kg body wt. dose level, as it did not create any toxicological effects on selected body organs, haematological and serum biomarkersof mice during the sub-acute toxicity study.So, the findings suggest that

ethanolic and water extracts of*Calotropisgigantea latex* do not produce any effective sub-acute toxicity in mammalian system and may be considered as phytomedicinal therapeutic agents.

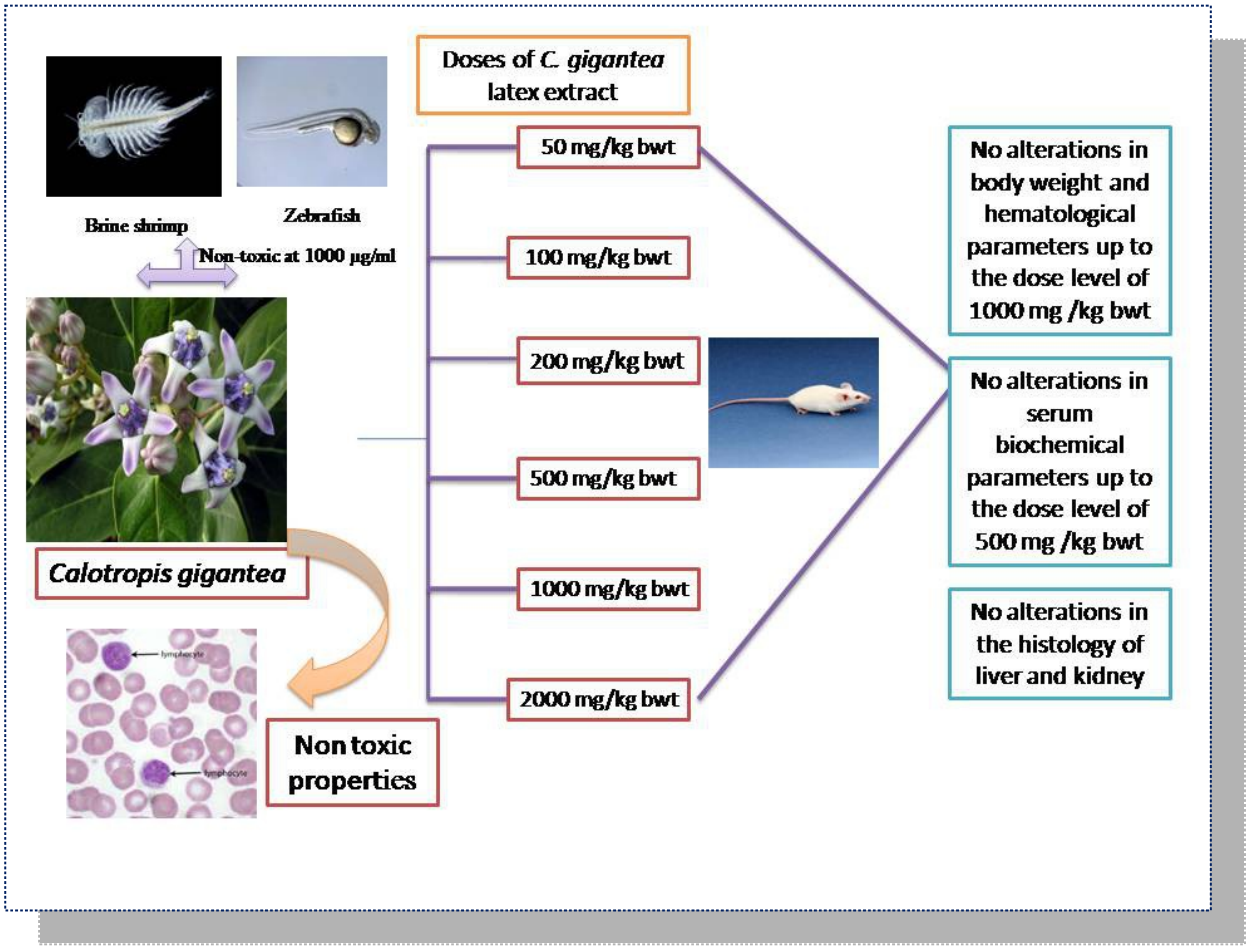


Figure 3.17. Schematic diagram of toxicity study of *calotropis gigantea* latex extract.

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