# 2a: Toxicity Evaluation of *Carica papaya* leaf extract in Murine Model

## 2a.1 INTRODUCTION:

From ancient times plants have been used for medicinal purpose. The practice of use of plants for medicinal purposes is as old as human civilization (Thakur & Sharma, 2015). Plants and plant-derived medicines have played a critical role in health and disease management for many centuries. From ancient times, civilizations such as Mesopotamian, Chinese, many of Indian civilizations, Greek as well as Unani showed documented evidences for the use of plant and plant parts in the treatment of different ailments (Biggs, 1995; Shafqat, 1995). Indian "Ayurveda" deals with thousands of herbal origin medicines for healing and treatment of diseases. Many African tribes are still using their knowledge of traditional medicine for healing and this knowledge is passed from generation to next by indigenous practices and teachings (Abdullahi, 2011). Continuous efforts are being made towards the improvement of the herbal products ( Firenzuoli & Gori, 2007; Tilburt & Kaptchuk, 2008; Mosihuzzaman, 2012 ) Till date over 2,00,000 natural products of plant origin are known and many more are being identified from higher plants and microorganisms (Kinghorn et al, 2011; Patwardhan & Mashelkar, 2009). Some plant-based drugs such as ASPIRIN and TAXOL have been of great importance in modern day medicine as well. For some, like cardiac glycosides, there is no alternative conventional medicine available. As a result there has been great demand of medicinal plant, plant parts and their bioactive molecules. Thus, medicinal plants have been central point of research as they have remained useful not only as remedy for different diseases that affect humans and animals, but also as good starting points for the discovery of bioactive molecules for drug development.

Wide range of compounds that have been identified and developed from herbs are used for pain relief, wound healing, abolishing fevers, newer therapies for cancer, hypertension, diabetes and as anti-infective (Harvey, 2008). However there is no scientific rationale to assume that plants, their parts and/or derived products, including those of long-standing popular use, are intrinsically safe and/or beneficial. Galen, a pharmacist from Greece, published the earliest report of the toxicity of herbs, which suggested that herbs do not contain only medicinally beneficial constituents, but may also be constituted with harmful substances (Cheng & Zhen, 2004). Thus for the sake of coherence in drug regulations, all medicines, regardless of their origin and development, should meet equally rigorous safety and efficacy standards for marketing authorization.

Many plants secrete an array of secondary metabolites as their natural defense against adverse conditions. Some of these phytochemicals are harmful to humans in addition to herbivores and insects. This effect could be due to highly conserved cell signaling pathway shared between two taxa. Human neuro-signaling chemicals with similar biological function are reported to be present in insects as well. Many of these functional molecules are mimicked or antagonized by alkaloids, terpeniods, flavonoids etc ( Rattan, 2010; Kennedy & Wightman, 2011).

Medicinal plants have been studied using modern scientific approaches for various biological components that can be used to treat different diseases (Pieme *et al.*, 2006), Similarly the harmful effects of plants are also common clinical occurrence (Nwafor, 2004).

Hence, one of the medicinal plants *C. papaya* leaves are used in the present study to find out the toxicity of leaves in Murine model.

Classification of papaya

**Kingdom** - Plantae

**Order** - Brassicales

**Family** - Caricaceae

**Genus** - Carica

**Species** - papaya

Binomial name (Gledhil, 2009).

Carica papaya is indigenous to the tropical region of Mexico, Central America and northern South America. *C. papaya* is distributed throughout the tropical and subtropical regions, where it is extensively cultivated. The leaves of this plant contains characterized metabolites viz. chitinase, glutaminyl cyclase and cysteine endopeptidases of class-II and III from Carica latex (Azarkan *et al.*, 2006). Linalool is present in fruit pulp, and alkaloids such as carpaine, pseudocarpaine, dehydrocarpaine I and II (Lim, 2012); and kaempferol and quercetin (Miean & Mohamed, 2001). The *Carica papaya* is claimed for its use in many different diseases and conditions. Some of the uses of the *Carica papaya* are mentioned in Table 2a.1.

**Table 2a.1:** Beneficial uses of *Carica papaya*

| Plant part | Method of use | Medicinal use and locality |
|---|---|---|
| Ripe fruit | Fruit juice, topical ulcer dressings, cosmetic (ointment, soap) | Warts, corns, sinuses, and chronic forms of skin induration (scaly eczema, cutaneous tubercles) in Caribe, Philippines; chronic skin ulcers in Jamaica Stomachic, digestive, diuretic, expectorant, sedative and tonic, bleeding piles, and dyspepsia in India |

| Plant part | Method of use | Medicinal use and locality |
|---|---|---|
| Green fruit | Juice | Contraceptive and abortifacient in Pakistan, India, and Sri Lanka<br>Malaria, hypertension, diabetes mellitus, hypercholesterolemia, jaundice, intestinal helminthiasis in Nigeria |
| Latex | Topical use | Dermatitis and psoriasis in Africa, Asia, Europe<br>Abortion in India, Malaysia |
| Seeds | Chewing, juice, powdered, paste, pessaries | Abortifacient, anthelmintic, thirst quencher, pain alleviator, bleeding piles, and enlarged liver and spleen in West Indies and India |
| Leaves | Fine paste, smoke, juice, infusion, decoction | Heart tonic, febrifuge, vermifuge, colic, fever, beriberi, abortion, asthma in India<br>Rheumatic complaints in Philippines<br>Stomach troubles, cancer in Australia |
| Flowers | Infusion, decoction | Jaundice, cough, hoarseness, bronchitis, laryngitis, and tracheitis in Asia |
| Roots/barks | Decoction, poultice, infusion | Digestive, tonic, abortifacient in Australia, sore teeth in India, syphilis in Africa |

Fresh papaya leaves are efficacious in the treatment of gonorrhea, syphilis and amoebic dysentery (Gill, 1992). Traditional healers in Malaysia use *Carica papaya* leaf formulations in palm oil (Vehicle) to increase platelet count in dengue fever (Malaysian folk medicine reports). *Carica papaya* leaf extract have shown to help in wound healing (Nayak *et al*, 2007), and as an antisickling agent (Imaga *et al.*, 2009). Reports also suggest the anti – tumor and immunemodulatory effects (Otsuki *et al.*, 2010).

A number of studies have reported the toxic effects of medicinal plants (Kaplowitz, 1997; Calixto, 2000; Taziebou *et al.*, 2007). The toxicity studies for papaya fruit, seed and latex is well documented ( Schmidt, 1995; Adebiyi *et al.*, 2003). Nevertheless, in spite of various uses over long time periods, little toxicological information is available regarding safety, following repeated exposure to *Carica papaya* leaf extract.

## 2a.2Material and Methods:

### Plant material:

Fresh green mature leaves of *C. papaya* were collected from local fields from Baroda District, Gujarat, India during the months of September-October and were identified by Prof. M. Daniel (Taxonomist), Dept. of Botany, The M.S. University of Baroda. The voucher specimen (BARO/51/2010) is deposited at the herbarium in Dept. of Botany, The M.S.University of Baroda, Vadodara, India, for future reference.

### Preparation of aqueous extract

The leaves of *C. papaya* L. (CP) were shade dried and were grounded in a food grinder. Around 100 g of powder was boiled for 30 min and the filtrate was concentrated to semisolid form. Final concentration achieved was 35 % w/w. The extracts were stored at 4°C in dried form and used for the subsequent experiment.

### Experimental Animals:

Animals were procured from Sun Pharma Advanced Research Centre, Vadodara, India and were housed in poly- polyproplene cages under standard 12-h light/dark cycle. The animals were maintained at Dept. of Zoology Animal house facility at The M.S. University of Baroda, were fed with standard diet for laboratory animals (Pranav Agro Pvt. Ltd., Vadodara, Gujarat) and water *ad libitium*,. The maintenance of animals and experimental protocols, for carrying animal test, were approved by CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals) ethical committee of Department of Zoology, The M.S. University of Baroda (827/ac/04/CPCSEA).

## Acute Tocixity:

Acute Toxicity testing was carried out as per OECD 423 and 425 guideline for Acute Toxicity. Twelve, 4 month old albino rats of *Wistar* strain weighing 200 – 225 g, were acclimatized for period of 5 days and were divided in Two Groups (1) control (2) CP at a dose of 1000 mg/Kg B.W (Acute). The dose was suspended in 0.5 % sodium carboxy methyl cellulose (vehicle) and fed orally with gastric intubation tube. Control group was fed with vehicle only. Animals were checked for abnormal behavioral responses, convulsions, abnormal breathing and death. After 24 hrs the animals were anaesthetized with Di-ethyl ether, blood was withdrawn for Complete Blood Count (CBC) and Serum Biochemistry, and animals were sacrificed to harvest vital organs.

The Organs were cleaned with PBS and were fixed in 10% formalin for histology studies.

## Sub Chronic Toxicity:

The Toxicity testing was done according to OECD guidelines test No. 407. Twenty four animals of 200-225 g were acclimatized for period of 5 days and were randomly divided into 4 groups. Group 1 served as control and was fed with Vehicle only , Group 2 , 3 and 4 were treated with CP leaf extract dissolved in CMC at dose of 10mg/Kg B.W., 100 mg/Kg B.W., 1000 mg/Kg B.W. respectively for the period of 28 days. Blood was collected under mild anesthesia from orbital sinus on every 7th day. Finally on 29th day, blood was withdrawn for CBC and Serum Biochemistry under mild anesthesia and animals were sacrificed to harvest the organs after washing in 10% formalin for histology.

## Biochemical Analysis:

Blood was collected in both K3 EDTA (2 ml) vacuities and plain vacuities for CBC analysis and serum analysis respectively. CBC (Complete Blood Count) analysis was done using Mindray 2800E Automated Haematoanalyser, while 1 ml serum was collected in the plain vacutainers for liver and kidney function tests such as Serum Glutamate Pyruvate Transaminase, Serum Glutamate Oxalate Transaminase, Acid Phosphatases, Alkaline phosphatases, Urea and Creatinine were measured using Reckon Diagnostic kits following manufacturer's instruction on Perkin Elmer (Lambda 25 UV/Vis) Spectrophotometer.

## Histopathological Examination:

Histopathological profile of vital organs such as liver, kidney, spleen and ovary was carried out in order to find out histopathological changes. Five micron thick sections were cut using Leica microtome and routine Haematoxylin and Eosin staining was performed for histopathological observations.

## Statistical Analysis:

Data were digitally analyzed using the statistical software package Graph Pad Prism version 6.0 for Windows, Graph Pad Software, San Diego California USA. All values are expressed in mean ± SEM. Treatment effects over the time are compared between control and treatment groups by analysis of variance (one way ANOVA) followed by Dunnett's Post hoc test. Pearson's correlation value (P-value) value less than 0.05 were considered statistically significant.

## 2a.3 RESULTS

***Acute toxicity:***

Twenty four hours after the administration of aqueous extract of CPLE at a dose of 1000 mg/kg, no adverse effect on the behavioural responses of the tested rats were observed. Physical observations indicated no signs of changes in the skin, fur, eyes mucous membrane, behaviour patterns, tremors, salivation, and diarrhoea of the rats. There was no mortality observed at the tested dose in the rats. There were generally no significant differences observed in the haematological and biochemical parameters in the CPLE treated group compared to the normal control group (Table 2a.2). Gross examination at autopsy and histopathological evaluations of the various organs (both treated and non treated), stained with haematoxylin and eosin, revealed no significant differences.

**Table 2a. 2: Serum Biochemical Parameters of Acute studies**

| Parmeters | Control | Treatment |
|---|---|---|
| **SGPT (IU/l)** | 26±1.5 | 31.67±1.62 |
| **SGOT (IU/l)** | 58.2±1.23 | 52.17±3.53 |
| **Alk PO4 (IU/l)** | 269.67±21.23 | 266.33±33.39 |
| **Acid PO4 (IU/l)** | 2.4±0.32 | 2.52±0.19 |
| **Urea (mg/dl)** | 47.06±1.83 | 51.79±2.16 |
| **Creatinine (mg/dl)** | 1.06±0.026 | 1.04±0.015 |

***Results are expressed*** as mean ± S.E. for n=6. *p<0.05, ** p<0.01, *** p<0.001
Control is compared treatment.

***Sub-chronic toxicity:***

***Clinical Signs, Necropsy Findings:***

Daily oral administration of *CPLE* extract for 28 days did not induce any obvious symptom of toxicity in rats, including the highest dose tested at 1000 mg/kg body weight. No deaths or obvious clinical signs were found in any groups throughout the experimental period. Physical observation of the treated rats throughout the study indicated that none of them showed signs of toxicity in their skin, fur, eyes, mucus membrane or behavioural changes, diarrhoea, tremors, salivation, sleep, and coma. Normal body weight gains were observed during the study period compared to the control group. No abnormal gross findings were observed in the necropsies of any of the rats.

***Haematology and Clinical Biochemistry Analysis***

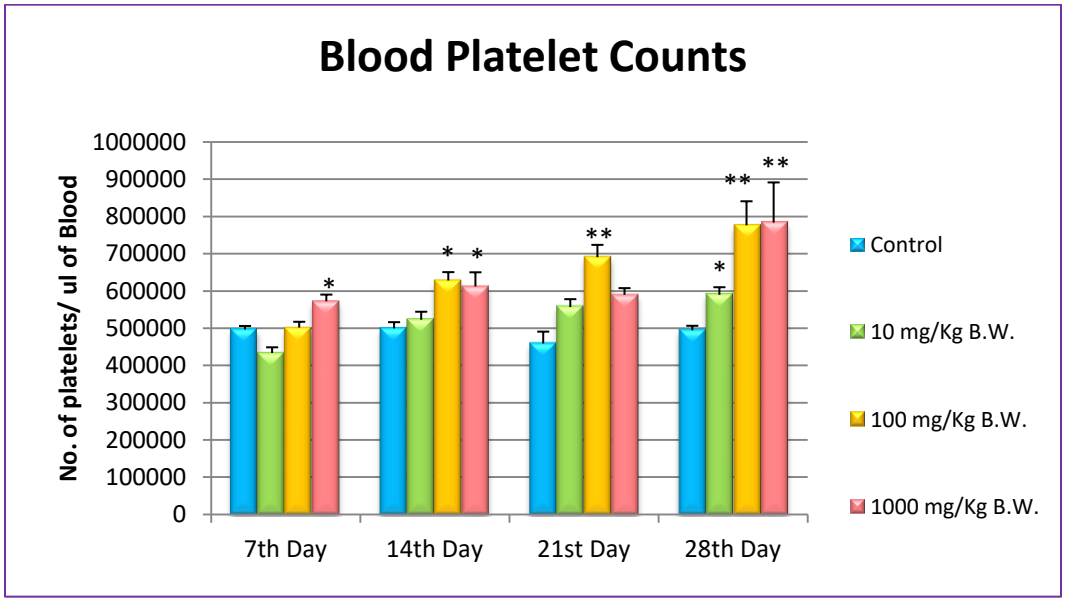
The effects of subchronic administration of *CPLE* extract on haematological and biochemical parameters are presented in Table 2a.2. The analyzed haematological parameters, which included red blood cell count (RBC), haemoglobin concentration (Hb), haematocrit (Ht), white blood cell count (WBC), and white blood cell differential count, in treated rats were not significantly different from the controls. However the platelet count showed a time dependent steady and significant increase (Fig. 2a.1). Moreover, the *CPLE* had no effect on the kidney function parameters (urea and creatinine) nor any statistically significant differences in liver function parameters (SGOT, SGPT and alkaline phosphatase) were noted.

**Table 2a.3:** Complete Blood count of rats treated with aqueous extract of *Carica papaya* leaf extract at 28th day

| Dose Treatment | Platelets | RBCs | HbG | HCT | MCV | MCH | MCHC | WBCs |
|---|---|---|---|---|---|---|---|---|
| **Control** | 4.98±0.13 | 8.20±0.20 ns | 15.47±0.47 | 49.27±1.68 | 57.63±1.05 | 18.05±0.32 | 31.38±0.14 | 5.50±0.40 |
| **24 hrs Acute (1000mg/kgBW** | 5.12±0.43 ns | 7.24±0.33 # | 14.97±0.17 ns | 47.65±0.43 ns | 60.30±1.15 ns | 18.85±0.37 ns | 31.35±0.20 ns | 6.15±0.54 |
| **10mg/kg BW** | 5.92±0.13 # | 6.75±0.14 ## | 13.68±0.55 # | 47.77±2.42 ns | 59.97±1.03 ns | 18.27±0.78 ns | 30.45±0.89 ns | 4.17±0.16 ns |
| **100 mg/kg BW** | 7.82±0.38 # | 7.13±0.12 # | 14.70±0.59 ns | 48.57±2.17 ns | 56.95±0.82 ns | 17.25±0.51 ns | 30.35±0.85 ns | 6.93±0.60 ns |
| **1000 mg/kgBW** | 7.95±0.29 ## | 8.18±0.14 ns | 14.52±0.39 ns | 53.87±1.58 ns | 57.62±1.36 ns | 15.53±0.75 # | 26.95±0.65 ## | 7.00±1.26 ns |

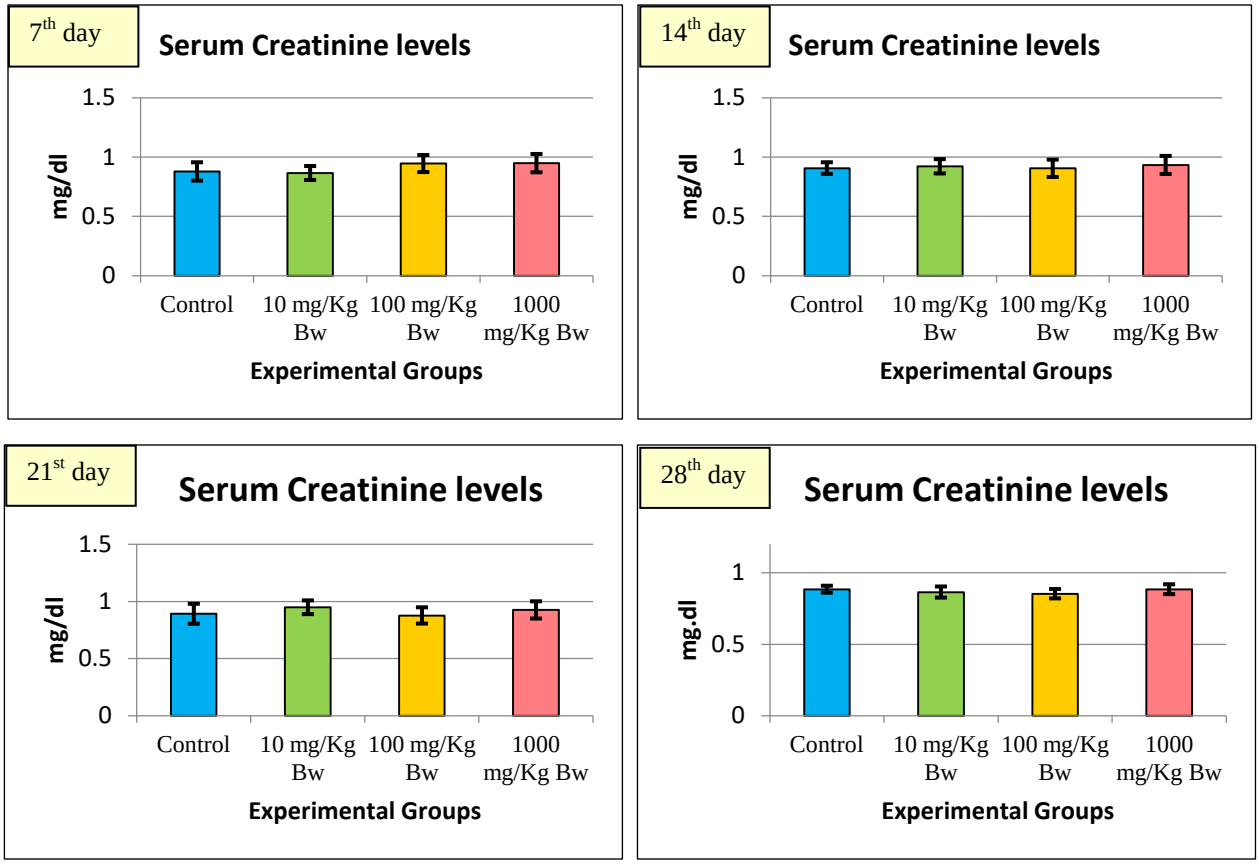
***Results are expressed*** as mean ± S.E.M. for n=6. #p<0.05, ## p<0.01, ### p<0.001
Control is compared treatment groups.

**Fig 2a.1:** Effect of CPLE on blood platelet counts of animals in dose dependent and time dependent manner.




***Results are expressed*** as mean ± S.E. for n=6. *p<0.05, ** p<0.01, *** p<0.001
Control is compared treatment groups.

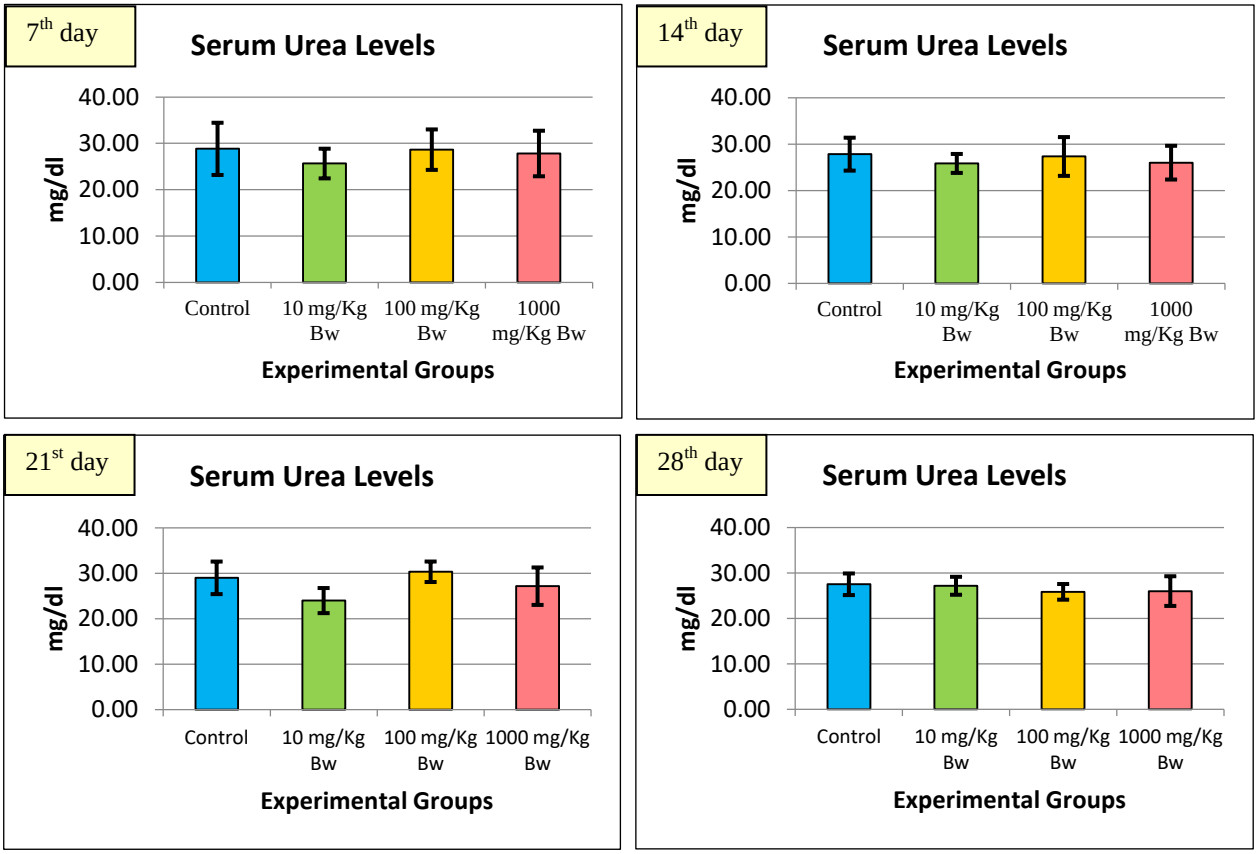
**Fig 2a.2:** Effect of Aqueous extract of Carica papaya leaves extract Serum Creatinine levels in rats




| | 7th day | 14th day | 21st day | 28th day |
|---|---|---|---|---|
| **Control** | 0.88±0.08 | 0.91±0.05 | 0.89±0.09 | 0.89±0.02 |
| **10 mg/Kg Bw** | 0.87±0.06 | 0.92±0.06 | 0.95±0.06 | 0.87±0.04 |
| **100 mg/Kg BW** | 0.95±0.07 | 0.91±0.07 | 0.88±0.07 | 0.85±0.03 |
| **1000mg/Kg Bw** | 0.95±0.08 | 0.93±0.08 | 0.93±0.08 | 0.89±0.03 |

***Results are expressed*** as mean ± S.E.M. for n=6. *p<0.05, ** p<0.01, *** p<0.001
Control is compared treatment groups.

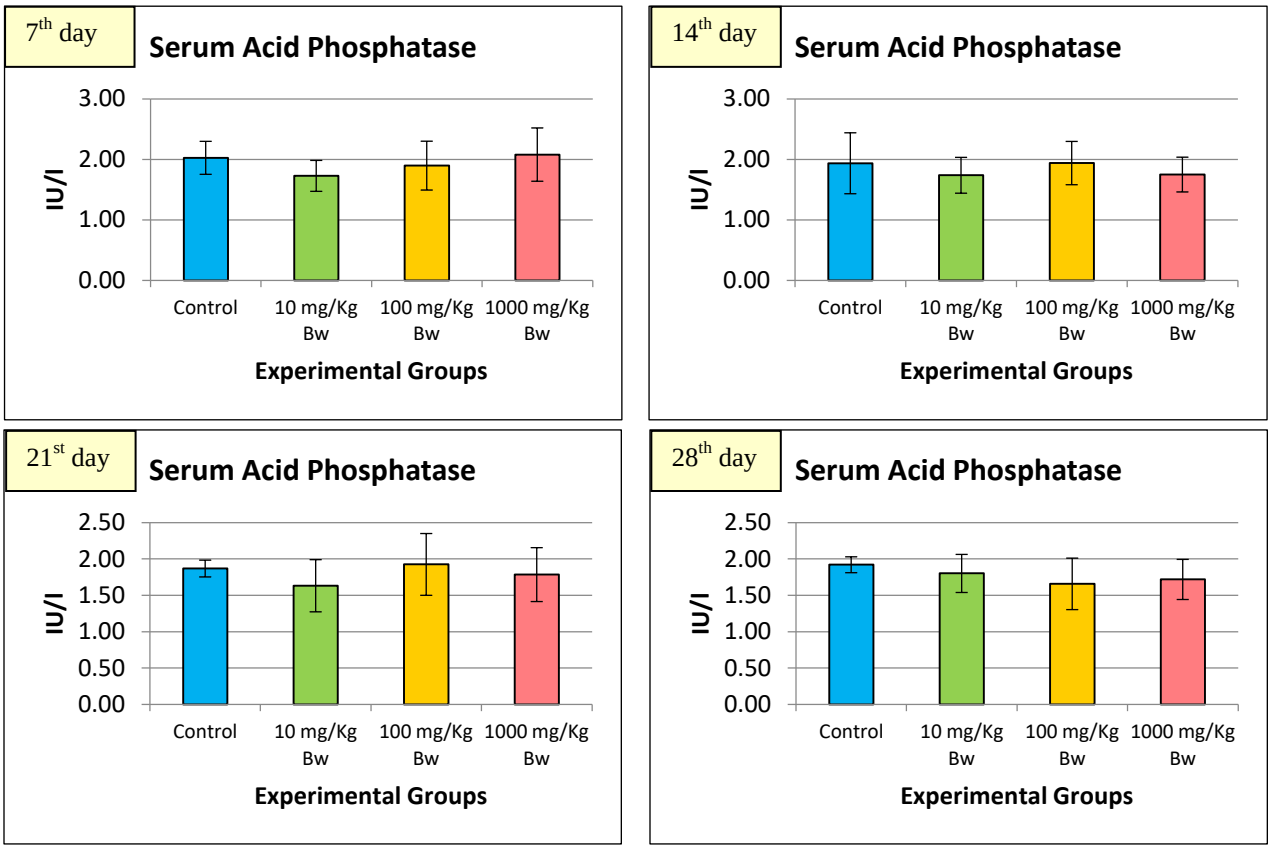
**Fig 2a.3:** Effect of Aqueous extract of Carica papaya leaves extract Serum Urea levels in rats




| | 7th day | 14th day | 21st day | 28th day |
|---|---|---|---|---|
| **Control** | 28.83±5.64 | 27.83±3.54 | 29±3.58 | 27.5±1.38 |
| **10 mg/Kg Bw** | 25.67±3.2 | 25.83±2.04 | 24±2.76 | 27.17±1.47 |
| **100 mg/Kg BW** | 28.67±4.37 | 27.33±4.18 | 30.33±2.25 | 25.83±1.72 |
| **1000mg/Kg Bw** | 27.83±4.92 | 26±3.63 | 27.17±4.12 | 26±1.26 |

***Results are expressed*** as mean ± S.E.M. for n=6. *p<0.05, ** p<0.01, *** p<0.001
Control is compared treatment groups.

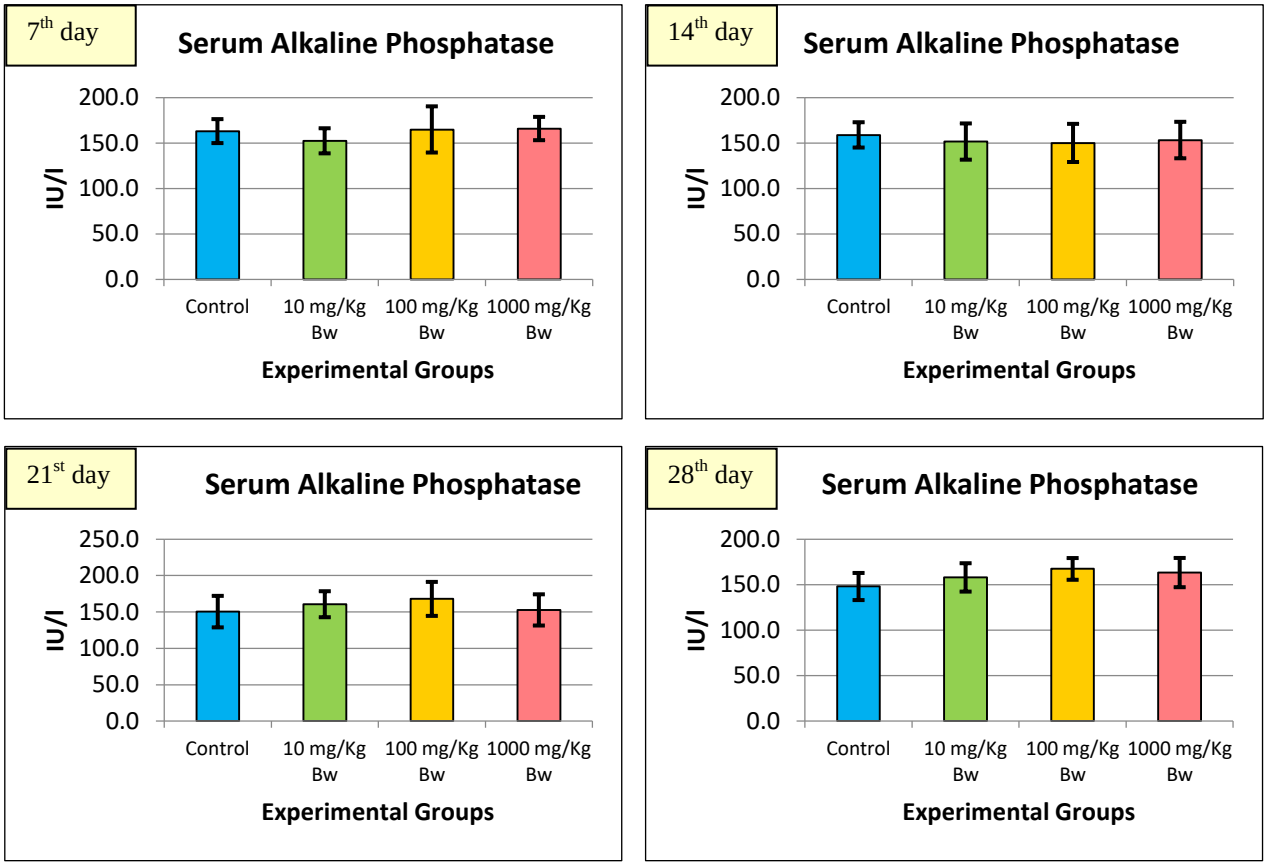
**Fig 2a.4:** Effect of Aqueous extract of Carica papaya leaves extract Serum Acid Phosphatase levels in rats




| | 7th day | 14th day | 21st day | 28th day |
|---|---|---|---|---|
| **Control** | 2.03±0.27 | 1.94±0.5 | 1.87±0.12 | 1.12±0.91 |
| **10 mg/Kg Bw** | 1.73±0.26 | 1.74±0.3 | 1.63±0.36 | 1±0.77 |
| **100 mg/Kg BW** | 1.9±0.4 | 1.94±0.36 | 1.93±0.42 | 1.16±0.84 |
| **1000mg/Kg Bw** | 2.08±0.44 | 1.75±0.29 | 1.79±0.37 | 1.12±0.83 |

***Results are expressed*** as mean ± S.E.M. for n=6. *p<0.05, ** p<0.01, *** p<0.001
Control is compared treatment groups.

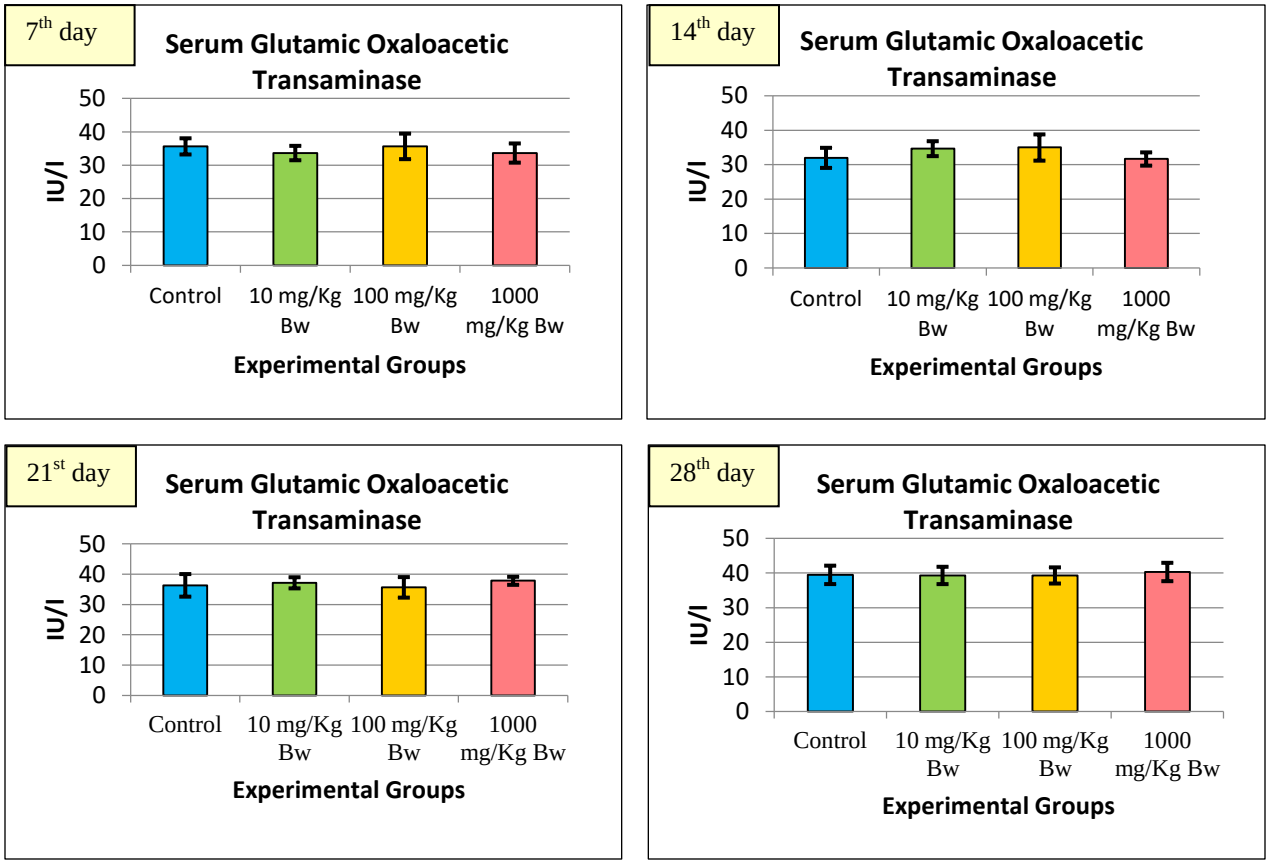
**Fig 2a.5:** Effect of Aqueous extract of Carica papaya leaves extract Serum Alkaline Phosphatase levels in rats




| | 7th day | 14th day | 21st day | 28th day |
|---|---|---|---|---|
| **Control** | 163±13.2 | 158.8±13.9 | 150.5±21.6 | 148±14.9 |
| **10 mg/Kg Bw** | 152.3±13.8 | 151.5±19.9 | 160.7±17.9 | 158±15.6 |
| **100 mg/Kg BW** | 164.8±25.4 | 150±21 | 168±23.4 | 167.3±11.9 |
| **1000mg/Kg Bw** | 165.8±12.8 | 153.2±20.1 | 152.8±21.4 | 163.3±16.1 |

***Results are expressed*** as mean ± S.E.M. for n=6. *p<0.05, ** p<0.01, *** p<0.001
Control is compared treatment groups.

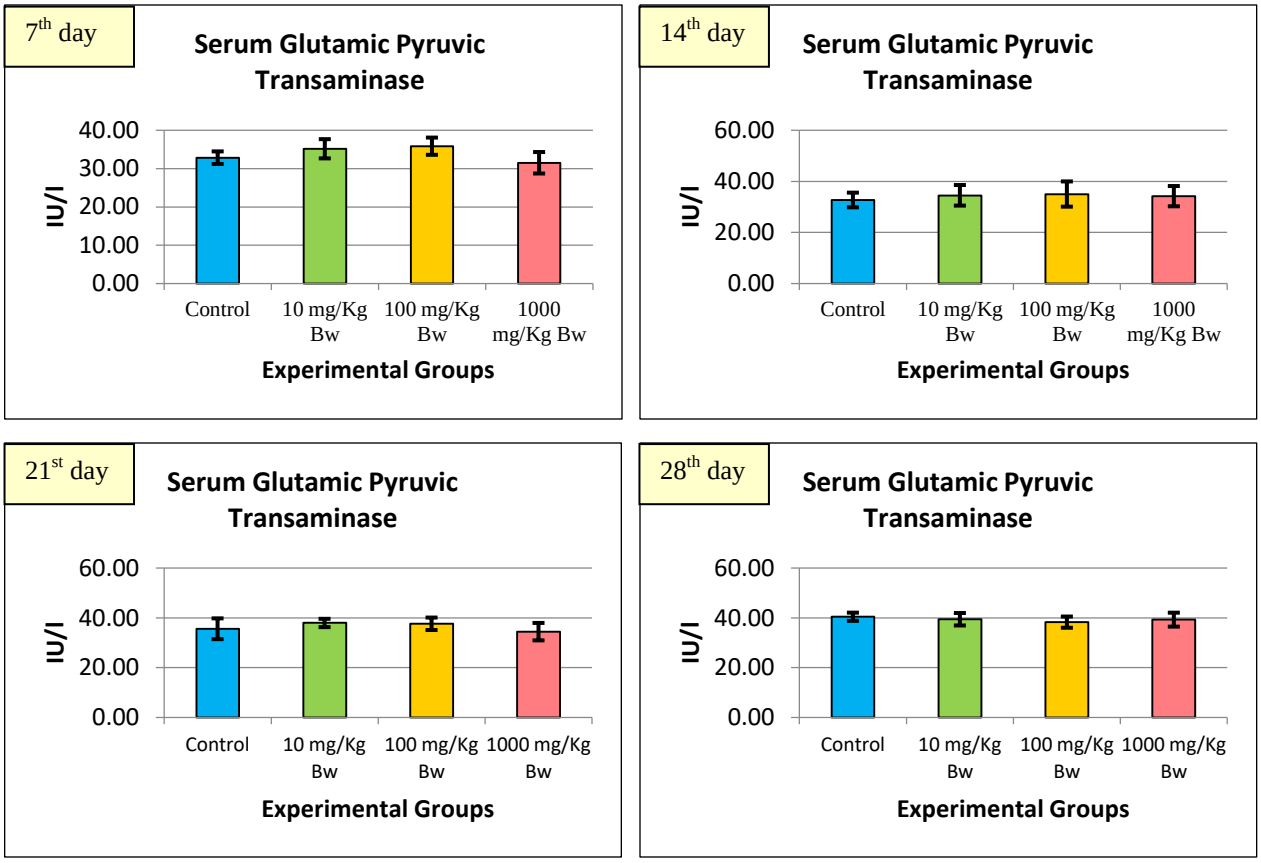
**Fig 2a.6:** Effect of Aqueous extract of Carica papaya leaves extract SGOT levels in rats




| | 7th day | 14th day | 21st day | 28th day |
|---|---|---|---|---|
| **Control** | 35.67±2.42 | 32±2.92 | 36.33±3.72 | 39.5±2.66 |
| **10 mg/Kg Bw** | 33.67±2.16 | 34.67±2.17 | 37.17±1.83 | 39.33±2.5 |
| **100 mg/Kg BW** | 35.67±3.83 | 35±3.81 | 35.67±3.39 | 39.33±2.34 |
| **1000mg/Kg Bw** | 33.67±2.88 | 31.67±1.92 | 37.83±1.33 | 40.33±2.66 |

***Results are expressed*** as mean ± S.E.M. for n=6. *p<0.05, ** p<0.01, *** p<0.001
Control is compared treatment groups.

**Fig 2a.7:** Effect of Aqueous extract of Carica papaya leaves extract SGPT levels in rats




| | 7th day | 14th day | 21st day | 28th day |
|---|---|---|---|---|
| **Control** | 32.83±3.19 | 32.67±2.88 | 35.67±4.18 | 40.50±1.64 |
| **10 mg/Kg Bw** | 35.17±3.43 | 34.50±4.04 | 38.00±1.67 | 39.50±2.51 |
| **100 mg/Kg BW** | 35.83±3.66 | 35.00±4.94 | 37.67±2.5 | 38.33±2.25 |
| **1000mg/Kg Bw** | 31.50±2.81 | 34.17±3.97 | 34.50±3.51 | 39.33±2.8 |

***Results are expressed*** as mean ± S.E.M. for n=6. *p<0.05, ** p<0.01, *** p<0.001
Control is compared treatment groups.

**Fig 2a.8:** Histopathological studies of Acute Toxicity

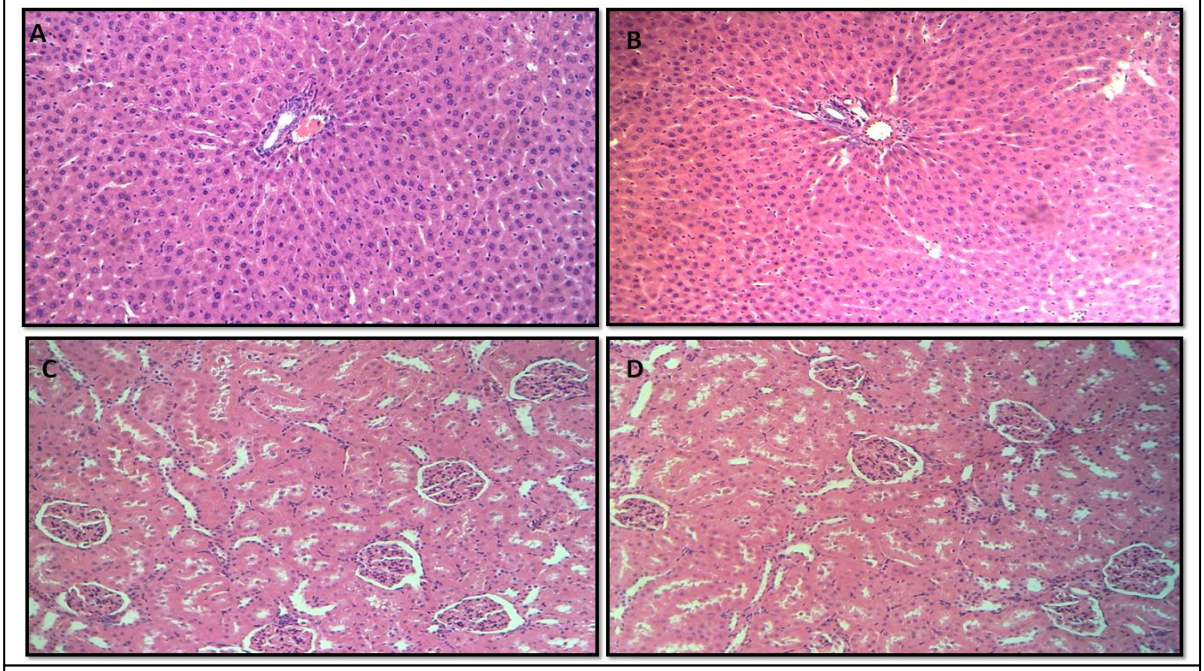



**Fig. (A)** & **(B)**: Representative histological section of Liver stained by (H-E) Control group and CPLE treated groups. Normal histo-architecture is observed. Magnification: 200×. **(C)** & **(D)** Representative histological section of Kidney stained by (H–E) from control and CPLE treated group No difference in glomerular morphology is seen. No significant histo-pathological changes were observed. Magnification: 200×.

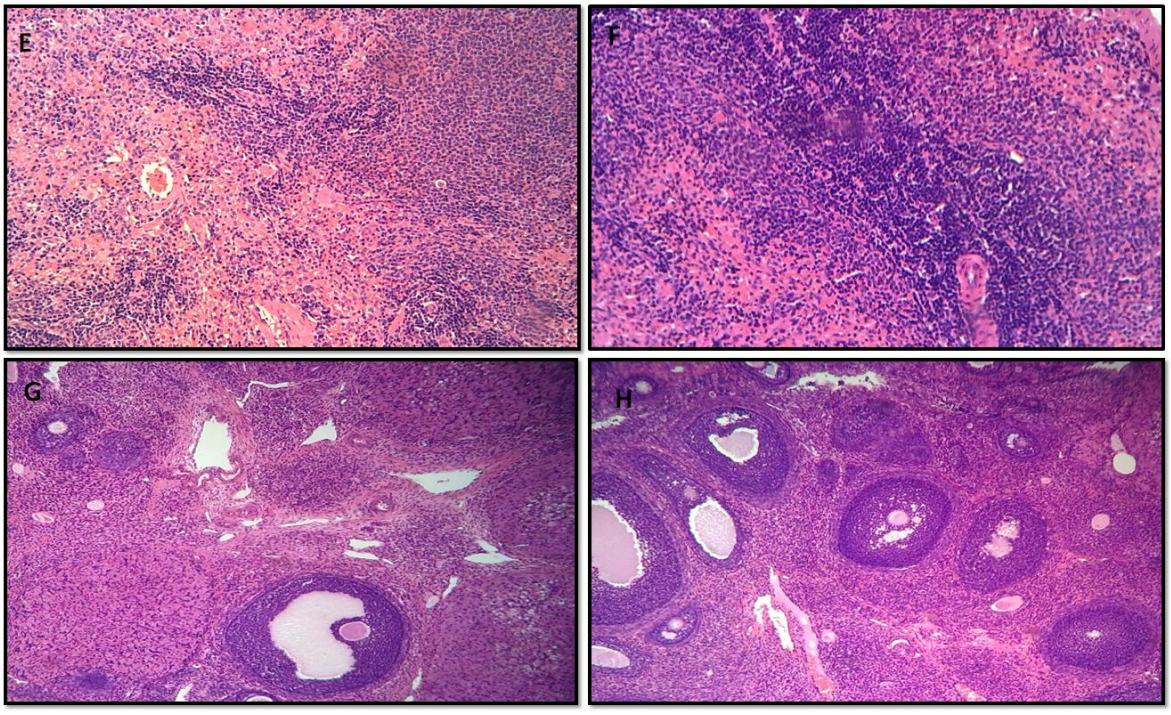
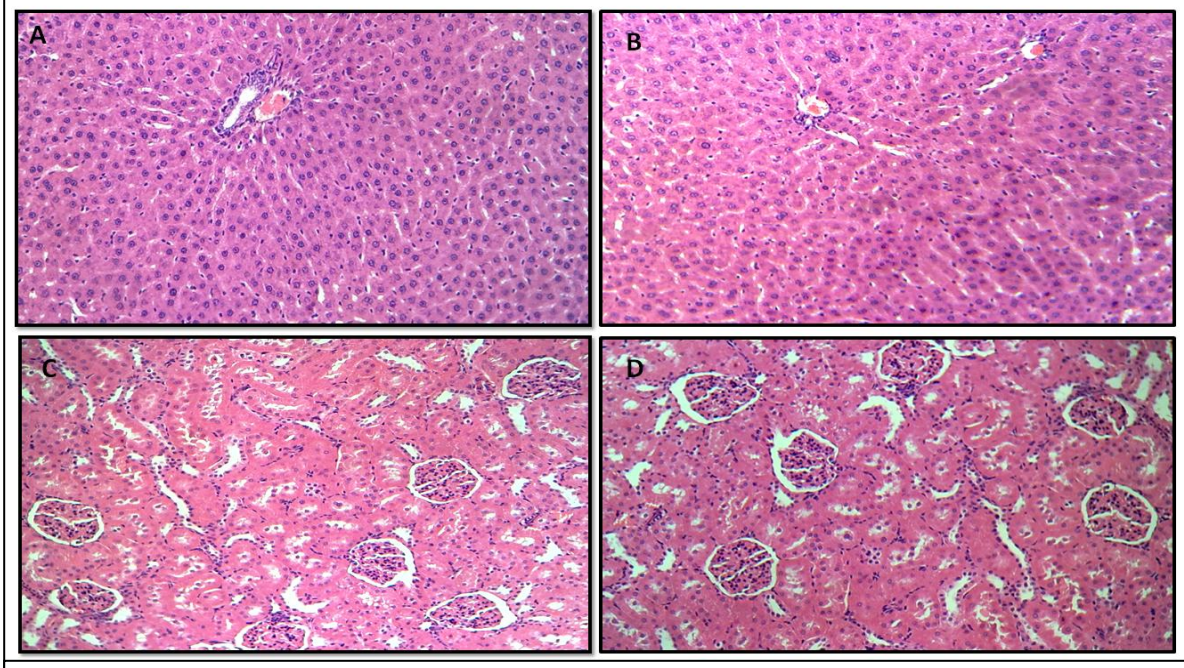



**Fig. (E) & (F)** Representative histological section of Spleen of Control and CPLE treated group respectively, stained by (H-E) No significant difference was observed as compared with control histology. Magnification: 100×. (G) & (H) Representative histological section of ovary stained by (H-E) from Control and CPLE-treated group respectively. Magnification: 100×.

**Fig. 2a.9 :** Histopathological studies in Sub-chronic Toxicity

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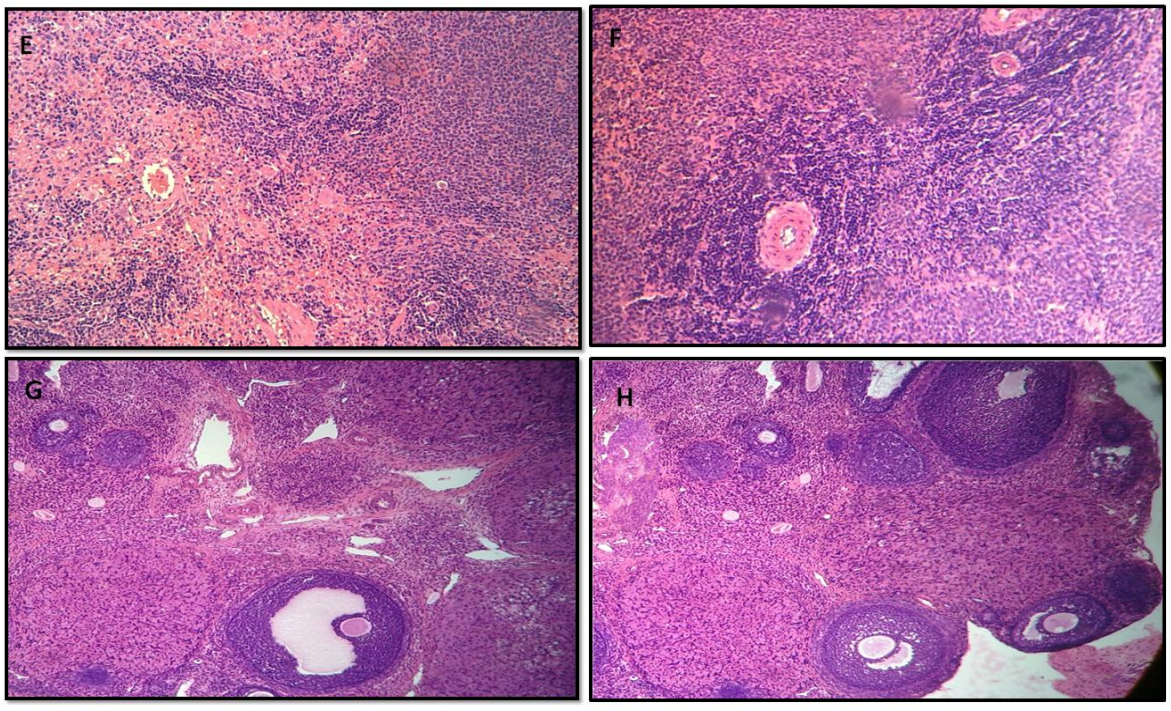


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## 2a.4 Discussion:

For centuries, natural products, such as medicinal plants have been the basis for the treatment of various ailments (Ridtitid *et al.* 2008). In screening natural products for the pharmacological activity, assessment and evaluation of the toxic characteristics of a natural product extract, fraction, or compound are usually an initial step. Regardless of the pharmacological beneficial effects of CPLE, detailed knowledge about the chronic toxicology of CPLE is lacking. Hence, the current study was undertaken to evaluate and focus on the acute and chronic toxicity of CPLE in an animal model.

During the evaluation of the toxic characteristics of medicinal plants, the determination of $LD_{50}$ is usually an initial step to be conducted. Data from the acute toxicity study may (i) serve as the basis for classification of substances (recommended by OECD); (ii) provide initial information on the mode of toxic action of a substance; (iii) help arrive at a dose of a new compound; (iv) help in dose determination in animal studies; and (v) help determine $LD_{50}$ values that provide many indices of potential types of drug activity (Ukwuani *et al.* 2012). Moreover, if the animals with a high dose (e.g.1000 mg/kg) are found to survive, without any side effects, no further acute testing will be required (National Research Council, 2006). In this study, CPLE at a dose of 1000 mg/kg had no adverse effect on the tested rats. This was confirmed both by haematological and biochemical results as well as histological observations. Therefore, this study indicates that CPLE does not cause acute toxicity effects at the dose tested and has an $LD_{50}$ value greater than 1000 mg/kg. In principle, the limit test method is not intended for determining a precise $LD_{50}$ value, but it serves as a suggestion for classifying the crude extract based on the expectation at which dose level the animals are expected to survive.

According to the study by Kennedy *et al.* 1986 substances with $LD_{50}$ values higher than 5000 mg/kg by oral route are regarded as being safe or practically nontoxic. However, many medicinal plants have also been reported to be toxic to both humans and animals. Therefore, it should be emphasized that the traditional use of any plant for medicinal purposes, by no means, guarantees the safety of such plant. Furthermore, the data of the acute and subchronic toxicity studies on medicinal plants or preparations derived from them should be obtained in order to increase the confidence in its safety to humans, particularly for use in the development of pharmaceuticals (Ukwuani *et al.*, 2012). Choosing the appropriate tests and dosing regimens that will demonstrate an adequate margin of exposure is a critical step in establishing human safety. Since no toxic effects were found during the acute toxicity study, further evaluation was conducted to evaluate the subchronic toxicity of CPLE up to 28 days in rats to prepare the comprehensive toxicology data of this ancient medicinal plant.

Subchronic studies assess the undesirable effects of continuous or repeated exposure of plant extracts or compounds, over a time period, to the experimental animals. Specifically, they provide information on target organ toxicity and are designed to identify No Observable Adverse Effect Level (NOAEL) (National Research Council, 2006). Subchronic evaluation can also help to determine appropriate dose regimens for long-term studies. Consequently, in this study the subchronic toxicity CPLE was evaluated in rats at doses of 10–1000 mg/kg/day for 28days. Administration of extracts of CPLE for 28 days produced no clinical signs for toxicity or mortality in rats. In addition, the treated rats did not show any significant alteration in water or food consumption (data not shown). Significant reduction in food and water intake is

suggested as being responsible for the observed decrement in body weight gain. Loss of appetite is often synonymous with weight loss due to disturbances in carbohydrate, protein or fat metabolisms. Moreover, at higher doses, crude plant extracts may metabolize to a toxic end product, which could interfere with gastric function and decreased food conversion efficiency (Chokshi, 2007). Interestingly, the food and water intakes were found to be unaltered during the 28-day treatment period when compared to a control group in this study. Furthermore, the diets and water were well-accepted by the rats treated with CPLE suggesting the extract did not possibly cause any alterations in carbohydrate, protein or fat metabolism in these experimental animals. It also shows that the CPLE did not adversely interfere with the nutritional benefits, such as weight gain and stability of the appetite, which are expected for animals that are continually supplied with food and water *ad libitum*. Also, the extract is safe by oral route in relation to its folkloric practice in the administration of medicinal herbs through oral route to treat the patients. Therefore, CPLE can be considered as non-toxic up to the said dose.

The body weight changes serve as a sensitive indication of the general health status of animals (El Hilaly *et al.,* 2004). However, weight gains were observed in all animals administered with CPLE. It can be stated that the CPLE did not interfere with the normal metabolism of animals as corroborated by the non-significant difference from animals in the vehicle control group. As mentioned earlier the loss of appetite is often synonymous with weight loss due to disturbances in the metabolism of carbohydrate, protein, or fat (Ezeonwumelu *et al.,* 2011). Therefore, the normal food and water intake without loss of appetite are suggested as being responsible for the observed increment in body weight in this study.

Similarly, no significant changes in the weights of the heart, liver, spleen, kidneys, or lungs were observed, suggesting that administration of CPLE at the subchronic oral doses had no effect on the normal growth. The usefulness of weighing organs in toxicity studies includes their sensitivity to predict toxicity, enzyme induction, physiologic perturbations, and acute injury; it is frequently a target organ of toxicity; it correlates well with histopathological changes; there is little interanimal variability; historical control range data are available. The relative change in organ weights have been observed in toxicity studies to be a relatively sensitive indicator for particular organs, and thereafter to define toxicity as significant changes observed in those particular organs (Michael *et al.*, 2007). The results of this study revealed that the essential organs, such as heart, liver, spleen, kidneys, and ovary, neither were adversely affected nor showed clinical signs for toxicity throughout the treatment. Since there was no reduction in body and relative organ weights of the treated animals at any of the doses tested, we conclude that the extract is nontoxic to the analyzed organs.

The serum hematology and clinical biochemistry analyses have been done to evaluate the possible alterations in hepatic and renal functions influenced by the extracts. Liver and kidney function analysis is very important in the toxicity evaluation of drugs and plant extracts as they are both necessary for the survival of an organism (Olorunnisola *et al.* 2012). High levels of SGOT, SGPT, acid and alkaline phosphatase are reported in liver diseases or hepatotoxicity (Brautbar & Williams, 2002). The nonsignificant changes in SGOT, SGPT, acid and alkaline phosphatase in rats at all doses suggests that subchronic administration of CPLE does not affect the hepatocyte function in the rats. Renal dysfunction can be assessed by concurrent measurements of urea, creatinine and uric acid

and their normal levels reflect at reduced likelihood of renal problems (Davis & Bredt, 1994). In the present study, changes in plasma urea and creatinine levels in CPLE treated groups showed non-significant differences indicating a normal renal function.

Evaluation of haematological parameters can be used to determine the extent of the deleterious effect of CPLE on the blood of an animal. It can also be used to explain blood relating functions of a plant extract or its products. Furthermore, such analysis is relevant to risk evaluation as changes in the haematological system have higher predictive value for human toxicity when the data are translated from animal studies (Yakubu *et al.*, 2007). A haematogram was undertaken for all the CPLE treated and control groups and the results show no significant effects. The non-significant effect of the extract on total red blood cells, mean corpuscular volume and mean corpuscular Hb, indicates that the CPLE does not affect the erythropoiesis, morphology, or osmotic fragility of the red blood cells. Leukocytes are the first line of cellular defense that respond to infectious agents, tissue injury, or inflammatory process. Our studies reports no significant changes in the WBC count, which further confirmed the above findings. Interestingly, however, we observed a dose dependent and time dependent increase in the platelet count. This finding is in agreement with the earlier findings (Sathasivam *et al.*, 2009). Therefore, a normal haematological profile of CPLE treated groups further establishes its non-toxic nature.

The macroscopic examinations of the organs of rats treated with various doses of CPLE did not show any changes in color compared with control group rat's organs. Hypertrophy of organs is first hand indication of toxicity of chemical or biological substance. However, no hypertrophy of organs was observed in this study amongst all the

groups studied. In addition, the microscopic examination revealed that none of the organs from the extract treated rats showed any alteration in cell structure or any unfavourable effects when viewed under the light microscope using multiple magnification powers. No pathologies were recorded in the histological sections of the vital organs (heart, liver, spleen, kidney, and ovary) of the control group either. Generally, any damage to the parenchymal liver cells results in elevations of both transaminases in the blood (Slichter, 2004). Thus, the non-significant changes observed in SGOT, SGPT activities strongly suggest that the subchronic administration of CPLE did not alter the hepatocytes and, consequently, the metabolism of the rats as observed in the histopathology observations of liver tissue. Equally, there were also no significant alterations in urea and creatinine levels in the subchronic administration of CPLE when compared to the control group. Any rise in urea and creatinine levels is observed only if there is marked damage to functional nephrons (Lameire *et al.,* 2005). This finding was further confirmed by histopathological observations of the kidney tissue in this study. Therefore, the results recorded in this study demonstrate that the CPLE did not alter the liver or renal function and further support its non-toxic nature.

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# 2b: Inflammation and Cytotoxicity Assessment of CPLE in HepG2 cell line.

**INFLAMMATION AND TOXICITY ASSESSMENT OF CPLE IN HEPG2 CELL LINE**

## 2b. 1 INTRODUCTION:

The use of non-animal test methods, viz. in vitro studies and computer-based model approach provides important and beneficial tool to enhance our understanding of hazardous effect manifested by chemicals, molecules or plant product, and for predicting these effects on humans and animals (Broadhead & Combes, 2000). Thus, identifying potential toxicity at an early stage in drug discovery can save both time and developmental cost, generating toxicological profiles and most importantly reduce the likelihood of late stage failure (Bowes *et al.*, 2012). Hence it is important to consider their application to other elements of the risk assessment paradigm. Non-animal test methods, including *in vitro* assays, provide important tools to enhance the extrapolation from *in vitro* to *in vivo* in humans. *In vitro* methods are invaluable in providing mechanistic information on toxicological findings both in experimental animals and in humans. It is anticipated that rapid advances in biomedical sciences results in the development of a new generation of mechanism-based *in vitro* test strategies for hazardous characterization that can be applied in risk assessment (Tice *et al.,* 2013). However, *in vitro* data have had no direct influence on the calculation of acceptable daily intake (ADI) values, as reviewed by International Life Sciences Institute (ILSI) Europe (Walton *et al.*, 1999).

Despite these limitations, *in vitro* systems are extremely useful in many ways. They can provide much more refined information on how food constituents interact with human cells and macromolecules. *In vitro* test systems are especially well suited for studying low molecular weight chemicals such as food additives, flavoring substances used in the

food products, contaminants, and pesticides as well as natural toxicants (Eisenbrand *et al.*, 2002).

Compounds that can be investigated using methods of *in vitro* toxicology can include food associated compounds such as: natural ingredients, secondary metabolites, permissible/authorized chemicals including additives, residues, and supplements, chemicals from processing and packaging and contaminants (Eisenbrand *et al.*, 2002)

The most commonly and primarily used tests include testing cytotoxicity in cells. The rapid response of cells to toxic stress is by altering metabolic rates and cell growth or even gene transcription controlling basic functions. Cytotoxicity data have their own intrinsic value in defining toxic effects (e.g. as an indicator of acute toxic effects *in vivo*) and are also important for designing more in-depth *in vitro* studies (Blum & Speece, 1990). The novel approaches of *in vitro* toxicology are, however, focused on the development of molecular markers based on detecting effects at levels of exposure to potentially toxic chemicals lower than those that cause the onset of clinically observable pathological responses (Campion *et al.*, 2013). Expression of stress response or other genes and ensuing biochemical alterations may be potential markers for compound-induced toxicity. Over last two decades there has been considerable interest in using basal cytotoxicity data to predict the acute effects of compounds *in vivo*. If a compound is acutely toxic, it may be anticipated, in most cases, to reflect an insult to the intrinsic functions of cells. The increased intracellular levels of ROS, frequently referred to as oxidative stress, represents a potentially toxic insult, which if not counteracted leads to membrane dysfunction, lipid peroxidation, DNA damage and inactivation of certain proteins (Valko *et al.*, 2007). These studies for ROS generation should be used in *in vitro*

toxicity assessment. Similarly number of compounds can directly or indirectly activate inflammatory cells such as neutrophils and macrophages with the production of reactive oxygen species and possibly other free radicals or can induce generation of inflammatory cytokines (Martin *et al.*, 1997).

There are several tests to detect the ROS generation and inflammatory cytokines

**MTT Assay:** MTT assay is most widely used method to measure cytotoxicity. Measurement of cell viability and proliferation forms the basis for numerous *in vitro* assays of a cell population's response to external factors. The yellow tetrazolium MTT (3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) is reduced by metabolically active cells, in part by the action of dehydrogenase enzymes, to generate reducing equivalents such as NADH and NADPH. The resulting intracellular purple formazan can be solubilized in DMSO and quantified by spectrophotometric means. The MTT Cell Proliferation Assay measures the cell proliferation rate and conversely, when metabolic events lead to apoptosis or necrosis, the reduction in cell viability (Mosmann, 1983).

Advantages of using MTT Reagent are that, it yields low background absorbance values in the absence of cells. For each cell type the linear relationship between cell number and signal produced is established, thus allowing an accurate quantification of changes in the rate of cell proliferation (Mosmann, 1983).

**DCFDA assay**: ROS induced Oxidative damage plays a major role in many human diseases including cancer, atherosclerosis, other neurodegenerative diseases and diabetes. Thus, establishing their precise role requires the ability to measure ROS accurately and

the oxidative damage that they cause. There are many methods for measuring free radical production in cells. However the most uncomplicated method is to use cell permeable fluorescent dye 2′-7′-Dichlorodihydrofluorescein diacetate (DCFH-DA). It is one of the most widely used techniques for directly measuring the redox state of a cell (Eruslanov & Kusmartsev, 2010).

Advantages: It is very easy to use, extremely sensitive to changes in the redox state of a cell, inexpensive and can be used to follow changes in ROS over time. It can be measured quantitatively via spectrophotometry or flowcytometry and qualitatively via microscopy.

Quantification of cytokine production is a valuable adjunct to standard immunologic assays in defining several pathologic processes. Cytokines are proteins which play an integral role in the human immune response. The functions of these proteins are diverse and include roles in normal T-cell-mediated immunity, the inflammatory response, cancer, autoimmunity, and allergy (Borish & Rosenwasser, 1996).

**Interleukin 6:** IL-6 (Interleukin-6) is a pleiotropic cytokine that influences antigen-specific immune responses and inflammatory reactions (Kishimoto, 2006). It contributes to chronic inflammation in obesity, insulin resistance, inflammatory bowel disease, arthritis, sepsis, and atherosclerosis. IL-6 signals elicits its response through a receptor complex of IL-6 R alpha and gp130 (Heinrich *et al.*, 2003; Rose-John *et al.*, 2006). gp130 is also a component of the receptors for cytokines CLC, CNTF, CT-1, IL-11, IL-27, LIF, and OSM (Taga & Kishimoto, 1997). Soluble forms of IL-6 R alpha are generated by both alternative splicing and proteolytic cleavage (Rose-John & Heinrich, 1994). In a mechanism known as trans-signaling, complexes of soluble IL-6 and IL-6 R alpha elicit

responses from gp130-expressing cells that lack cell surface IL-6 R alpha (Rose-John & Heinrich, 1994).

Thus in current studies we have assessed the *invitro* toxicity of CPLE and have utilized MTT assay to assess cyto-toxicity, DCFDA assay to evaluate ROS effect in Human liver carcinoma cells (HepG2) and levels of IL6 to know the inflammation results to get entire picture of toxicity of CPLE.

## 2b. 2 MATERIALS AND METHODOLOGY:

### Preparation of Caripill dosage:

- Caripill (Commercially available Carica papaya leaf extract tablet) was dissolved in 0.9% Saline. The resultant suspension was strained using 100uM Cell strainer and was used for various dosages.

### Assessment of Cytotoxicity of CPLE and Caripill (Mosmann T., 1983)

- Thiazolyl Blue Tetrazolium Bromide (MTT)
- 1x Trypsin-EDTA (0.05% / 0.02% in PBS) (Himedia Laboratories)
- *Carica Papaya* Leaf Extract (CPLE: 1mg/ml)
- Dulbecco's Modified Eagle Media, High Glucose (Himedia Laboratories)
- DMSO (Dimethyl Sulphoxide)

### Preparation Instructions

- MTT is soluble in water (10 mg/ml), ethanol (20 mg/ml) and is also soluble in buffered salt solutions and culture media (5 mg/ml). Reconstituted MTT solution is stable for at least 6 months when stored at -0$^{o}$C.
- Storage at 4$^{o}$C for more than four days results in decomposition and yield erroneous results.
- MTT Solution: 5 mg/ml MTT in PBS. Solution must be filtered and sterilized after adding MTT.
- MTT Solvent: Dimethyl Sulphoxide (DMSO)

**Proliferation Assay**

For most tumour cells, hybridomas, and fibroblast cell lines, 5,000 cells per well are taken to perform proliferation assays. 12 well plates need 1 ml of solution and have about 400,000 cells while 24 well plates have 0.5 ml and 200,000 cells at confluency.

**Cells Details:**

HEPG2 (Human Liver Carinoma procured from NCCS, Pune)

**Procedure:**

1. HEPG2 Cells were trypsinized (T-25 flask) and 5 ml of complete media was added to trypsinized cells.
2. Cells were centrifuged in a sterile 15 ml Falcon tube at 1000 rpm in the swinging bucket rotor (~400 x g) for 5 min.
3. Media was removed and cells were resuspended into 1.0 ml complete media.
4. Cells were counted taking care to maintain the sterile conditions when counting.
5. Cells were diluted to about 75,000 cells per ml. using complete media
6. 100 µl of cells (7500 total cells) were added into each well and incubated overnight in $CO_2$ incubator.

**DAY TWO**:

7. Media was removed and cells were treated with CPLE (5ug/ml to 640 ug/ml) fresh incomplete media (after filtration via 0.22um)
8. Final volume was made 100µl per well.
9. Cells were allowed to incubate with the CPLE at various concentration

**DAY THREE**:

10. Media containing CPLE was removed
11. 5 mg/ml MTT (in incomplete media) was added to each well.
12. MTT was also added to blank wells for blank reading.
13. Cells were again incubated for 4 hours at 37$^{o}$C
14. Media was removed carefully without disturbing the cells and without rinsing the with PBS.
15. 150 µl DMSO (MTT solvent) was added and Cells were agitated manually for Five min.
16. The plate was read at 490 nm with a reference filter of 620 nm (absorbance).

**Calculations:**

1. All the calculations were performed with Microsoft Excel (2010)
2. Blank readings were substracted from each readings
3. Average of 3 readings were taken for each concentration (CPLE)
4. Percentage viability was calculated by given formula.

$$\textbf{\% Viability} = \frac{\boldsymbol{Average\ OD\ of\ Treated\ Cells - Blank\ OD}}{\boldsymbol{Average\ of\ Untreated\ Cells\ (control) - Blank\ OD}}\ \boldsymbol{X}\ \textbf{100}$$

5. Readings were plotted on standard bar plots with respective SD values.

**Detection of Intracellular Reactive Oxygen Species.**

Materials:

1. 1x Trypsin-EDTA (0.05% / 0.02% in PBS) (Himedia Laboratories)
2. Dulbecco's Phosphate Buffered Saline with Calcium & Magnesium (Himedia Laboratories)
3. Hank's Balanced Salt Solution (HBSS) with calcium and magnesium and without Red Phenol (Himedia Laboratories)
4. $H_2$DCFDA - 2',7' –dichlorofluorescin diacetate (Sigma Aldrich)
5. Cell Culture Plates
6. 2 x 75 mm, 5 ml polystyrene round bottom tubes (Tarsons, India)

Protocol:

1. HEPG2 cells were plated at a density of 2.5 x $10^5$ cells per well into six-well plates in complete culture medium.
2. Next day the cells (90% confluence) were washed with DPBS and CPLE was dosed at 50ug/ml and 100 µg/ml. Cells were incubated for 24 hrs in $CO_2$ incubator
3. On third day cells were harvested by trypsinization (1000 µl of trypsin / Well).
4. 1 ml of complete culture medium (10% FBS) was added to stop trypsin activity and cells were resuspended by gently pipetting up and down.
5. Cells were collected into 15 ml Falcon tubes by centrifugation (5 min at 400 *x g*) at room temperature. Supernatant was discarded.
6. Cell pellet was washed with 5 ml DPBS and centrifuge 5 min at 400 *x g* at room temperature.

7. Cell pellet was resuspended in HBSS with 25 μmol/L $H_2$DCFDA and by gently pipetting up and down (500 μl/tube).
8. Resuspended cells in tubes were incubated, in $CO_2$ incubator (37°C), high relative humidity (95%), and controlled $CO_2$ level (5%) in dark for 45 min.
9. Cells were analysed on Flow Cytometer.
10. A total of 10,000 events are analyzed in flow cytometry (Beckman Coulter FC 500 Flow cytometer, Toprani Advanced Lab). Fluorescence of DCF on FL-1 channel (525 nm) was recorded.

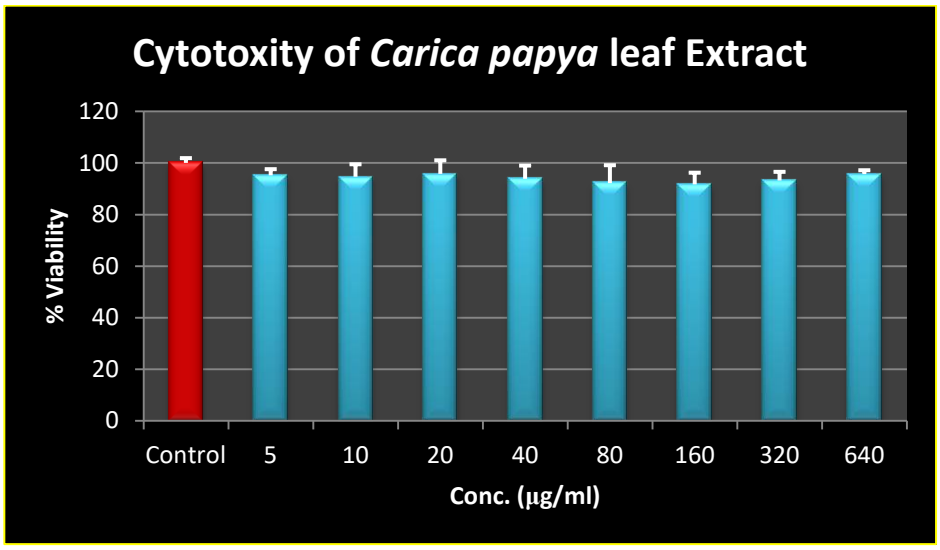
**Analysis of Interleukin 6 levels:**

1. Levels of IL-6 were analysed with ELISA kit (Human IL-6 ELISA: Ray Biotech, ELH-IL6)
2. 0.25 million Cells were plated in each well of 12 well plate and incubated for 24 hrs.
3. Cells were treated with CPLE at dose of 50μg/ml and100μg/ml.
4. Culture media (supernatant) was collected at 2 hrs, 6 hrs and 24 hrs post treatment and stored at -20$^{o}$C until analyzed.
5. The Cell culture supernatant was given to Pathology lab for measuring IL-6 levels (outsourced).

## 2b. 3 RESULTS:

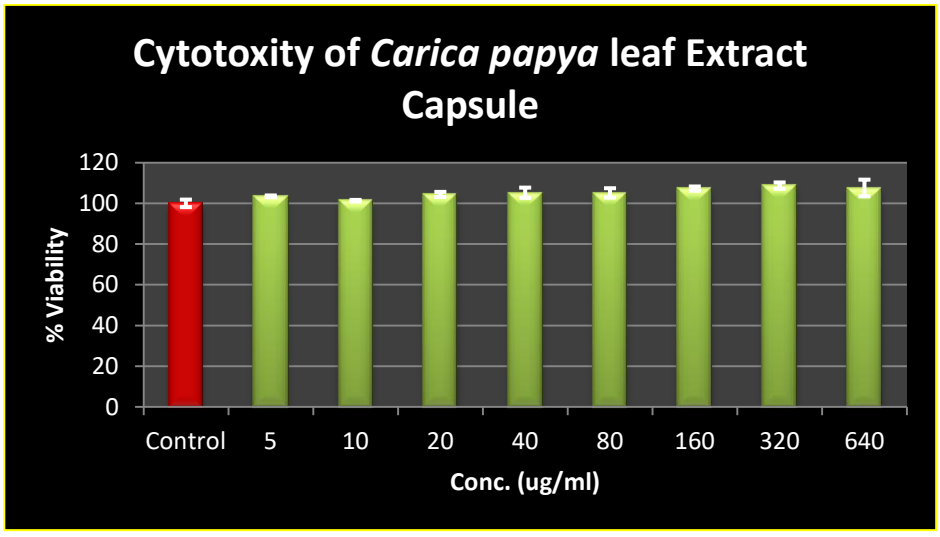
### Assessment of Cytotoxicity of CPLE and Caripill using MTT assay

**Fig. 2b.1: Graph representing assessment of cytotoxicity of CPLE using MTT assay**




***Results are expressed*** as mean ± S.E.M. for n=6. *p<0.05, ** p<0.01, *** p<0.001
Control is compared treatment groups.

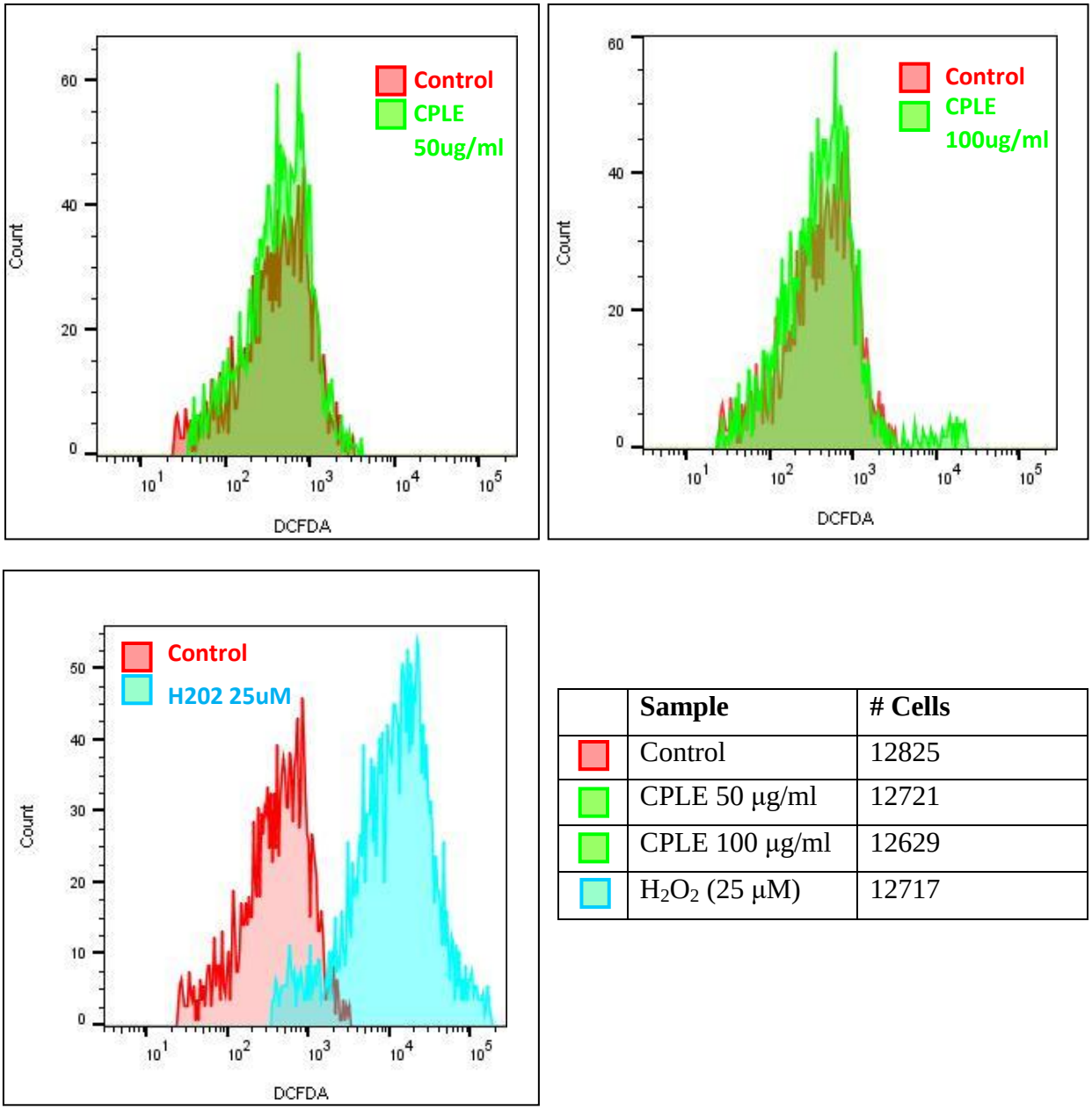
**Fig.2b.2: Graph representing assessment of cytotoxicity of Caripill using MTT assay**




***Results are expressed*** as mean ± S.E.M. for n=6. *p<0.05, ** p<0.01, *** p<0.001
Control is compared treatment groups.

Results of MTT assay revealed that there was no significant cytotoxicity recorded in HEPG2 cells exposed to CPLE (Fig. 2b.1) and Caripill (Fig. 2b.2) at any of the dosage levels i.e 5µg/ ml to 640 µg/ml. However, slight proliferation activity was observed in Caripill dosed group but the activity was non-significant

**Evaluation of Intracellular Reactive Oxygen Species.**

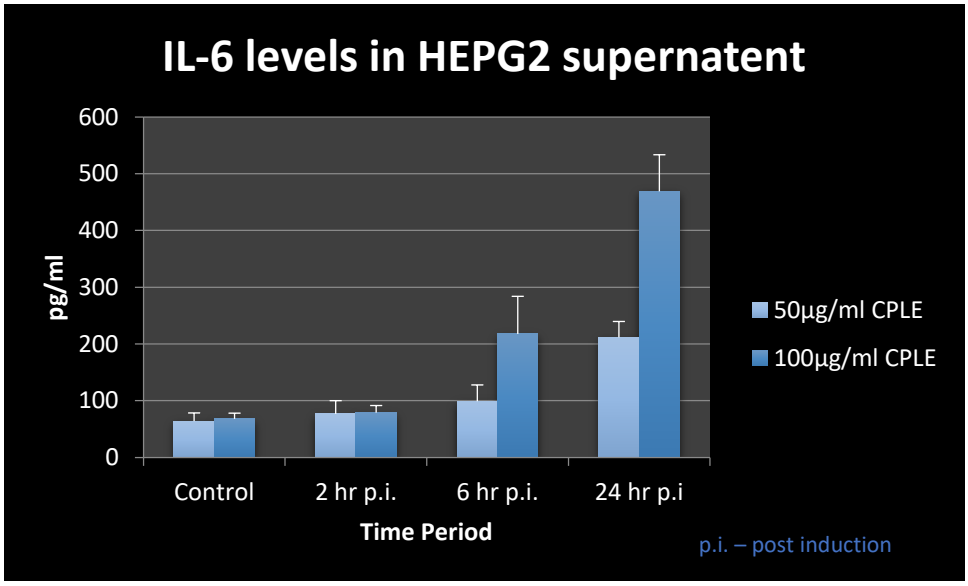



| | Sample | # Cells |
|---|---|---|
| | Control | 12825 |
| | CPLE 50 µg/ml | 12721 |
| | CPLE 100 µg/ml | 12629 |
| | $H_2O_2$ (25 µM) | 12717 |

**FIGURE 2b.3: Graph representing quantification of Intracellular levels of ROS.**

Quantification of intracellular levels of ROS revealed that there was no significant increase in the ROS generation when HepG2 cells were exposed to CPLE with respect to control group. However in CPLE 100µg dosage there was slight fluorescence observed than control group. $H_2O_2$ (25µM) was used as positive control, where HepG2 cells were exposed to $H_2O_2$ showed significantly high intensity of fluorescence depicting more breakdown of DCFDA to DCF.

Fig. 2b.4: Graph representing IL-6 levels in HEPG2 cells supernatant




***Results are expressed*** as mean ± S.E.M. for n=6. *p<0.05, ** p<0.01, *** p<0.001
Control is compared treatment.

Increased levels of IL-6 in supernatant are suggestive of inflammation or anti inflammatory activity as IL-6 is pleiotropic cytokine. There is time dependent increase in the levels of IL-6.

## 2b. 4 DISCUSSION:

The results of in vitro assessment of CPLE clearly showed that it does not exert any toxic effect on HepG2 cells. The MTT Cell Proliferation Assay measures the cell proliferation rate and conversely, when metabolic events lead to apoptosis or necrosis, the reduction in cell viability (Mosmann, 1983). Advantages of using MTT Reagent are that, it yields low background absorbance values in the absence of cells. For each cell type the linear relationship between cell number and signal produced is established, thus allowing an accurate quantification of changes in the rate of cell proliferation (Eruslanov & Kusmartsev, 2010). In our assessment with MTT, the viability of the HepG2 cells were not compromised when treated with CPLE (Fig. 2b.1).

Cells constantly generate reactive oxygen species (ROS) during aerobic metabolism or due to treatment with some of the test compounds. The ROS generation plays an important protective and functional role in the immune system of the cell. The cell is equipped with a powerful antioxidant defence system in both enzymatic and non enzymatic forms are able to combat excessive production of ROS, yet oxidative stress can occur in cells when the generation of ROS overwhelms the cell's natural antioxidant defence property. ROS and the oxidative damage are thought to play an important role in many human diseases including cancer, atherosclerosis, other neurodegenerative diseases and diabetes (Eruslanov & Kusmartsev, 2010). Thus, establishing their precise role requires the ability to measure ROS accurately. In the present study, HepG2 treated cells with CPLE did not generate any excess ROS was evident in the DCFDA assay (Fig. 2b.3) which confirmed that CPLE was non-toxic.

Also the levels of IL-6 were high in HEPG2 cells which were stimulated with CPLE 100 µg/ml, suggesting the inflammation, however IL-6 acts as both pro-inflammatory cytokine and an anti-inflammatory myokine. It has been also reported that IL-6 levels stimulate hematopoesis (Patchen *et al*., 1991; Hauser *et al.,* 1997). In our studies we hypothesize that CPLE increases IL-6 levels in HepG2 which in turn would increase thrombopoetin levels, thus helping to increase platelets in the blood (Fig. 2b.4) which is in accordance with the studies reported by Wolber & Jelkmann, 2000 and Kaser *et al.*, 2001. However, this is a primary study and needs further investigation.

## 2b.5. CONCLUSION:

In light of our findings (both *in vitro* as well as *in vivo*), we may conclude that CPLE is not toxic in all the doses studied herein and did not produce any toxic signs or evident symptoms at acute and sub-chronic oral toxicity administration. CPLE did not cause any lethality or produced any remarkable histopathological signs or serum biochemical alterations. It however showed significant increase in platelet counts with increasing dose and time of exposure. The preliminary results therefore suggest promising alternatives for exploring therapeutic and pharmaceutical interest in CPLE with a reduction of possible adverse effects.

## 2b.6. BIBLIOGRAPHY

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