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INVIVO AND INVITRO SAFETY EVALUATION OF CARICA PAPAYA LEAF EXTRACT FOR POTENTIAL THERAPEUTIC APPLICATION

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ABSTRACT

Carica papaya Leaf Extract (CPLE) has emerged as a promising Phyto therapeutic agent for dengue. However, its potential toxic effects still remains weakly understood. The current study aims at toxicity assessment of CPLE. For acute toxicity, maximum dose of 1000 mg/kgBW was given to female wistar rats. Furthermore, for the sub-chronic toxicity test 24 rats were divided in four group (n=6) and were given dose of 1000mg/kg BW, 100mg/kg BW and 10 mg/kg BW respectively, fourth group served as control. Results indicated that there was no obvious effect at tested doses during the test period. The study showed that oral administration of CPLE had no alteration in serum biochemistry as well as histo-pathology of vital organs in acute as well as sub-chronic toxicity studies. The *in vitro* study indicated that there was no effect of CPLE on cell viability of HepG2 when treated with various concentrations from 5µg/ml to 640µg/ml. Further there was no abnormal ROS generation in HepG2 cells with respect to control. Levels of IL-6 in cell supernatant showed marked time dependent increase which may be correlated with increased thrombopoietin production in HepG2. Thus HepG2 cells did not show any sign of toxicity and CPLE did not cause apparent lethal alterations to HepG2 cells. Taken together, our results provide a useful evaluation for the use of CPLE as the Phyto therapeutic agent against Dengue and other platelet disorders.

KEYWORDS: Acute Toxicity, Subchronic toxicity, Carica papaya, MTT DCFDA, IL-6.

INTRODUCTION

Plants and plant-derived medicines have played a critical role in health and disease management for many centuries. There are wide range of compounds identified and developed from herbs and are used for pain relief, wound healing, abolishing fevers, cancer treatment, hypertension, diabetes and as anti-invectives (Harvey, 2008). Though western medicine drifted away from use of plants, 75 % to 90 % of the rural population in other parts of the world still rely on plant products and herbal medicine as their only means of health care (Robinson & Zhang, 2011). However, systematic scientific studies, evaluating the medicinal properties of regional plants are still lacking, causing issues in authorization and marketing of the drug derived from these plants. Hence, basic scientific investigations on medicinal plants using indigenous medical systems become imminent (Kong *et al.*, 2003).

Carica papaya of Caricaceae family is one such plant, known for its various therapeutic uses such as anti-inflammatory agent, wound healing properties, antitumour, antioxidant and treatment in dengue fever (Owoyele *et al.*, 2008; Gurung & Skalko-Basnet, 2009; Otsuki *et al.*, 2010; Imaga *et al.*, 2010). Nevertheless, in spite of its various uses from time immemorial, studies pertaining to its toxicological effects and safety limits are still lacking.

Therefore the systematic *in-vitro* and *in-vivo* evaluation of *C. papaya* leaves is essential for understating the etiology, mechanism, toxicity profile and safety limits, in order to derive proper drug molecules using suitable models. The non-animal test methods, such as *in-vitro* assays and computer base models act as powerful tools to enhance the extrapolation from *in-vitro to in-vivo* in animals and finally to humans (Broadhead & Combes, 2000).

Hence the present study aims at evaluating the toxicity profile of CPLE (Carica papaya leaf extract), using *in-vitro* and *in-vivo* assays using suitable models.

MATERIAL AND METHODS

Plant material

Fresh green mature leaves of *C. papaya* were collected from local fields, Vadodara, Gujarat, India, during the months of September-October. After the scientific identification by the experts, the voucher specimen (BARO/51/2010) was 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

The animals were maintained at Dept. of Zoology Animal house facility at The M.S. University of Baroda. They 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 and ethical committee of Department of Zoology, The M.S. University of Baroda (827/ac/04/CPCSEA).

2.1 Tocixity Studies

All toxicity evaluations were carried out as per Organization for Economic Co-operation and Development (OECD) guideline. Four month old albino rats of *Wistar* strain weighing 200 – 225 g were selected after acclimatization of 5 days. For acute toxicity (OECD guideline 423/425) twelve animals were divided in two Groups (1) Control (2) CP leaf extract at a dose of 1000 mg/Kg B.W (Acute) whereas in case of sub-chronic toxicity. (OECD guideline 407 test) assessment, Twenty four animals 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. The dose of CPLE was suspended in 0.5 % sodium carboxy methyl cellulose (vehicle) and fed orally with gastric intubation tube. Animals were checked for abnormal behavioral responses, convulsions, abnormal breathing and death. Animals were sacrificed after treatment period under mild Di-ethyl ether anesthesia. Blood was withdrawn for Complete Blood Count (CBC) and serum biochemistry, and vital organs were harvested for histopathology. The Organs were cleaned with PBS and were fixed in 10% formalin for histology studies.

2.2 Biochemical Analysis

Blood was collected in both K3 EDTA (2 ml) vacutainers and plain vacutainers 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 from plain vacutainers for liver and kidney function tests (Serum Glutamate Pyruvate Transaminase, Serum Glutamate Oxalate Transaminase, Acid Phosphatases, Alkaline phosphatases, Urea and Creatinine) using Reckon Diagnostic kits following manufacturer's instruction on Perkin Elmer (Lambda 25 UV/Vis) Spectrophotometer.

2.3 Histopathological Examination

Five micron thick sections were cut using Leica microtome and routine Haematoxylin and Eosin staining was performed for histopathological observations. Histopathological profile of liver, kidney, spleen and ovary was examined.

Assessment of Cytotoxicity of CPLE in HepG2
MTT ASSAY

MTT Assay was performed using Mosmann, (1983). Briefly HEPG2 Cells were seeded at 7500 cells/well in 96 well and incubated overnight. Cells were treated with CPLE (5ug/ml to 640µg/ml) in fresh incomplete media and were incubated for 24 hrs. After incubation media was removed and 5 mg/ml MTT in incomplete media (DMEM) was added to each well and incubated for 4 hours at 37^{o}C. Finally MTT media was removed and 150 µl DMSO (MTT solvent) was added and plates were agitated manually 5 min. The plates were then read at 490 nm with a reference filter of 620 nm (absorbance) on ELISA Reader (ELx-800, Biotek Ins., USA). Percentage viability was calculated and readings were plotted on standard bar plots with respective SD values.

DCFDA: Detection of Intracellular Reactive Oxygen Species.

CPLE was dosed at 50ug/ml and 100 ug/ml to 2.5 x 10^6 cells per HepG2 Cells in 6-well plate. Cells were incubated for 24 hrs in CO_2 incubator. Further cells were harvested after trpsinization and were resuspended in 500ul HBSS with 25 µmol/L H_2DCFDA. Cells were incubated in the dark for 45 min. Finally cells were analysed with flow cytometer (Beckman Coulter FC 500 Flow cytometer, Toprani Advanced Lab) and total of 10,000 events are analyzed and recorded FL-1 channel (525 nm).

Analysis of Interleukin 6 levels

Interleukin -6 (IL-6) levels in cell supernatant were analysed with Elisa kit (Ray Biotech, ELH-IL6.) Briefly 0.25 million Cells were plated in each well of 12 well plate and incubated for 24 hrs. Cells were Treated with CPLE @ dose of 50µg/ml &100µg/ml. Finally culture media (supernatant) was collected at 2 hrs post treatment 6 hr and 24 hr post treatment and stored at -20^{o}C until analysis. Supernatant were analysed with ELISA and results were calculated and plotted with bar graph.

2.4 Statistical Analysis

Data wherever necessary 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 were expressed in mean ± SEM. Treatment effects over the time were compared between control and treatment groups by analysis of variance (one way ANOVA) followed by Dunnett's Post hoc test. P values less than 0.05 were considered statistically significant.

RESULTS

Toxicity Evaluation

No adverse effect were observed in acute as well as sub chronic treatment regime in either of the dose. No observable change in behavioral responses such as tremors, salivation in tested rats was observed. Physical observations indicated no signs of changes in the skin, fur, eyes mucous membrane in either regime. There was no mortality observed in any of the tested dose in acute as well as subchronic regime. No significant differences observed in the haematological and biochemical parameters in the CPLE treated group compared to the normal control group (Table 1) in acute dose regime. Similarly there were no significance differences in serum biochemistry of liver function tests (Table 2) and renal function tests (Table 3) in sub chronic dosage *viz.* 10mg/kg BW, 100mg/kg BW and 1000mg/kg BW with respect to control group. Hematological parameters also did not differ from control group in subchronic toxicity studies (Table 4). 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 in acute as well as subchronic dosage (Fig. 1 & 2).

Table 1: Serum Biochemical Parameters of Acute studies.

Parmeters	Control	Treatment
SGPT (IU/l)	26±1.5	31.67±1.62
SGOT (IU/l)	28.5±1.23	52.17±3.53
Alk PO4 (IU/l)	169.67±21.23	266.33±33.39
Acid PO4 (IU/l)	2.4±0.32	2.52±0.19
Urea (mg/dl)	37.06±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

Table 2: Serum Hepatic Parameters of Acute studies.

Hepatic Function Tests				
	7th day	14th day	21st day	28th day
Serum Glutamic Pyruvic Transaminase				
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
Serum Glutamic Oxaloacetic Transaminase				
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
Serum Alkaline Phosphatase				
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
Serum Acid Phosphatase				
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

Table 3: Serum Renal Parameters of Acute studies

Renal Function Tests				
	7th day	14th day	21st day	28th day
Serum Urea				
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
Serum Creatinin				
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

Table 4: Complete Blood count of rats treated with aqueous extract of *Carica papaya* leaf extract.

Days of 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	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/kgBW	6.29±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	6.32±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	8.05±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.

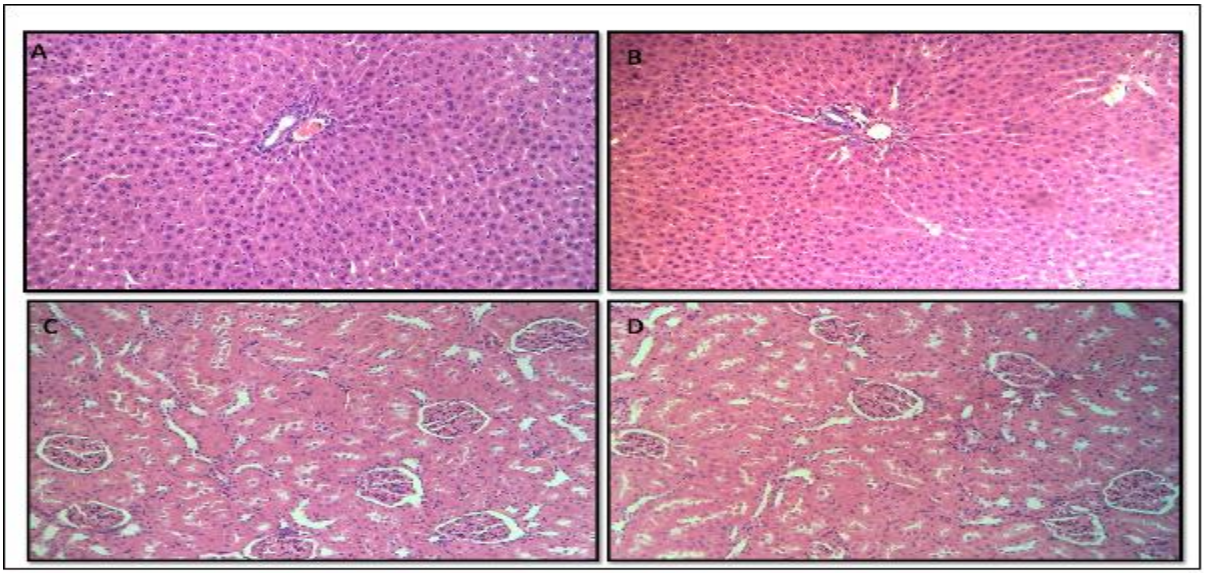


Fig. 1: Histopathological studies of acute toxicity (A) & (B): Representative histological section of Liver stained by (H-E) Control group and CPLE treated groups. Normal histo-architecture is observed. Magnification: 200×. (C) & (D) Representative histological section of Kidney stained by (H–E) from control and CPLE treated group No difference in glomerular morphology is seen. No significant histo-pathological changes were observed. Magnification: 200×.

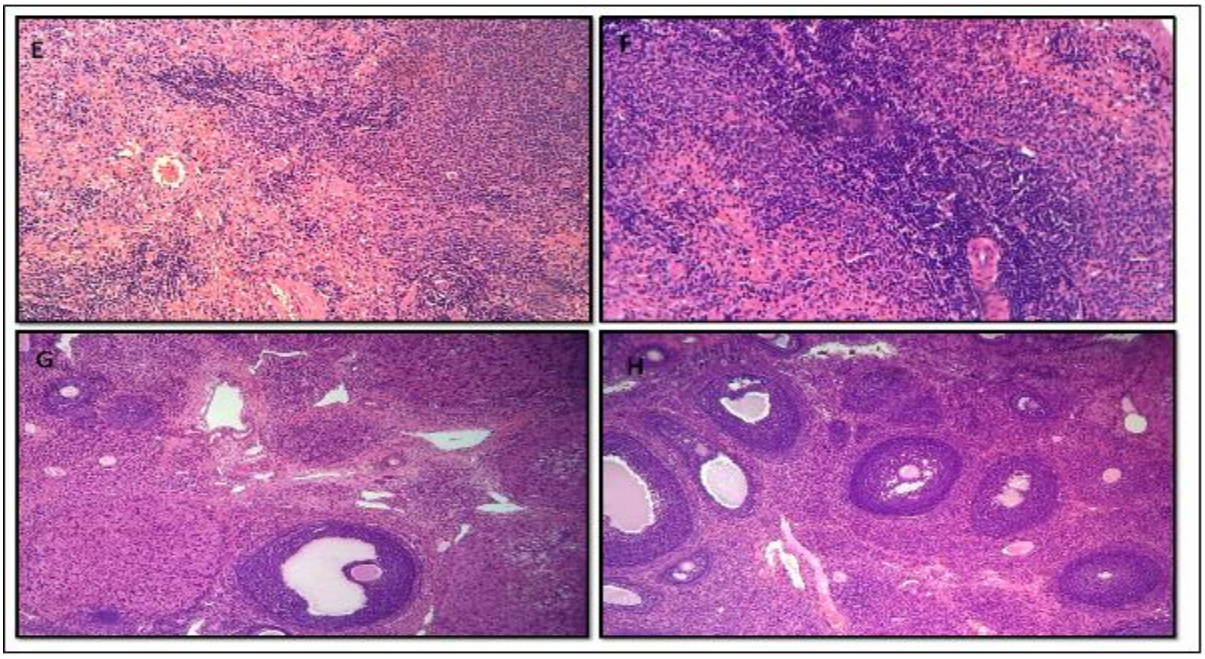


Fig. 2: Histopathological studies of subchronic Toxicity (E) & (F) Representative histological section of Spleen of Control and CPLE treated group respectively, stained by (H-E) No significant difference was observed as compared with control histology. Magnification: 100×. (G) & (H) Representative histological section of ovary stained by (H-E) from Control and CPLE-treated group respectively. Magnification: 100×.

Assessment Cytotoxicity of CPLE using MTT assay
Results of MTT assay revealed that there was no significant cyto-toxicity recorded in HEPG2 cells exposed to CPLE (Fig. 3) at any of the dosage levels i.e. 5ug/ ml to 640µg/ml.

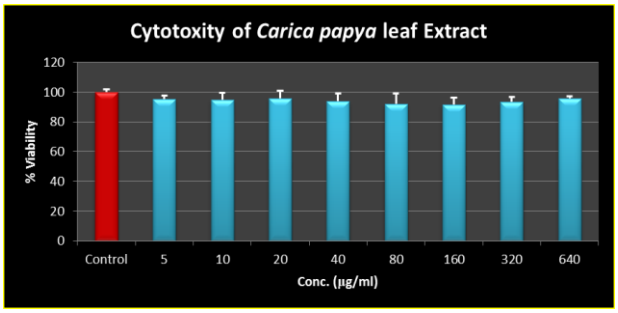
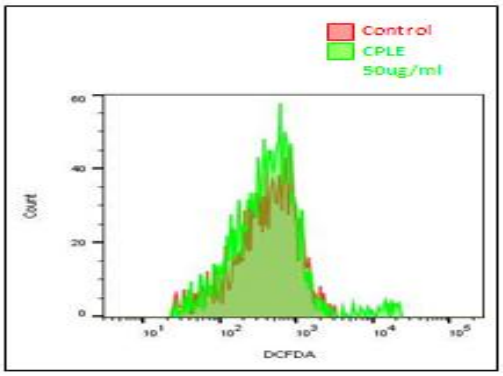
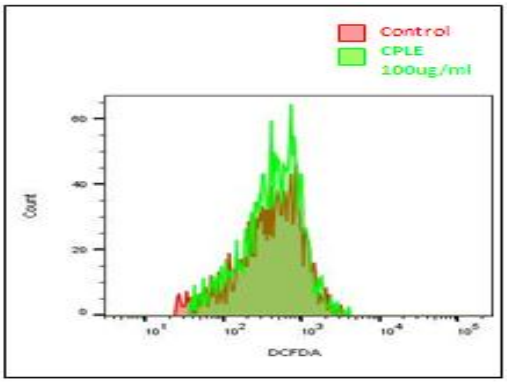


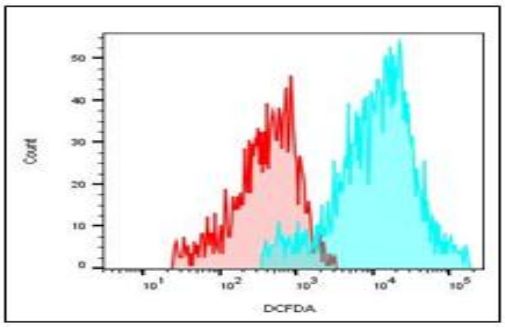
Fig. 3: Represenating results of MTT assay.

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.

Evaluation of Intracellular Reactive Oxygen Species.
Intracellular levels of ROS quantification revealed that there was no significant increase in the ROS generation when HepG2 cells were exposed to CPLE with respect to control group (fig. 4a). However in CPLE 100ug dosage there was slight fluorescence observed than control group (Fig 4b). H_2O_2 (25uM) 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 4c).







	Sample	# Cells
	Control	12825
	CPLE 50 µg/ml	12721
	CPLE 100 µg/ml	12629
	H_2O_2 (25 uM)	12717

Fig. 4: Graph representing quantification of intracellular levels of ROS.

Evaluation of Interleukin-6 levels in HEPG2 Supernatant
Supernatant levels of IL-6 significantly showed increased levels of IL-6, suggesting inflammation or anti inflammatory activity, as IL-6 is pleiotropic cytokine. There is time dependent increase in the levels of IL-6 in both 50µg as well as 100µg dose of CPLE, however 24 hours post treatment showed moderately but significant increase in IL-6 levels and highly significant increase in the IL-6 levels in 100µg does (Fig 5).

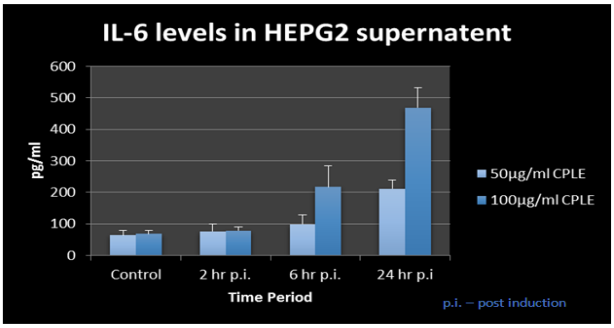


Fig. 5: Representing time depended and dose depended activity of Interleukin-6.
Results are expressed as mean ± S.E.M. for n=6.
*p<0.05, ** p<0.01, *** p<0.001
Control is compared treatment.

DISCUSSION

The current study evaluates the acute and chronic toxicity of CPLE in an animal model. The hematological, histological and biochemical estimation revealed that CPLE dose of 1000mg/kg has no adverse effect on the tested animals, thus establishing LD_{50} value greater than 1000 mg/kg. Studies carried out by Kennedy *et al.* (1986) has suggested safe use of substance with LD_{50} values higher than 5000 mg/kg by oral route and are considered as nontoxic. However, studies evaluating the toxicity levels of medicinal plants especially used by traditional healers are still lacking. Therefore, data pertaining to acute and subchronic toxicity studies on medicinal plants are required in order to increase the confidence in its safety to humans, particularly for use in the development of pharmaceuticals (Ukwuani *et al.*, 2012).

In the current study, the results from acute as well as sub-chronic toxicity tests of CPLE (10–1000 mg/kg/day) were in accordance with each other. No clinical signs of toxicity or mortality, or variation in the consumption of food and water were observed. Chokshi (2007) has reported decreased food conversion efficiency when higher dose of crude plant extracts were given. Hilaly *et al.* 2004, has reported, the variations in the body weight as a sensitive indicator for the general health status of an animal. The relative weight gain in the organs also serves as an sensitive indicator of toxicity (Michael *et al.*, 2007). In the current study a normal weight gain was observed in all the animals administered with CPLE. There were no signs of clinical toxicity nor were any noteworthy changes in the weight of heart, liver, spleen, kidneys and lungs or signs of hypertrophy, observed. Thus, establishing the non toxic profile of CPLE even after subchronic treatment and confirming the safe use of extract via oral route, as used in the folkloric practices.

The above results were substantiated by serum biochemistry assays, where no significant changes in SGOT, SGPT, alkaline & acid phosphatase were observed in the treated groups. Serum biochemistry assays specially pertaining to liver and kidney are considered essential tools in evaluating the toxicity of drugs and plant extracts, the reason could be attributed to their association with removal of toxic substances from an organism (Olorunnisola *et al.* 2012). Studies by Brautbar & Williams (2002) suggested high levels of SGOT, SGPT, acid and alkaline phosphatase in liver as an indicator of diseases and/or hepatotoxicity. As no significant changes were observed in hepatocyte functioning, the present study suggests that CPLE is safe for administration.

Similarly non-significant differences were observed in serum urea and creatinine levels, there by indicating no adverse effect on renal functioning. 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). The results from serum biochemistry assays were supported by the histopathological studies were no changes were observed in the histoarchitecture of liver, kidney, spleen and ovary in both treatment regimes. Generally, any damage to the parenchymal liver cells results in elevations of both transaminases in the blood (Slichter, 2004). Results from current study showed non-significant changes in SGOT, SGPT activities, there by strongly suggesting non interference of CPLE in hepatocytes functioning, consequently in the metabolism of the rats when administrated with sub chronic dosage. Similarly, there were 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, where no alternation in the histo-architecture was observed.

Hematology analysis plays an essential role in risk evaluation and higher predicting values, pertaining to human toxicity, when the data are translated from animal studies (Yakubu *et al*., 2007). Thus a hematogram was undertaken for all the CPLE treated and control groups in subchronic toxicological profile regimes. There were no significant effects of the extract on total red blood cells, mean corpuscular volume and mean corpuscular Hb, indicating that the CPLE does not affect the erythropoiesis, morphology or osmotic fragility of the red blood cells. Similarly, no significant changes in the WBC count were observed, which further confirmed the above findings. Interestingly, however, we observed a dose dependent and time dependent increase in the platelet count. Studies carried out by Sathasivam,*et al.*, 2009 are in accordance with the present findings, there by further establising non-toxic nature on CPLE based on normal haematological profile of treated group.

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, reducing the cell viability (Mosmann, 1983). MTT assay evaluates an accurate quantification of changes in the rate of cell proliferation because each cell type has the linear relationship between cell number and signal produced (Eruslanov & Kusmartsev, 2010). Results from the current study indicted no compromisation in the viability of the HepG2 cells when treated with CPLE as observed in MTT profiling.

Reactive Oxygen Species (ROS) molecules are generated as a response to aerobic metabolism or in case of drug treatment. The ROS generation plays an important protective and functional role in the immune system of the cell. Though the cell is equipped with a powerful antioxidant defence system, both in enzymatic and non

enzymatic form, to combat excessive production of ROS, the cells can nevertheless suffer from oxidative stress specially in the case when generation of ROS overwhelms the cell's natural antioxidant defence system. ROS and the oxidative damages play an important role in many human diseases including cancer, atherosclerosis, other neurodegenerative diseases and diabetes (Eruslanov & Kusmartsev, 2010). Accordingly, establishing their precise role requires the ability to measure ROS accurately. The results from DCFDA assay showed no excess generation of ROS in HepG2 cells and did not increased the ROS load on the cells, when treated with CPLE. Thus the results from DCFDA assay once again confirmed the non toxic nature of CPLE.

IL-6 levels which indicate inflammation and related stress were also tested. In our study the levels of IL-6 were found to be high in HepG2 cells stimulated with CPLE 100 ug/ml, suggestive of inflammation. However, this could be attributed to pleiotropic cytokine nature of interleukin 6, which acts as both a pro-inflammatory and an anti-inflammatory myokine. We believe that CPLE increases IL-6 levels in HepG2 which inturn would increase thrombopoetin levels, thus helping to increase the platelets in the blood. Similar findings has been reported by Patchen *et al* (1991) and Hauser *et al* (1997), where the authors have reported stimulation of hematopoiesis by the activity of IL-6. Finding from the present study are also in accordance with the results of Wolber & Jelkmann (2000) and Kaser *et al.* (2001) there by suggesting the role of CPLE in thrombopoesis and increased levels of thrombopoetin in hepatocytes.

The results from the present study establish the non toxic nature of CPLE, however, as these are priliminary studies, further evaluation in the same direction will be helpful in deducing the exact role of CPLE in thrombopoesis.

CONCLUSION

CPLE did not show any toxic effects in acute toxicity as well as sub-chronic toxicity studies, in Wistar rats. Moreover there were no signs of ROS generation, inflammation associated cytotoxicity in *invitro* studies using HepG2 cells. However, we recorded significant dose dependent increase in blood platelet count of rats exposed to CPLE which we hypothesize could be due to changes in IL-6 cell signalling axis.

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