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Toxicity Evaluation of *Anisomeles indica* Kuntze Leaf Flavonoid Fraction

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Abstract

Anisomeles indica (L.) Kuntze belongs to Lamiaceae, is used against inflammation and gastric dysfunctions. Till-date there are no reports on toxicological evaluation of *A. indica* leaf flavonoids. In the present study, *in vivo* and *in vitro* toxicity evaluation of *A. indica* leaf flavonoid fraction was carried out to assess its safety. Acute toxicity results in mice suggest that, LFF is non-toxic, as the treated animals survived beyond 14-day observation period and with no mortality and toxic signs or symptoms. Subacute oral toxicity study was carried out for 28 days in Swiss albino mice both sexes. Data revealed no statistically significant dose-related effects on food consumption, body weight gain, clinical signs in LFF treated animals. Urine analysis, hematological parameters, serum biochemical examination and histopathology revealed no adverse effects even at 1000 mg/kg body weight/day. *In vitro* chromosome aberration assay in human lymphocytes revealed no clastogenic effect at 24 h and 48 h LFF treatments. Cytotoxicity results against human cancer cell lines KB, HepG2, HT-29 and HEK-293 suggests, LFF is cytotoxic to four human cancer cell lines with IC_{50} values 36.57, 40.0, 48.6, 68.58 µg/mL respectively. In conclusion *A. indica* LFF appears to be safe for oral administration in humans, may be used in traditional medicine.

Keywords: *Anisomeles indica*; Acute toxicity; Subacute toxicity; Genotoxicity; Cytotoxicity

Introduction

Custom practiced medicines have a very tedious record, and are the whole of practices founded on speculative thought, trusts and practical acquaintance with facts. They are being used in keeping up of health, such as in the hindering, identification of disease, improvement and treatment of illnesses. Plants have been used in diverse traditional treatment for many years and manifest to be efficient by current medical science. The opinion that, herbal medicines are reliable and independent of side effects as they are "natural" promote the public to use natural extracts for self-treatment [1]. Herbal treatment, comprising folk herbal recipes are a prevalent trend in the area of Complementary and Alternative Medicine (CAM) [2,3]. Most of the plants used in traditional medications have shown to be efficient by the upright support of modern science [4]. Side effects of synthetic chemical drugs are great worry in modern society, thus there is increasing popularity in folk medicine. Scientific studies are being actively conducted on ethnobotanical medicines [5]. However, there are no available facts of degree of excellence, reliability and efficacy of majority of the generally used herbal recipes. Recently it has been established that, poisonous side effects combined quite with diverse beneficial herbs [6-8]. These features need to consider among the visible effects of absolute examinations carried out for the toxicity assessment of these folk medicines and herbal recipes.

Anisomeles indica (L.) Kuntze belongs to the family Lamiaceae/Labiatae, under the Magnoliopsida class and order Lamiales. The plant grows as a weed in wild. In India it is commonly called as 'Indian Cat mint' and 'Kalabhangra'. The other vernacular names for the plant are, English-Malabar catmint; China-Fang feng cao; Malaysia-Pokok Ati-ati; Indonesia-Ramput Ati-ati (Bangra); Congo-Sauang-sauang (Mbo.); Philippines-Kabling lalake; Hindi-Kalabhangra, Gobara; Marathi-Gopali; Malayalam-Chedayan; Telugu-Adabeera; Kannada-Mangamari soppu.

A. indica aerial parts are used in rheumatism, paralysis, epilepsy, convulsions, spasm, pregnancy, stomach trouble, cold, fever, intermittent fever and dyspepsia. Aqueous extracts are used in ethnobotany to treat disorders like inflammation, gastric dysfunction. Leaves are chewed for toothaches, considered useful in chronic rheumatism, psoriasis and other chronic skin eruptions. Bruised leaves are applied locally in snake bites. *A. indica* has significant gastro-protective activity that is mediated via an increase in the thickness of the protective mucous layer and by antioxidant activity [9-11]. Published reports in pertinent literature suggest that, *A. indica* contains secondary metabolites including flavonoids, diterpenoids, phenyl propanoids, steroids and yields essential oil [9-10,12-19]. Ethanol extract of *A. indica* is active against *Helicobacter pylori* infection [10]. Ovatodiolide, a diterpenoid of *A. indica* has been shown to anti-HIV activity (IC_{50} of 1.2 µg/mL) with maximum cellular protection upto 80-90%, tumor cell proliferation and inflammatory mediators [20-22]. Ovatodiolide pre-treatment to Triple-Negative Breast Cancer (TNBC) cells sensitizes the cells for doxorubicin cytotoxicity, and make the cells to lose their cancer stem cell-like phenotype [23]. However, in spite of its immense medicinal and pharmacological potential, till-date there are no published reports in the literature on toxicity evaluation of leaf flavoinds of *A. indica*. Therefore, our present study is aimed to carry out the toxicity evaluation of *A. indica* Leaf Flavonoid Fraction (LFF) through *in vivo* oral acute and sub-acute toxicity in mice, *in vitro* genotoxicity in human lymphocytes and cytotoxicity in human cancer cell lines. The investigations were carried out to determine the safety limit of this extract.

Materials and Methods

Collection and identification of plant materials

A. inidca Kuntze plants at the pre-flowering stage (between June-August, 2013, during monsoon season) were collected from a field area

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of Davangere University campus, lies in the Maidan region on Deccan Plateau (western part of south India), positioned at geographical centre of the state Karnataka $14^{0}28^{1}$N latitude and 602.5 meters (1977 ft) above sea level and receives 644 mm annual rain fall. Plant was authenticated by plant taxanomist Prof. Pushpalatha, Department of Botany, Sahyadri Science College, Kuvempu University, Shivamogga, India. Leaves (aerial parts) were used for the flavonoid fraction extraction. Leaves were separated, shade dried and pulverized in a mechanical grinder. The coarse powder was sieved passing through sieve No. 40, and used for the solvent extraction.

Isolation of Leaf Flavonoid Fraction (LFF)

Plant material (coarse powder) was defatted in petroleum ether at room temperature for about 24 hours, filtrate was collected and shade dried. The dried material was extracted with methanol and subsequently concentrated under reduced pressure at 30°C. Extract concentrates were combined, purified by subjecting to liquid-liquid partition against ethyl acetate to remove chlorophylls, stilbenoids, less polar flavonoids and other non-polar compounds from the mixture. Aqueous extracts obtained after the partition step combined. Further the water soluble non-aromatic compounds other than flavonoids like free sugars, aliphatic acids in the extract were removed by adsorption chromatography using Amberlite XAD-4, which adsorbs aromatic compounds including flavonoids, anthocyanins. While free sugars and non-aromatic compounds were removed by washing the column with distilled water until eluted or eluent water pH resulted neutral. The adsorbed flavonoids were eluted using methanol [24]. Thus obtained LFF was concentrated under reduced pressure, dried at room temperature and powder was used for further experiments. Yield of LFF or ratio of LFF obtained was 1.75 g per 50 g dried leaf powder, from 370 g of raw leaf material. LFF was dissolved in methanol (1 mg/mL), filtered through a 0.2 µm microfilter (Millipore) and subjected for HPLC analysis using Agilent 1260 infinity HPLC unit equipped with Diode Array Detector (DAD), C18 column at 30°C, methanol and water (30: 70) as mobile phase with flow rate 0.5 mL/min, and detected at 270-340 nm by DAD.

Acute toxicity of LFF in mice

Study design: Acute toxicity study was carried out for 14-day period in accordance with OECD Guideline 420 [25]. The repeat-dose toxicity study was conducted in mice (5 mice/group) of single sex, dosed with LFF dissolved in distilled water at 5, 50, 300, 2000 mg/kg body weight/10 mL DW by oral gavage once daily for 14 days, and equal volumes of sterile water as a control. Weight of each animal in group was recorded before administration of LFF. After administration of LFF animals were observed individually for behavioural pattern. Changes in the skin, eyes, respiratory and behaviour pattern were recorded. Animals were observed individually after dosing for every 30 min for 4 h and daily once thereafter for 14 days, and body weight of each animal was recorded [26]. All the animals in LFF treated group withstood 2000 mg/kg body weight of LFF, appeared to be healthy. Hence the animals were administered LFF 5000 mg/kg b.w. to a group of 5 female mice, which is the highest dose tested for the toxic symptoms. The animals were observed for behavioural changes and toxicity signs for 14 days.

Sub-acute toxicity

Experimental design: Subacute oral toxicity study was carried out by following OECD Guideline 407 [27]. The 28-days repeated dose study was carried out in Swiss albino mice (5/sex/group) administered with LFF, daily a dose of 100, 500 or 1000 mg/kg body weight by oral gavage. Equal volume of sterile distilled water administered for control group.

Clinical observations, body weight and food consumption: All the groups of animals were observed for toxicity and mortality behaviour two times daily throughout the study period. Meanwhile animals were observed for changes in skin, fur, eyes, mucus, membranes possessing of any abnormal oral secretion as well as urine and faeces, presence of involuntary functioning, behaviour for handling, presence of healthy elasticity of muscles and any strange behaviour has been recorded before and after the dose. Periodically body weights were recorded at before starting of treatment and throughout the study once in a week. Daily food consumption was recorded and means food consumption determined.

Urine analysis: Mice urine samples were collected before 16 h of sacrifice and subjected for analysis. Physical appearance of urine samples recorded and pH were determined. Further samples were analysed for the presence of glucose, bilirubin, ketones, protein and blood [28].

Blood clinical parameters: Mice were sacrificed by cervical dislocation. Blood samples were collected from direct heart puncture. Blood samples were subjected for clinical biochemistry parameters and hematological parameters analysis. Quantitative determination of White Blood Cell (WBC), Red Blood Cell (RBC), Hemoglobin (HGB), Hematocrit (HCT), Mean Corpuscular Volume (MCV), Mean Corpuscular Hemoglobin (MCH), Mean Corpuscular Haemoglobin Concentration (MCHC), Platelets (PLT), Mean Platelet Volume (MPV), Neutrophils (NEUC), Lymphocytes (Ly), Monocyte (Mo), Eosinophil (EOS), and Basophil (BASO) were performed using automated haematology analyser.

Serum biochemistry: To determine clinical parameters serum has been separated and used. Clinical chemistry parameters like Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST), γ Glutamyl Transferase (γ-GT), Albumin (ALB), Globulin (GLO), Total Protein (TPRO), Total Bilirubin (TBIL), Creatine (Cr), Blood Urea Nitrogen (BUN), Glucose (Glu), High Density Lipoprotein (LDL), urea, uric acid, Total Cholesterol (TOTCHOL) and Triglycerides (TRIG) were determined by using semi autoanalyzer (Prie-test Touch, RobonicK, India).

Organ weights, gross necropsy and histopathology studies

After sacrifice of all mice, vital organs like liver, spleen, kidney, heart and testis (only males) were excised and weights of individual organ determined and they were preserved in phosphate buffered 10% formalin and submitted for histopathology analysis to department of pathology laboratory, S.S. Institute of Medical Sciences and Research Centre, Davangere, India.

In vitro chromosome aberration (genotoxicity) assay

The chromosomal aberration (*in vitro* genotoxicity) assay was carried out [29] using human lymphocytes isolated from healthy non-smoking male volunteer aged 27 years. Lymphocytes were isolated from heparinized blood using HiSepTM LSM 1077 (Himedia, India). Blood was diluted 1:1 with PBS and layered onto HiSepTM LSM 1077 at a ratio of 4:3 (Blood+PBS:HiSep) and subjected to centrifugation at 1200 rpm for 45 min at room temperature. After careful removal of lymphocytes, subjected to wash with PBS twice at 1200 rpm for 10 min. Lymphocyte cultures were set up with 5 ml of RPMI 1640 medium supplemented with 10% fetal calf serum, $NaHCO_3$ (7.5% w/v), 200 mM glutamine, pencillin (100 units/mL) and streptomycin (100 µg/mL) in a 15 mL conical tube. To initiate cell division in lymphocyte culture 100 µL phytohaemagglutinin (PHA). Cultures were incubated in humidified 5% CO_2 atmosphere at 37°C. 24 h old lymphocyte culture was taken for the study.

Cultured lymphocytes (for 24 h) were treated with different concentrations of LFF in PBS (w/v) 100, 500, 1000 µg/mL, for 24 h and 48 h individually, and a control group of lymphocytes were maintained treating with PBS. 2 h before harvest, 0.2 µg/mL colcemid was administered, and metaphase chromosomes were prepared [30]. Briefly, harvested cells subjected to hypotonic treatment with KCI (0.075 M) for 20 min and fixed in acetic acid:methanol (1:3) solution. Cell lysates were added to pre-cleaned slides, air-dried and chromosomes stained with diluted Giemsa (1:20). Assay was repeated for three times and the data were summarized as mean number of chromosome aberrations. Slides were coded, but not scored, blind. One observer scored for aberrations. The number of cells with chromosomal aberrations among 100 well spread metaphases were recorded [31]. Negative (-) result was decided if the frequency of cells with structural or numerical aberrations in both untreated and treated group did not exceed 4%. Similarly positive (+) result was decided by that the frequency of aberrant cells or polyploidy was ≥ 10%.

Cytotoxicity assay

Cell lines and culture conditions: Four human cell lines were investigated for cytotoxicity effect of *A. indica* LFF. HEK-293 (kidney), HepG2 (liver), KB (mouth), HT-29 (colorectal adenocarcinoma) cell lines obtained from NCCS, Pune, India. Cells were grown in MEM medium supplemented with 10% heat inactivated Fetal Calf Serum (FCS), containing 5% mixture of gentamicin (10 µg/mL), penicillin (100 units/mL) and streptomycin (100 µg/mL). Cultures were incubated at 37°C in humidified atmosphere of 95% air and 5% CO_2.

In vitro cytotoxic activity

The proliferation rates of human cell lines, HEK-293 (kidney), HepG2 (liver), KB (mouth), HT-29 (colorectal adenocarcinoma) were determined after treatment with LFF by colorimetric assay using 3-(4,5-dimethyl thiazol-2-yl)2,5-diphenyl tetrazolium bromide (MTT), that works with the principle, yellow colored compound MTT is reduced by mitochondrial dehydrogenases to the water insoluble blue compound formazan, depending on the viability of cells [32]. Briefly cells were seeded into 96 well microplate at a density of 2.5×10^4 viable cells per well, and LFF dissolved in DMSO (w/v) was added at different concentrations ranging from 10, 20, 25, 30 and 50 µg/mL. To evaluate cell survival after 48 h, 20 µL of MTT solution (5 mg/mL) was added to each well and incubated for 4 h at 37°C in a CO_2 incubator. Supernatants were carefully aspirated, the precipitated crystals of formazan blue were solubilized into 100 µl DMSO and optical density was measured at 570 nm using microplate reader (LISA Plus). All tests and analyses were run in triplicate and averaged.

The percentage of viability was determined as formulated below:

$$I\% = (A_{blank} - A_{sample} / A_{blank}) \times 100$$

where, A_{blank} is absorbance of the control (without test compound), and A_{sample} is absorbance of the test compound.

The IC_{50} LFF of value (the drug concentration reducing the absorbance by 50% in treated cells, with respect to untreated cells) was calculated from the graph generated by plotting the percentage of cytotoxicity (cell death) versus the tested concentrations of LFF.

Statistical analysis

Results were presented as mean ± SD. In order to make comparisons between the groups, data were analyzed by one-way analysis of variance and Duncan's test. p-values less than 0.05 indicated a significant difference among groups.

Results

Isolation of leaf flavonoid fraction

A. indica LFF was successfully isolated and HPLC analysis from 270 nm to 340 nm revealed the presence of four compounds (Figure 1). The total phenolic content was 328 mg gallic acid equivalent/g LFF, and total flavonoid content was 241 mg Catechin equivalent/g LFF.

Acute toxicity

Animals treated with LFF (5, 50, 300, 2000 mg/kg b.w.) were remained alive, showed normal appearance and no death was recorded during 14 days of observation. All the animals showed body weight gain and results of mean body weight gain presented in Table 1. Results of acute toxicity study suggested that, the maximum tolerated dose of LFF was more than 5000 mg/kg b.w., and in this group of animals some adverse effects like abdominal contraction, piloerection, tachypnoea, catatonia and hypothermal were observed (Table 2). However, these symptoms gradually disappeared within 12 h, and there was no treatment-related mortality observed in the animals treated at this dose.

Subacute toxicity

Clinical observation and mortality: Clinical exceptional and loss of life has not been recorded between the animal groups throughout the study.

Food consumption and body weight: Oral administration of LFF for 28 days did not show any noticeable effect on the body weights of treated mice compared to control group (Table 3). Body weight gain was observed to be normal in all the animals administered with LFF over the treatment period as compared to control group of animals, further there was no significant difference in food consumption observed between male and female mice in all the groups (Table 4).

Urine analysis: Results of 28-day repeated toxicity study of LFF on urine parameters are presented in Table 5. Urinary parameters such as pH, protein, glucose, ketone bodies and bilirubin were found to be within the physiological range and there were no significant differences observed among LFF treatment and control groups of animals.

Hematological parameters: Results of hematology are presented in Table 6. There are no significant differences observed between LFF treated and control groups of animals. Hematological parameters studied including total Red Blood Cells (RBC), White Blood Cell (WBC), Hemoglobin (HGB), Hematocrit (HCT), Mean Corpuscular Volume (MCV), Mean Corpuscular Hemoglobin (MCH), Mean Corpuscular Hemoglobin Concentration (MCHC), Platelets (PLT), Mean Platelet Volume (MPV) and white blood cell differential count were found to be within the normal physiological range in LFF treated groups and control group of mice.

Clinical chemistry: Outcome of clinical chemistry was briefly accounted in Table 7. Oral administration of LFF for 28 days did not cause any significant changes in biochemical parameters including, ALB, GLO, TPRO, TBIL, Cr, BUN, Glu, HDL, LDL, UREA, URIC ACID, TOTCHOL, TRIG, and the activities of the marker enzymes of liver ALT, AST, γ-GT in the treatment group of animals relative to control group of animals.

Gross necropsy, organ weights and histopathology: Results of histopathology confirm that, no gross lesions were observed at necropsy in LFF treatment groups as compared to control group of animals. Data on organ weights recorded in Table 8. No statistically significant differences in absolute organ weights observed among the treatment groups relative to control group of animals. Results of histopathological

CFF Dose (mg/kg b.w.)	Body weight (g)		
	Day 0	Day 7	Day 14
Control	18.16 ± 0.68	18.88 ± 0.51	19.44 ± 0.84
5	18.48 ± 0.84	19.33 ± 1.23	20.18 ± 1.04
50	19.74 ± 0.46	21.06 ± 0.81	22.38 ± 0.80
300	19.12 ± 1.02	20.96 ± 2.20	22.8 ± 0.790
2000	18.80 ± 0.50	20.47 ± 0.40	22.14 ± 0.64
5000	18.96 ± 0.58	20.53 ± 1.30	22.1 ± 0.850

Values are means ± SD.
No statistically significant differences observed between control and the treatment groups.

Table 1: Mean body weights of mice during acute toxicity study.

CFF Dose (mg/kg b.w.)	Behavior				
	Abdominal contraction	Piloerection	Tachypnea	Catatonia	Hypothermal
Control	-	-	-	-	-
5	-	-	-	-	-
50	+	-	+	-	-
300	+	+	+	+	+
2000	+	+	+	+	+
5000	+	+	+	+	+

(+) present; (-) absent

Table 2: Behavioral patterns of mice in different dose groups after CFF administration.

Groups	Control	Leaf flavonoid fraction (LFF)		
		100 mg/kg b.w.	500 mg/kg b.w.	1000 mg/kg b.w.
Females				
Day 0	31.158 ± 1.42	27.412 ± 2.52	28.394 ± 1.57	30.340 ± 0.88
Day 7	29.308 ± 1.46	25.906 ± 2.63	28.054 ± 1.70	29.982 ± 0.58
Day 14	31.280 ± 2.33	24.768 ± 3.38	24.728 ± 2.35	31.124 ± 0.67
Day 21	29.850 ± 1.25	24.386 ± 3.48	25.670 ± 1.43	30.120 ± 0.69
Day 28	33.156 ± 1.76	24.114 ± 3.17	30.370 ± 1.39	31.860 ± 0.90
Males				
Day 0	36.810 ± 0.93	31.676 ± 1.01	34.442 ± 0.78	33.706 ± 1.03
Day 7	32.972 ± 4.18	32.376 ± 0.85	33.388 ± 1.41	31.908 ± 3.25
Day 14	35.446 ± 3.38	34.482 ± 0.86	34.904 ± 2.48	33.218 ± 2.42
Day 21	36.786 ± 2.96	31.620 ± 0.97	32.112 ± 1.491	32.948 ± 1.76
Day 28	37.886 ± 3.24	35.130 ± 1.39	35.734 ± 2.52	38.520 ± 2.470

Values are means ± SD.
No statistically significant differences observed between control and treatment groups.

Table 3: Mean body weights (g) of females and males mice during subacute toxicity.

examination were recorded in Table 9. Histopathological examination of vital organs, including heart, liver, spleen, kidneys, epididymis revealed that, no abnormal symptoms induced by the LFF administered at different doses as compared to control group (Figure 2).

***In vitro* chromosome aberration assay:** The frequencies of aberrant metaphase were 2.0 per 100 metaphases in the control and also in LFF treated lymphocyte cultures for 24 h and 48 h (Figure 3). Microscopic examination confirm that, there were no statistically significant increase in frequencies of metaphases in the treatment groups as compared to control group (Table 10).

Cytotoxicity assay: Results of cytotoxicity assays carried out in human cell lines HEK-293 (kidney), HepG2 (liver), KB (mouth), HT-29 (colorectal adenocarcinoma) suggest that, *A. indica* LFF is detrimental to human cancer cell clines, inhibited the cell proliferation in a dose dependent manner (Figure 4). The inhibition was found to be significantly stronger ($p<0.001$) in KB mouth cells with IC_{50} of 36.57 µg/mL, followed by HepG2 cells (IC_{50} value of 40 µg/mL), HEK-293 cells (IC_{50} value of 68.58 µg/mL) and HT-29 cells (IC_{50} value of 48.6 µg/mL).

Groups (CFF mg/kg b.w.)	I week	II week	III week	IV week
Females				
Control	29.2 ± 1.3	28.2 ± 1.4	30.3 ± 1.4	32.3 ± 1.4
100	25.3 ± 1.4	27.4 ± 1.3	27.3 ± 1.3	28.2 ± 1.4
500	27.5 ± 1.3	29.3 ± 1.3	30.2 ± 1.4	30.3 ± 1.3
1000	29.5 ± 1.3	29.3 ± 1.4	31.2 ± 1.4	31.5 ± 1.5
Males				
Control	35.4 ± 1.4	36.3 ± 1.3	38.3 ± 1.3	40.4 ± 1.4
100	29.2 ± 1.4	30.8 ± 1.7	33.3 ± 1.4	34.5 ± 1.3
500	31.4 ± 1.3	33.4 ± 1.4	34.3 ± 1.4	36.3 ± 1.3
1000	30.2 ± 1.3	32.3 ± 1.4	35.3 ± 1.4	38.4 ± 1.4

Values are means ± SD.
No statistically significant differences between control and any of the treatment groups.

Table 4: Mean food consumption (g/animal/week) by female and male mice during subacute toxicity.

Parameter	Dose groups (mg/kg b.w.)			
	Control	100	500	1000
Females				
Appearance				
Light yellow (n)	4	4	5	3
Dark yellow (n)	1	1	0	2
pH	7.8 ± 0.07	7.5 ± 0.07	7.9 ± 0.07	7.6 ± 0.07
Glucose				
Negative (n)	5	5	5	5
Bilirubin				
Negative (n)	5	5	5	5
Ketone				
Negative (n)	5	5	5	5
Protein				
Negative (n)	5	5	5	5
Blood				
Negative (n)	5	5	5	5
Males				
Appearance				
Light yellow (n)	4	5	3	4
Dark yellow (n)	1	0	2	1
pH	7.4 ± 0.07	7.3 ± 0.07	7.5 ± 0.07	7.4 ± 0.07
Glucose				
Negative (n)	5	5	5	5
Bilirubin				
Negative (n)	5	5	5	5
Ketone				
Negative (n)	5	5	5	5
Protein				
Negative (n)	5	5	5	5
Blood				
Negative (n)	5	5	5	5

Values are means ± SD.
No statistically significant differences between control and any of the treatment groups.

Table 5: Urinalysis data of female and male mice during subacute toxicity study.

Parameter	Dose groups			
	Control	100 mg/kg b.w.	500 mg/kg b.w.	1000 mg/kg b.w.
Females				
WBC (×10^9/L)	11.18 ± 2.5	12.3 ± 2.9	10.5 ± 2.5	10.9 ± 1.4
RBC (×10^{12}/L)	6.3 ± 0.75	6.2 ± 0.59	6.5 ± 0.26	5.9 ± 0.67
HGB (G/L)	127 ± 11.4	121 ± 16.2	11 ± 13.3	123 ± 19.1
HCT (%)	32.1 ± 4.3	30.4 ± 2.4	31.3 ± 1.2	31.2 ± 5.2
MCV (fL)	49.6 ± 1.4	50.5 ± 0.2	50.5 ± 1.7	50.4 ± 1.6
MCH (pg)	19.5 ± 0.5	19.6 ± 0.4	19.3 ± 0.7	19.4 ± 0.3
MCHC(g/L)	392 ± 19.4	396 ± 12.4	389 ± 13.3	388 ± 14.3
PLT (×10^9/L)	267 ± 31.3	217 ± 46.7	232 ± 29.3	242 ± 18.4
MPV (fL)	9.4 ± 0.7	9.4 ± 0.3	9.5 ± 0.01	9.6 ± 0.09
NEUC (%)	34.2 ± 9.7	33.6 ± 8.9	29.1 ± 10.4	35.6 ± 6.8
LY (%)	59.1 ± 8.1	50.9 ± 9.7	64.5 ± 6.6	59.2 ± 7.4
MO (%)	5.8 ± 0.7	5.3 ± 0.9	4.8 ± 1.0	4.6 ± 1.4
EOS (%)	0.9 ± 0.2	0.8 ± 0.5	0.8 ± 0.7	0.6 ± 0.2
BASO (%)	0	0	0	0
Males				
WBC (×10^9/L)	14.8 ± 6.4	14.3 ± 8.2	14.7 ± 5.7	14.6 ± 7.7
RBC (×10^{12}/L)	6.73 ± 0.36	6.53 ± 0.67	6.66 ± 0.43	6.11 ± 0.22
HGB (G/L)	128 ± 1.3	123 ± 2.4	120 ± 5.2	125 ± 3.3
HCT (%)	35.2 ± 2.5	34.2 ± 3.1	34.9 ± 2.6	34.5 ± 3.7
MCV (fL)	54.3 ± 6.2	55.9 ± 2.7	55.5 ± 3.8	55.8 ± 4.4
MCH (pg)	19.8 ± 0.6	20.4 ± 0.2	20.2 ± 0.5	20.2 ± 0.7
MCHC(g/L)	356 ± 14.3	356 ± 21.6	349 ± 24.2	347 ± 22.4
PLT (×10^9/L)	238 ± 43.5	231 ± 51.1	230 ± 23.4	203 ± 19.3
MPV (fL)	9.8 ± 0.7	9.6 ± 0.7	9.5 ± 0.2	9.4 ± 0.6
NEUC (%)	37.1 ± 5.7	39.3 ± 4.2	40.2 ± 3.1	39.1 ± 6.6
LY (%)	56.2 ± 5.3	54.9 ± 6.2	54.3 ± 3.7	54.9 ± 10.3
MO (%)	5.8 ± 1.2	5.1 ± 2.2	4.7 ± 2.7	5.3 ± 1.6
EOS (%)	0.8 ± 0.07	0.7 ± 0.07	0.8 ± 0.07	0.7 ± 0.07
BASO (%)	0	0	0	0

Values are means ± SD.
No statistically significant differences between control and any of the treatment groups.

Table 6: Hematological values of females and males mice during subacute toxicity study.

Discussion

Medicinal plants commonly contain various bioactive compounds that have the potential to cause either beneficial or detrimental effects. To optimize the safe use of a plant-based medicine, one should take into account the historical applications on animals and humans, as well as perform a toxicity evaluation of the medicinal herbs and their bioactive components [33]. To this perspective acute and subacute oral toxicity evaluation is necessary for the dosage fixation and safety assessment of bioactive compounds.

A. indica Kuntze extracts have been shown to possess analgesic and anti-hyperalgesic activities. The plant is used for the treatment of inflammatory skin diseases, liver disease and protection, gastrointestinal disease, hypertension and immune system deficiencies [34-35]. Extracts and isolated constituents of *A. indica* has been demonstrated to possess, anti-inflammatory, anti-tumor cell proliferation, bactericidal function against *H. pylori* [21,22,36]. Despite the medicinal and pharmacological properties of *A. indica* extracts and isolated constituents, there are no data available in the pertinent literature on the toxicological concerns of this plant, its extracts and isolated constituents that would be useful for the safety assessment and dosage fixation. Therefore, in the present study leaf flavonoid fraction of *A. indica* was subjected for toxicity evaluation in mice, human lymphocytes and human cancer cell lines. In a 14-day oral dose study no mortality, toxic signs or symptoms observed in the LFF treated mice. Body weights of both treated and control groups of mice showed no differences (Table 1), and the determined LD_{50} exceeds 5000 mg/kg b.w (Table 2). Substances having LD_{50} of range 5000 and 15,000 mg/kg b.w. are considered as non-toxic [37].

In the 28-day repeat oral dose subacute toxicity study, the parameters of general behaviour, body weight and food intake showed no differences between LFF treated mice and control group of both sexes. Change over in the animal body weight point outs the toxic consequence of drugs and chemicals [38]. The results of the two oral toxicity studies in mice indicated that, LFF is well tolerated at all the doses tested. In the present study, the safety limit was evaluated for LFF as up to 1000 mg/kg b.w. Results of the study confirm that, LFF is safe for human consumption.

Hematopoietic complex is one of the most acutely affected by

Parameter	CFF Dose groups			
	Control	100 mg/kg	500 mg/kg	1000 mg/kg
Female				
SGOT (U/L)	21.08 ± 1.90	18.39 ± 0.78	19.65 ± 0.30	11.64 ± 1.25
SGPT (U/L)	8.14 ± 0.14	13.55 ± 0.59	13.73 ± 0.17	13.74 ± 0.69
GGT (U/L)	15.35 ± 0.39	15.85 ± 0.38	15.72 ± 0.28	15.61 ± 0.19
TBIL (mg/dL)	0.37 ± 0.05	0.63 ± 0.25	0.29 ± 0.01	0.28 ± 0.06
DBIL (mg/dL)	0.35 ± 0.23	0.56 ± 0.32	0.26 ± 0.18	0.07 ± 0.02
TP (mg/dL)	6.19 ± 0.38	5.81 ± 0.37	6.13 ± 0.43	6.23 ± 0.37
GLB (g/dL)	2.53 ± 0.25	2.38 ± 0.26	2.71 ± 0.37	2.79 ± 0.31
ALB (mg/dL)	3.65 ± 0.14	3.43 ± 0.13	3.41 ± 0.08	3.44 ± 0.12
Glu (mg/dL)	99.15 ± 0.71	120.03 ± 13.69	118.2 ± 1.08	80.88 ± 10.98
HDL Chol(mg/dL)	44.73 ± 3.33	44.49 ± 1.781	45.55 ± 2.12	44.16 ± 1.65
LDL CHOL(mg/dL)	55.87 ± 15.24	33.18 ± 3.66	57.97 ± 18.42	93.15 ± 32.70
TRIG(mg/dL)	44.62 ± 6.98	64.13 ± 9.85	48.75 ± 7.13	62.54 ± 21.19
UREA (mg/dL)	11.80 ± 2.47	12.28 ± 2.44	11.05 ± 1.92	12.57 ± 2.39
URIC ACID(mg/dL)	1.75 ± 0.14	1.59 ± 0.13	1.59 ± 0.16	1.71 ± 0.14
TOTCHOL(mg/dL)	104.43 ± 12.41	88.81 ± 7.31	95.35 ± 1.04	132.86 ± 29.93
A/G ratio	1.48 ± 0.10	1.59 ± 0.084	1.27 ± 0.19	1.30 ± 0.46
BUN(mg/dL)	5.40 ± 1.13	5.60 ± 1.12	5.07 ± 0.89	5.79 ± 1.08
Male				
SGOT (U/L)	21.76 ± 9.91	13.83 ± 1.40	12.00 ± 0.38	14.23 ± 2.06
SGPT (U/L)	11.59 ± 0.30	14.82 ± 2.09	12.50 ± 0.28	9.76 ± 0.92
GGT (U/L)	13.91 ± 1.35	14.67 ± 0.58	14.21 ± 0.28	12.30 ± 1.50
TBIL (mg/dL)	0.48 ± 0.18	0.54 ± 0.21	0.71 ± 0.30	0.50 ± 0.34
TP (mg/dL)	7.37 ± 0.85	6.17 ± 0.36	6.26 ± 0.36	6.32 ± 0.29
DBIL (mg/dL)	0.33 ± 0.21	0.11 ± 0.005	0.11 ± 0.009	0.40 ± 0.29
GLB (g/dL)	3.33 ± 0.70	2.42 ± 0.37	2.53 ± 0.27	2.69 ± 0.18
ALB (mg/dL)	4.03 ± 0.25	3.76 ± 0.25	3.72 ± 0.13	3.63 ± 0.19
Glu (mg/dL)	79.08 ± 3.56	89.25 ± 5.56	87.22 ± 3.39	98.94 ± 4.48
HDLChol (mg/dL)	41.69 ± 1.16	42.6 ± 1.50	42.67 ± 1.41	42.67 ± 0.65
LDLCHOL (mg/dL)	54.08 ± 4.39	61.36 ± 17.31	60.16 ± 20.30	59.67 ± 4.25
TRIG(mg/dL)	41.64 ± 3.39	74.48 ± 20.44	91.49 ± 57.14	56.05 ± 8.87
UREA (mg/dL)	14.11 ± 2.105	14.76 ± 2.36	13.27 ± 1.88	15.02 ± 2.37
URIC ACID(mg/dL)	2.38 ± 0.49	1.97 ± 0.45	6.07 ± 1.03	5.88 ± 1.01
TOTCHOL(mg/dL)	100.95 ± 4.14	116.40 ± 11.60	183.18 ± 63.05	175.3 ± 57.84
A/G	1.47 ± 0.34	1.79 ± 0.21	1.46 ± 0.11	1.37 ± 0.10
BUN (mg/dL)	6.45 ± 0.95	6.76 ± 1.10	6.09 ± 0.87	6.54 ± 1.26

Values are means ± SD.
No statistically significant differences between control and any of the treatment groups.

Table 7: Clinical biochemistry values of female and male mice during subacute toxicity study.

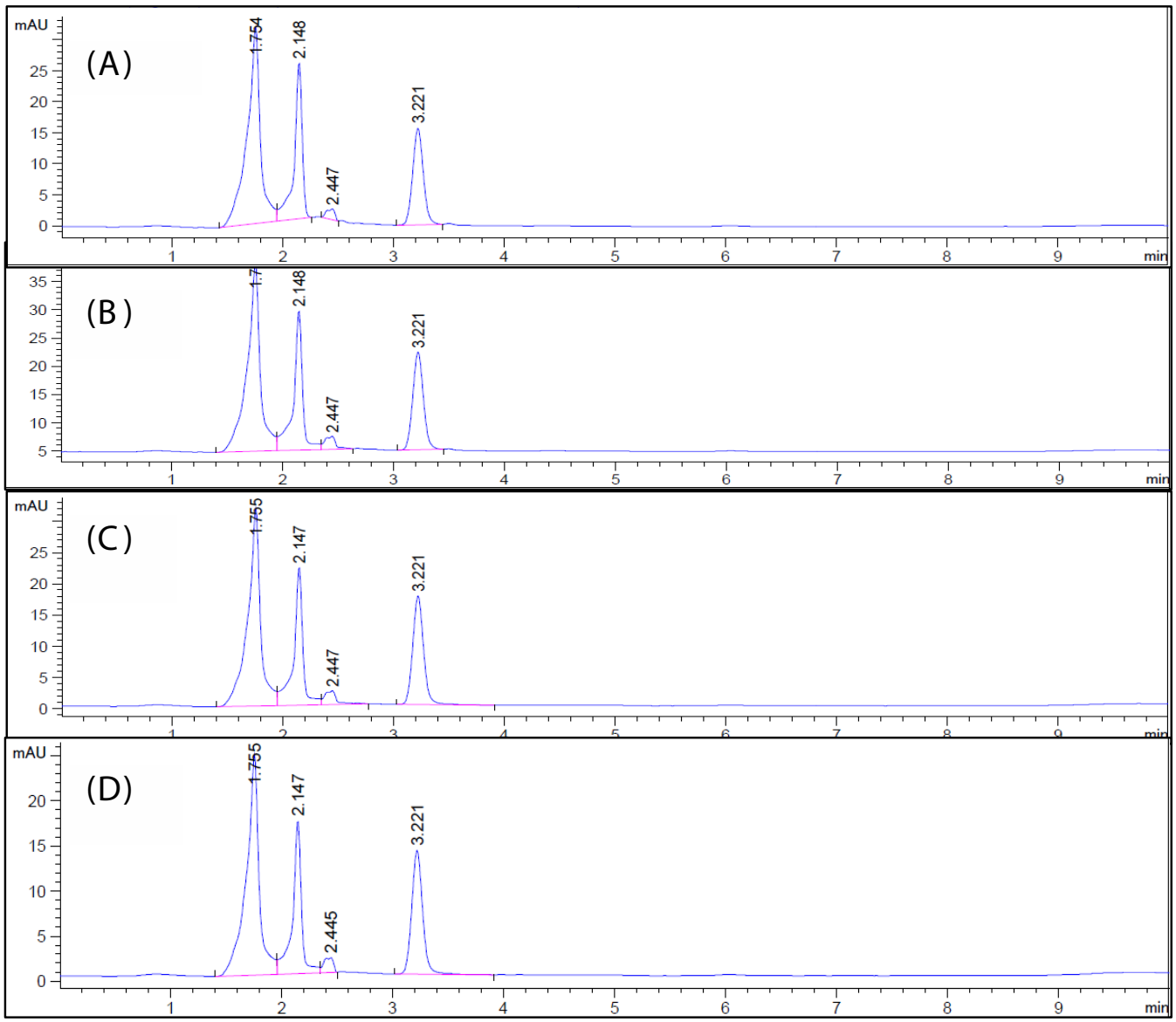
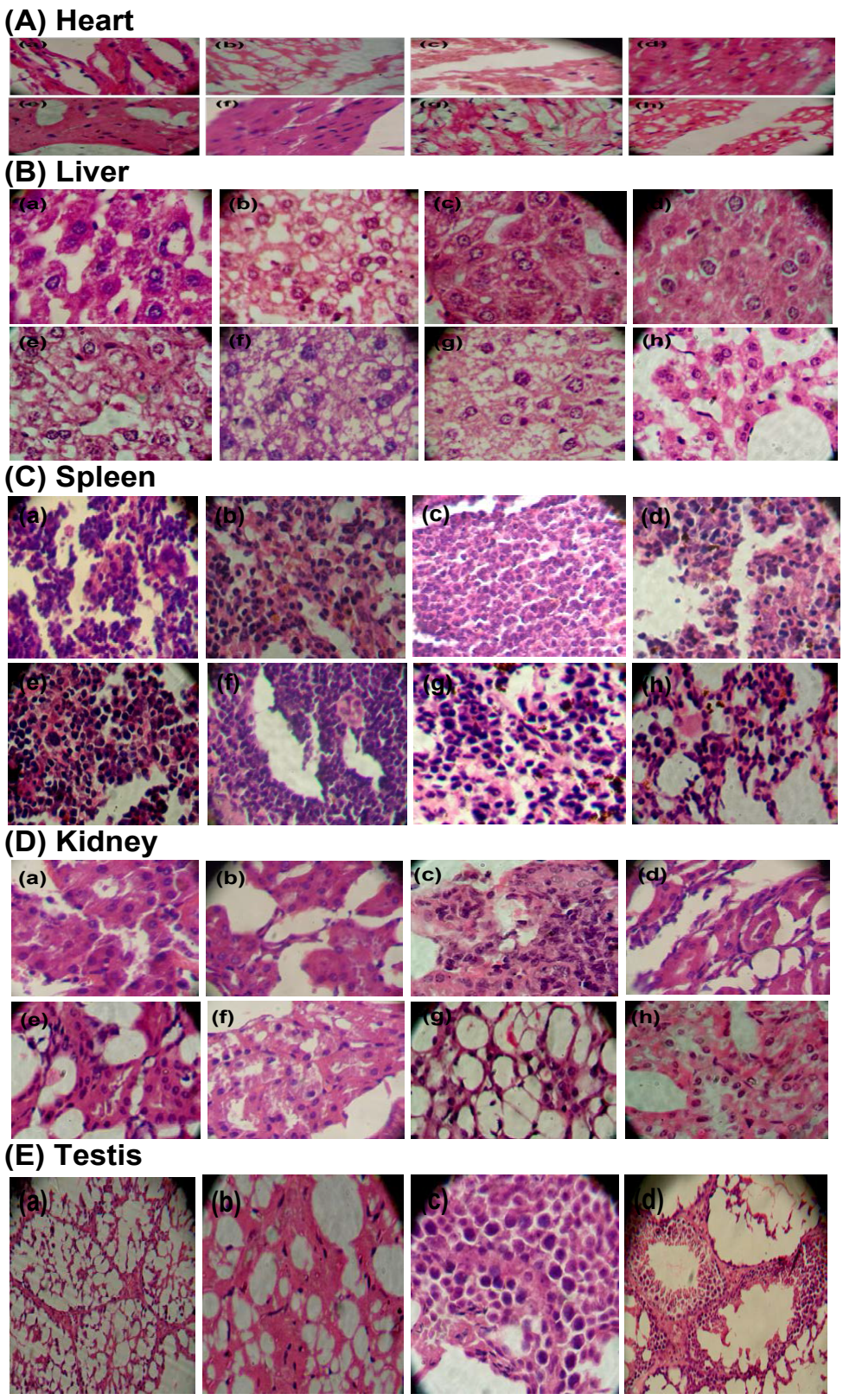


Figure 1: HPLC of *A. indica* leaf flavonoid fraction.

Organ	CFF Dose groups			
	Control	100 mg/kg b.w.	500 mg/kg b.w.	1000 mg/kg b.w.
Female				
Liver	1.564 ± 0.149	2.070 ± 0.045	1.696 ± 0.119	1.776 ± 0.059
Kidney	0.312 ± 0.028	0.278 ± 0.010	0.242 ± 0.028	0.266 ± 0.013
Spleen	0.148 ± 0.027	0.128 ± 0.008	0.132 ± 0.013	0.128 ± 0.012
Heart	0.144 ± 0.005	0.128 ± 0.007	0.132 ± 0.008	0.130 ± 0.004
Male				
Liver	1.670 ± 0.166	1.492 ± 0.125	1.992 ± 0.162	2.040 ± 0.039
Kidney	0.312 ± 0.033	0.284 ± 0.013	0.286 ± 0.022	0.312 ± 0.016
Spleen	0.192 ± 0.020	0.156 ± 0.012	0.224 ± 0.025	0.176 ± 0.026
Heart	0.180 ± 0.018	0.168 ± 0.005	0.224 ± 0.013	0.194 ± 0.008
Testes	0.128 ± 0.010	0.100 ± 0.01	0.142 ± 0.017	0.140 ± 0.013

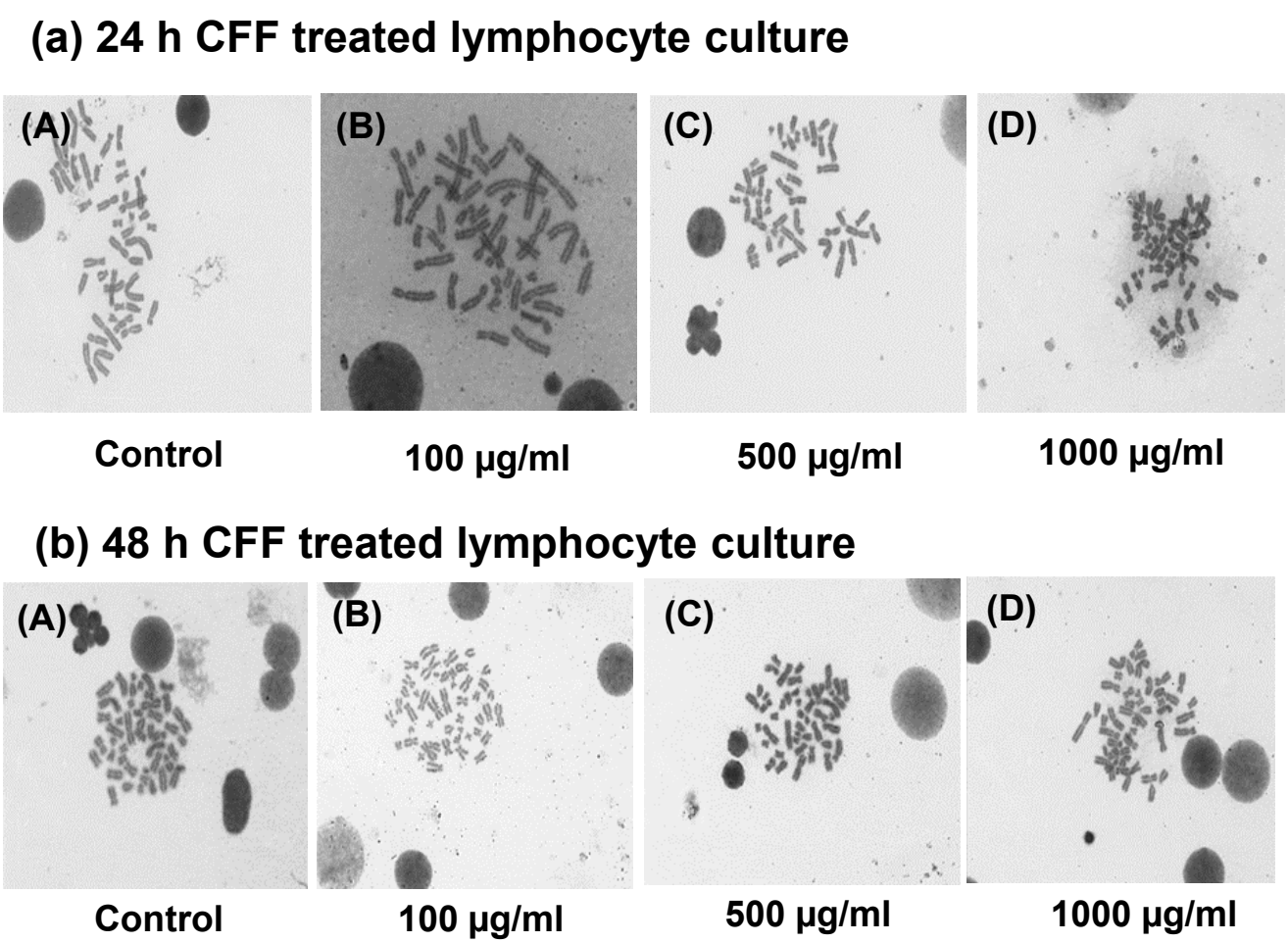
Values are means ± SD.
No statistically significant differences between control and any of the treatment groups.

Table 8: Absolute organ weight data of female and male mice during subacute toxicity study.



Control (a&e), LFF: 100 mg (b&f), 500 mg (c&g) 1000 mg/kg b.w. (d&h).
(A)Heart (B) Liver (C) Spleen (D) Kidney (E) Testis (only male).

Figure 2: Photomicrographs of histological sections of vital organs of male (A-D) and female (E-H) mice.



(a) 24 h LFF treated lymphocyte culture; (b) 48 h LFF treated lymphocyte culture.
(A) Control; (B-D) LFF treatments: (B) 100 µg/mL; (C) 500 µg/mL; (D) 1000 µg/mL.

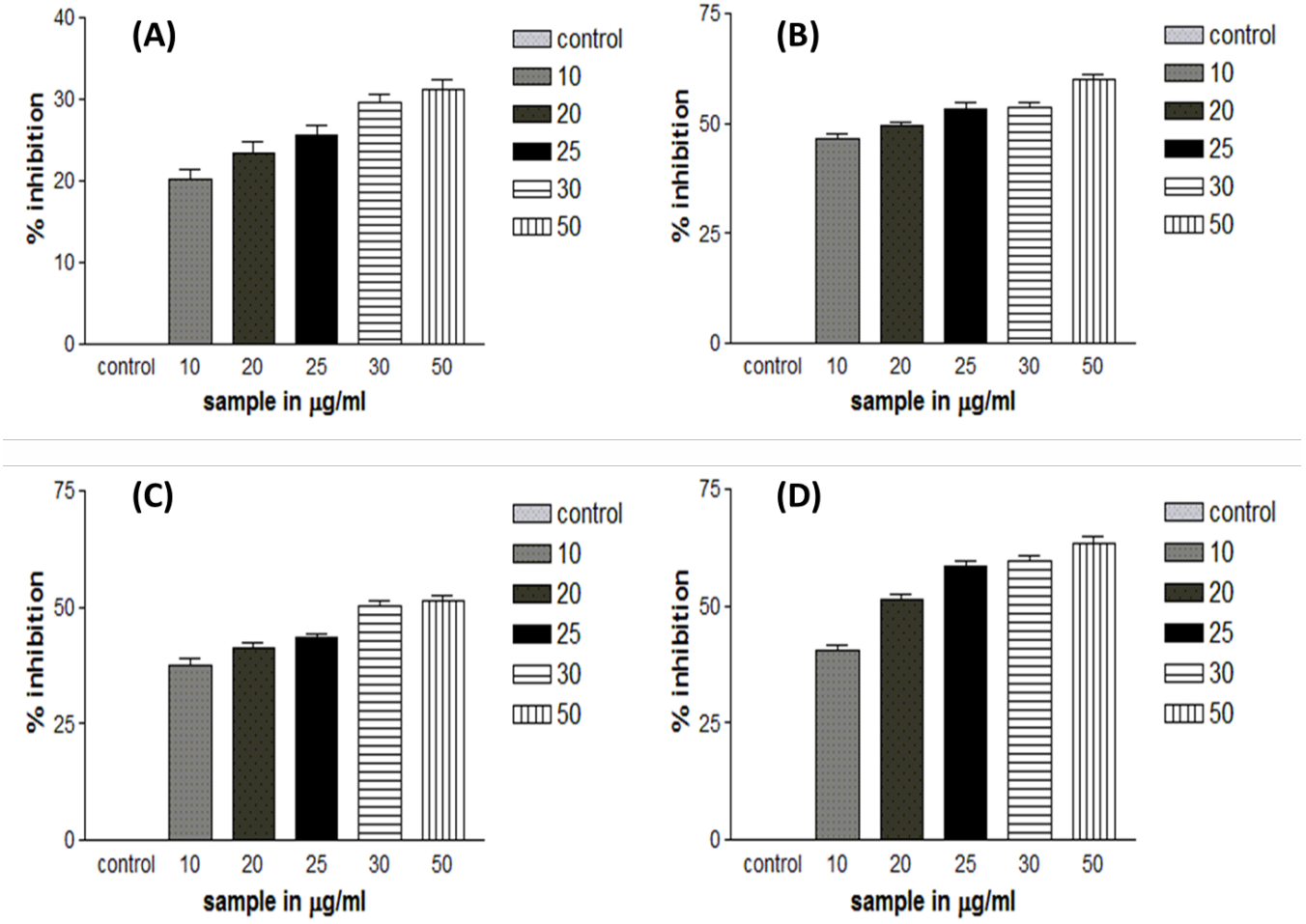
Figure 3: Chromosome aberration (genotoxicity) assay.

Organ: Finding(s)	Male				Female			
	Control	100 mg/kg b.w.	500 mg/kg b.w.	1000 mg/kg b.w.	Control	100 mg/kg b.w.	500 mg/kg b.w.	1000 mg/kg b.w.
Heart								
Examined	5	5	5	5	5	5	5	5
No abnormalities detected.	5	3	3	4	4	3	4	3
Fatty change	0	2	0	0	1	0	1	1
Smooth muscle separated by fat	0	0	1	0	0	2	0	0
Focal area of hemorrhage	0	0	1	0	0	0	0	0
Mild fatty change with epicardial hemorrhage.	0	0	0	1	0	0	0	1
Liver								
Examined	5	5	5	5	5	5	5	5
No abnormalities detected.	5	4	4	4	4	4	4	4
Congested liver parenchyma with occasional perivascular lymphocytes inflammatory infiltrate	0	0	0	0	1	0	0	0
Mild fatty change	0	1	1	1	0	1	1	0
Extensive fatty change with perivenular mononuclear infiltration.	0	0	0	0	0	0	0	1
Spleen								

Examined	5	5	5	5	5	5	5	5
No abnormalities detected.	4	4	4	4	4	4	4	4
Mild congestion	1	0	1	0	1	0	1	0
Mild congestion with extra medullary hemapoiesis.	0	1	0	1	0	1	0	1
Kidney								
Examined	5	5	5	5	5	5	5	5
No abnormalities detected.	5	5	4	4	4	4	4	4
Dilated collecting tubules, mild interstitial inflammation.	0	0	1	0	1	1	1	0
Well preserved renal parenchyma with focal interstitial inflammation comprised of lymphocytes.	0	0	0	1	0	0	0	0
Vascular degradation of proximal convoluted tubular cells, dilated collecting tubules interstitial inflammation with congestion.	0	0	0	0	0	0	0	1
Epididymides								
Examined	5	5	5	5				
No abnormalities detected.	4	5	5	4				
Leydig cell hyperplasia	1	0	0	1				

Values represent number of animals with findings.

Table 9: Histopathological findings of male and female mice vital organs during subacute toxicity study.



(A) HEK-293; (B) HepG2; (C) HT-29; (D) KB.
Results are mean ± S.D. of three parallel measurements.

Figure 4: *In vitro* cytotoxicity of LFF in human cancer cell lines.

Concentration of CFF (µg/mL)	24 h Treatment				48 h Treatment			
	Control	100 µg/mL	500 µg/mL	1000 µg/mL	control	100 µg/mL	500 µg/mL	1000 µg/mL
Total no. of metaphases	100	100	100	100	100	100	100	100
normal	99	100	99	98	100	100	98	99
Chromatid break	-	-	-	-	-	-	-	-
Chromosome break	-	-	-	-	-	-	-	-
Deletion	-	-	-	-	-	-	-	-
Ring	-	-	-	-	-	-	-	-
Gap	1	0	1	2	0	0	2	1
Dicentric	-	-	-	-	-	-	-	-
Total aberrations	1	0	1	2	0	0	2	1

Table 10: Chromosome aberration assay in lymphocytes treated with CFF for 24 h and 48 h.

external stimuli of poisonous chemicals and is a significant reference of physiological and pathological position in humans and animals. In the present study, the hematological parameters showed no significant differences among the control and treated groups (Table 6), indicating that, LFF had no toxic or deleterious effects on the circulating blood cells and on their production.

Liver and kidney are the two vital organs associated with metabolism and detoxification of metabolites and drugs. In the present study, there were no adverse effects observed in the usual markers of liver, AST and ALT, and kidney that confirm the toxicity (Table 7). Transaminases (AST and ALT) are extensively distinguished enzymes significantly referenced for proper activity of liver and are well known biomarkers prophesy capable of existing toxicity [38]. Usually, no matter which, injury to the parenchymal liver cells consequences in raise of both ALT and AST transaminases in blood. Irrespective of the source whether it is derived from mitochondrial or cytoplasmic, AST in serum exceeds normal level, if and only if there is any damage to cells that provide access enzyme into serum. Creatinine is an indicator of kidney function, and any change in normal range of creatinine levels observed it may be due to damage to functional nephrons [39]. No significant changes in serum ALT and AST levels and creatinine content were observed in animals of LFF treatment groups as compared to controls, suggesting that the repeated-dose administration of LFF did not alter the hepatocytes and renal cell function and metabolism. Results of these parameters further supported by the data of urine analysis and clinical parameters that showed no significant changes between the LFF treated groups as compared to control groups of animals. LFF did not produce any severe toxicity symptoms as confirmed from histopathological examination of vital organs of all groups of animals of both genders. Moreover, these findings are commonly observed in normal mice as suggested in the pertinent literature [40].

Results of chromosomal aberration assay in human lymphocytes suggest that, there was no statistically significant increase in the number of metaphases with structural aberrations observed in the LFF treated groups at the tested dose as compared to controls (Table 10), confirming that LFF is not clastogenic. The *in vitro* chromosome aberration test is serves to consider agents that persuade structural chromosomal aberrations in cultured mammalian cells. Structural aberrations can affect chromosomes or chromatids. The greater number of chemical mutagens persuade aberrations of the chromatid type, but chromosomal aberrations may occur [34].

Cytotoxicty assays using human cancer cell lines confirm that, LFF possesses anticancer property as the cell proliferation was affected in theses cell lines (Figure 4). When metabolites or drugs delivered through oral route they get in touch with cells and tissues of mouth, gastrointestinal tract, liver and kidney. In the present study, LFF was tested against four human cancer cell lines *viz*., KB (mouth), HT-29 (colorectal adenocarcinoma), HepG2 (liver) and HEK-293 (kidney) to confirm whether CFF is beneficial against these cancers. Results confirmed that, LFF suppress the cancer cell lines with stronger activity against KB (mouth) cell lines. The cytotoxicity of LFF against human cancer cell lines tested may be attributed to the flavonoids present in LFF. Many flavonoids have been shown to prevent carcinogenesis in many animal models [41]. Flavonoids are efficient scavengers of Reactive Oxygen Species (ROS), and their anticancer property determined intensely on their antioxidant and chelating abilities. Nowadays, practical approaches have supplied increasing obviousness for the advantageous action of flavonoids on various biological pathways relating cancer like carcinogenesis, cell-signaling, regulation of cell cycle, angiogenesis, oxidative stress, inflammation [42-45]. Over all the results of the present study confirm that, LFF is safe for oral administration as a drug, however further studies are need to be conducted to evaluate its bioavailability and pharmacokinetics.

In conclusion the present study is first of in its kind, was carried out for the toxicity evaluation of *A. indica* LFF in mice, human lymphocytes and human cancer cell lines, so as to assess the safety of LFF for human administration. LFF is non-toxic to mice at acute and subacute exposures under the experimental conditions, with the maximum tolerated dose evaluated as more than 5000 mg/kg b.w. *A. indica* LFF is not mutagenic or clastogenic, does not affect hematopoietic system, and suppress cancer cell proliferation. Thus the present study provides valuable data on toxicity profile of *A. indica* LFF. Further, the data generated may be useful for carrying out comprehensive *in vivo* clinical studies that would support for the development of suitable formulation for the human administration of *A. indica* LFF.

Compliance with Ethical Standards

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Ethical approval

All applicable international (OECD), national (CPCSEA, Delhi, India) and institutional guidelines for the care and use of animals were followed.

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