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Evaluation of acute and sub-acute oral toxicity of ethanolic root extract of *Tetracera akara* (Burm. f.) Merr., an ethnomedicinal plant used by the Kani tribe of Kerala.

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Abstract

The aim of the study was to evaluate the acute and sub-acute toxicity of ethanolic root extract of *Tetracera akara* in Swiss albino mice and Wistar rats. *Tetracera akara* (Burm. f.) Merr. has been used as traditional medicine by the Kani tribe of Kerala to cure liver diseases. In acute toxicity studies, four groups of mice (n = 5/group/sex) were orally treated with doses of 0.625 g, 1.25 g, 2.5 g and 5.0 g/kg and mortality were recorded. In the subacute toxicity study, animals received *T. akara* extract at the doses of 0.1 g, 0.5 g and 2.5 g/kg/day (n = 5/group/sex) for 28 days and biochemical, hematological, morphological and histopathological parameters were determined. *T. akara* did not produce any mortality in the acute toxicity studies, showing LD_{50} higher than 5 g/kg. Sub-acute treatment with *T. akara* didn't cause any changes in body weight gain, hematological, biochemical profiles when compared to normal control. In addition, no changes in morphological and histopathological aspect of organs were observed in the animals. Taking all factors into consideration, administration of *Tetracera akara* does not produce acute toxicity in Swiss albino mice and sub-acute toxicity in Wistar rats, suggesting it's safe use by humans.

Keywords: *Tetracera akara, Nennalvalli, Pattuvalli,* Kani tribe, Subacute toxicity, Dilleniaceae.

1. Introduction

Toxicology is an important aspect of pharmacology that deals with the adverse effect of bio active substance on living organisms prior to the use of it as a drug or chemical in clinical use. Traditionally used medicinal plants have occupied a vital place in the modern pharmaceutical industries which relies largely on the diversity of secondary metabolites in plants of which at least 12,000 have been isolated, a number estimated to be less than 10% of the total. According to WHO, about 80% of the world's population rely predominantly on plants and plant products for healthcare (Setzer *et al*., 2006). Toxicity studies in animals are necessary for any pharmaceutical intended for human use. The information obtained from these studies is useful in choosing doses for repeat-dose studies, providing preliminary identification of target organs of toxicity and occasionally revealing delayed toxicity. For developing Improved Traditional Medicines (ITM's) that are affordable, safe and efficient, it is proposed that preclinical testing strategies of botanicals should start with the *in vivo* examination of plant drug in relevant animal models to substantiate the ethnopharmaceutical use (Vilash *et al*., 2016).

Tetracera akara (Burm. f.) Merr. locally known as *Nennalvalli* or *'Pattuvalli'*, belongs to the family Dilleniaceae, a woody climber distributed in the Western Ghats region of Kerala and Tamil Nadu. Roots of *T. akara* (*Pattuvalli*) is used by the Kani tribes of Kerala to cure liver diseases and inflammatory conditions (Saradamma *et al*., 1987). Leaf decoction of *T. akara* is used to treat pulmonary haemorrhages and gargles for aphthae (Udayan *et al*., 2009). The safety of the plant extract is not verified so far and the present work is aimed to study the acute and sub-acute toxicity of ethanolic extract of *T. akara* (Burm. f.) Merr. in Swiss albino mice and Wistar rats.

2. Materials and Methods

2.1. Chemicals and instruments

Chemicals used for the study were purchased from Sigma-Aldrich, USA. The reagents used were of analytical grade and biochemical kits for plasma markers, urea and creatinine were purchased from Coral Clinical System, Goa, India. The rotary evaporator was from Buchi R-215, Switzerland and Auto hematology analyser- BC-2800vet was from Mindray China.

2.2. Collection and authentication of plant material

Tetracera akara (Burm. f.) Merr. rootswere collected from Kottoor (N08°35'03.8", E77°10'54.8" and altitude 585m), Thiruvananthapuram district of Kerala, India, and authenticated by the plant taxonomist of JNTBGRI, Palode. Voucher specimens were deposited at the Institute's Herbarium (TBGT 86868 dated 08 /08 /2015).

2.3. Preparation of ethanolic extract of *Tetracera akara* roots.

The collected roots were washed in running water, shade dried and powdered. The powder was extracted with 95% ethanol for 48 h, using a Soxhlet apparatus. The extract was then filtered and the filtrate was concentrated under reduced pressure in Rotary evaporator, to get the ethanolic extract with 11% w/w of yield. The dry residue was stored at 4ºC, and, at the time of use, was suspended in 0.5% v/v Tween-80. This ethanolic extract was referred to as TA.

2.4. Animals Used

Both sexes of Swiss albino mice (23 to 28 g) for acute toxicity study and Wistar rats (150 to 175 g) for sub-acute toxicity study was obtained from the Institute's Animal house. All the animals were housed in polypropylene cages under standard conditions with temperature 25 ± 2°C, relative humidity 60 ± 10%, room air changes 15±3 times/h and a 12 h light-dark cycles, were fed commercial rat feed and boiled water *ad libitum*. Animals were acclimatized for 1 week before the initiation of an experiment. The study was carried out according to NIH guidelines after getting the approval of the Institute's Animal Ethics Committee (No: B-17/02/2015/ EM&EP-16).

2.5. Qualitative phytochemical Analysis

Phytochemical analysis was performed according to the methods of Harbone (1984) to screen the *T. akara* ethanolic extract for the presence of flavonoids, saponins, alkaloids, triterpenoids, tannins, coumarins, phenols, cardiac glycosides, aminoacids, steroids, gum & mucilage, anthraquinone etc.

2.6. Acute toxicity

The acute toxicity test was performed according to the Organisation of Economic CO-operation and Development guideline (OECD). A total of five groups of 10 animals with equal numbers of male and female mice was used and each group received a single oral-dose of 0.625g, 1.25g, 2.5g and 5g/kg TA and the control group received 0.5% Tween-80 (vehicle). Animals were kept overnight fasting prior to oral drug administration and after administration of drug sample, food was withheld for a further 3 - 4 h. Animals were observed individually at least once during first 30 min after dosing, periodically during the first 24 h (with special attention during the first 4 h) and daily thereafter for a period of 14 days. Daily observations on the changes in skin and fur, eyes and mucus membrane (nasal), respiratory rate, circulatory signs (heart rate and blood pressure), autonomic effects (salivation, lacrimation, perspiration, pilo erection, urinary incontinence and defecation) and central nervous system (ptosis, drowsiness, gait, tremors and convulsion) changes were noted (OECD, 2001).

2.7. Sub-acute toxicity study

The sub-acute oral toxicity study was carried according to the OECD Test Guidelines (OECD, 2007). Wistar rats were divided into five groups of 10 animals (5 males and 5 females/group) each. Group 1, the control group is treated with 0.5% Tween - 80 (vehicle) for 28 days. Groups II, III and IV were treated with TA 0.1g, 0.5g and 2.5g/kg/day, p. o respectively. In order to assess reversibility effect, the extract at the dose of 2.5g/kg was given once daily to the Group V (satellite group) of rats for 28 days, and kept for another 14 days post treatment observation. Toxic manifestations such as signs of toxicity, mortality and the body weight changes were monitored daily. At the end of the study, all animals were fasted for 24 h and sacrificed by carbon dioxide inhalation on 29th day and satellite groups on 43rd day. Blood samples for haematological and biochemical analyses were collected by cardiac puncture. Detailed gross necropsy, including careful examination of the body external surface, orifices and cranial, thoracic and abdominal cavities and their contents were performed in all the groups. Liver samples were subjected to antioxidant assays. The internal organs (lungs, heart, liver, spleen, kidney, testis and ovary) were collected, weighed and preserved in 10% neutral buffered formaldehyde solution for histopathological examination.

2.8. Haematological and Biochemical analysis

Blood samples collected in EDTA coated vials were used for the estimation of haematological parameters like White Blood Cell (WBC) count, Lymphocyte (LYM) count, Monocyte (MONO) count, Granulocyte (GRAN) count, LYM%, MONO%, GRAN%, Red blood cell (RBC) count, Haemoglobin (HGB) concentration, Haematocrit HCT(%), Mean corpuscular volume (MCV), Mean corpuscular haemoglobin concentration (MCHC), Mean corpuscular haemoglobin (MCH), Red blood cell distribution width (RDW), Platelet (PLT) count, Mean platelet volume (MPV), Platelet distribution width (PDW) and Plateletcrit (PCT) % using Veterinary Auto Hematology analyzer.

The collected blood samples in test tubes without EDTA were allowed to coagulate for 1h at room temperature. It was centrifuged at 1500 rpm for 15 min at 37ºC to separate the serum which was then subjected to the assay of plasma markers of hepatic injury like Alkaline phosphatase (ALP), Alanine transaminase (ALT), Aspartate transaminase (AST), γ-Glutamyl transferase (GGT) and other serum parameters namely Total protein (TP), Serum Bilirubin (SB), Total Cholesterol (TC), Triglycerides (TGL), and Albumin using commercial kits. The kidney functions were evaluated by estimating Creatinine and Urea using the commercial kits from the serum collected. Glucose level in blood was directly measured by placing one drop of blood on the test strip of blood glucometer and values displayed were observed.

The anti-oxidative enzymes in liver like Catalase (CAT), Reduced glutathione (GSH), Malondialdehyde (MDA) and Superoxide dismutase (SOD) were estimated. Malondialdehyde is the end product of lipid peroxidation and was estimated by the procedure of Ohkawa *et al*. (1979). Catalase in the rat liver was assayed according to the method of Aebi (1974). The antioxidant enzyme SOD was analyzed by the method of Kakkar *et al*. (1984) and reduced glutathione by the procedure of Ellman *et al*. (1959).

2.9. Morphological and Histopathological investigations

Macroscopic and microscopic features of the organs of male and female rats were compared with that of the control. After the animals were euthanized with CO_2 inhalation, the macroscopic external features of the heart, liver, spleen, lungs, kidney and reproductive organs (ovary or testis) were observed. These organs were carefully removed and weighed individually. Organ weights were expressed in absolute and relative terms (g and g/100 g of body weight, respectively).

Histological examination was performed in two animals per group/sex, randomly selected in the group. A portion of the internal organ (Liver, kidney, heart, lungs, spleen, ovary and testis) obtained from all the groups was sliced into two pieces of approximately 6 mm^3 sizes and preserved in 10% formalin solution for 24 h. They were subjected to dehydration with acetone of strength 70, 80 and 100 % respectively, each for 1 h. Infiltration and impregnation were done by treatment with paraffin wax, twice each time for 1 h. Paraffin was used to prepare paraffin 'L' moulds. Specimens were cut into sections of 3-7 μm thickness and stained with haematoxylin and eosin. The thin sections of the liver were made in to permanent slides and examined under high resolution microscope with photographic facility and photomicrographs were taken.

2.10. Statistical analysis

All the data were expressed as mean ± standard deviation (SD). The significance of difference among the group was assessed using one-way analysis of variance (ANOVA) followed by Dunnett's post-test using GraphPad Prism 6.01. The $p \leq 0.001$ was considered statistically significant.

3. Results

3.1. Phytochemical analysis

Preliminary phytochemical analysis of *T. akara* ethanolic extract showed the presence

of important phytochemicals like flavonoids, alkaloids, triterpenoids, coumarins, phenols, cardiac glycosides, gum and mucilage etc.

3.2. Acute toxicity

No deaths or hazardous signs were recorded in mice during 14 days of observation after acute treatment by oral route with *T. akara* ethanolic extract in doses of 0.625g, 1.25g, 2.5g and 5.0 g/kg. Cage side observations like condition of the fur, skin, abdominal distension, eyes dullness & opacities, pupil diameter, colour and consistency of the faeces, condition of teeth, breathing etc. were normal in all groups of animals.

3.3. Sub-acute toxicity study

The animals under study did not show any signs of toxicity like piloerection, alteration in the locomotor activity, food and water consumption or deaths during the 28 consecutive days of treatment with TA orally in doses of 0.1g, 0.5g and 2.5g/kg. Likewise, no significant changes were recorded in body weight gain of drug treated rats when compared to the normal. (Fig. 1 and 2).

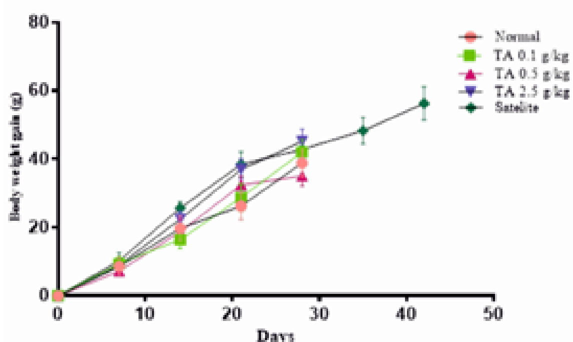

Fig. 1. Body weight gain of male rats treated orally with *Tetracera akara* ethanolic extract TA (0.1g, 0.5g and 2.5 g/kg) by oral route for 28 consecutive days. The values are expressed as mean ± SD (n = 5 animals/group).

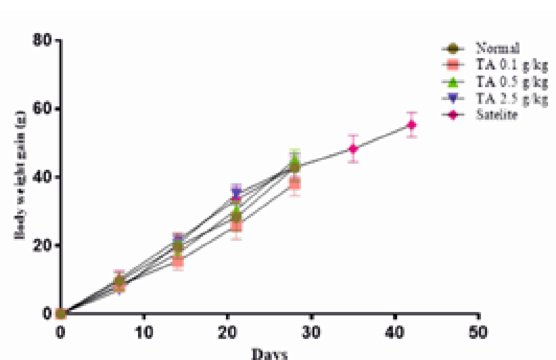

Fig. 2. Body weight gain of female rats treated orally with *Tetracera akara* ethanolic extract TA (0.1g, 0.5g and 2.5 g/kg) by oral route for 28 consecutive days. The values are expressed as mean ± SD (n = 5 animals/group).

The effect of TA on serum hepatic marker enzymes like AST, ALT, ALP, GGT and other serum parameters such as SB, TC, TP, TGL, Albumin, renal function markers like urea and creatinine values were estimated. The blood glucose level of all the animals (both male and female) was found to be within the normal range represented in Table 1 and 2. CAT, GSH, MDA and SOD of the liver in the entire plant drug treated groups (including satellite group) showed no significant difference from the normal control group at 28 days sub-acute toxicity study (Figs. 3) showing that there was no negative impact on the *in vivo* antioxidant status of the liver. In the same way, the haematological profile of male (Table 3) and female rats (Table 4) was not altered by treatment with TA. There was no significant ($p \leq 0.001$) difference, observed in the serum hepatic marker enzymes, renal function markers, blood glucose values and haematological studies of drug treated animals including satellite group when compared to normal control.

Table 1
Effects of *Tetracera akara* ethanolic extract on serum biochemical parameters of male Wistar rats treated for 28 consecutive days of sub-acute oral toxicity study.

Parameters	Normal control	TA (0.1 g/kg)	TA (0.5 g/kg)	TA (2.5 g/kg)	Satellite (2.5 g/kg)
ALP (U/L)	147.40 ± 7.86	142.73 ± 5.76^{ns}	152.40 ± 6.42^{ns}	148.20 ± 6.68^{ns}	154.42 ± .84^{ns}
ALT (U/L)	45.82 ± 3.56	43.56 ± 2.48^{ns}	47.67 ± 4.14^{ns}	46.58 ± 3.92^{ns}	48.95 ± 5.14^{ns}
AST (U/L)	138.26 ± 6.04	142.48 ± 5.72^{ns}	146.74 ± 6.54^{ns}	143.62 ± 7.18^{ns}	145.84 ± 7.62^{ns}
GGT (U/L)	7.13 ± 1.34	7.82 ± 1.49^{ns}	6.51 ± 1.25^{ns}	8.18 ± 1.68^{ns}	7.90 ± 2.04^{ns}
TP (g/dL)	7.02 ± 0.12	7.48 ± 0.22^{ns}	8.16 ± 0.14^{ns}	7.82 ± 0.13^{ns}	8.52 ± 0.16^{ns}
SB (mg/dL)	0.35 ± 0.06	0.33 ± 0.02^{ns}	0.36 ± 0.09^{ns}	0.41 ± 0.03^{ns}	0.38 ± 0.08^{ns}
TC (mg/dL)	64.65 ± 2.46	62.47 ± 3.04^{ns}	58.16 ± 2.87^{ns}	65.26 ± 3.62^{ns}	59.82 ± 3.44^{ns}
Triglycerides (mg/dL)	78.12 ± 4.72	75.54 ± 5.16^{ns}	80.52 ± 4.44^{ns}	82.28 ± 6.12^{ns}	81.78 ± 2.84^{ns}
Albumin (g/dL)	4.74 ± 0.10	4.93 ± 0.42^{ns}	4.18 ± 0.27^{ns}	5.02 ± 0.18^{ns}	5.42 ± 0.64^{ns}
Creatinine (mg/dL)	0.52 ± 0.02	0.50 ± 0.06^{ns}	0.56 ± 0.04^{ns}	0.46 ± 0.12^{ns}	0.53 ± 0.04^{ns}
Urea (mg/dL)	36.4 ± 2.48	40.03 ± 1.58^{ns}	36.56 ± 2.02^{ns}	38.26 ± 3.86^{ns}	42.02 ± 2.52^{ns}
Glucose (mg/dL)	75.34 ± 2.34	77.28 ± 3.24^{ns}	82.12 ± 2.34^{ns}	78. ± 3.64^{ns}	81.67 ± 3.46^{ns}

Values are expressed as mean ± SD, n = 5, one-way ANOVA followed by Dunnett's multiple comparison test, ns- no significant difference when the drug treated groups were compared to normal control.

Table 2
Effects of *Tetracera akara* ethanolic extract on serum biochemical parameters offemale Wistar rats treated for 28 consecutive days of sub-acute oral toxicity study.

Parameters	**Normal**	**TA (0.1 g/kg)**	**TA (0.5 g/kg)**	**TA (2.5 g/kg)**	**Satellite (2.5 g/kg)**
ALP (U/L)	132.24 ± 4.24	133.64 ± 6.18^{ns}	128.36 ± 5.82^{ns}	134.26 ± 6.22^{ns}	131.81 ± 7.24^{ns}
ALT (U/L)	49.32 ± 2.84	50.66 ± 2.58^{ns}	55.96 ± 3.87^{ns}	53.38 ± 3.22^{ns}	55.42 ± 3.82^{ns}
AST (U/L)	118.34 ± 3.02	122.34 ± 3.82^{ns}	127.02 ± 5.56^{ns}	121.92 ± 4.25^{ns}	127.14 ± 4.02^{ns}
GGT (`U/L)	6.45 ± 1.40	7.72 ± 1.86^{ns}	6.86 ± 1.57^{ns}	7.42 ± 1.48^{ns}	8.05 ± 1.83^{ns}
TP (g/dL)	6.28 ± 0.54	6.88 ± 0.27^{ns}	7.18 ± 0.12^{ns}	6.79 ± 0.34^{ns}	7.28 ± 0.22^{ns}
SB (mg/dL)	0.34 ± 0.05	0.32 ± 0.15^{ns}	0.47 ± 0.03^{ns}	0.43 ± 0.25^{ns}	0.50 ± 0.25^{ns}
TC (mg/dL)	96.62 ± 2.34	97.88 ± 3.54^{ns}	93.88 ± 4.14^{ns}	94.22 ± 3.82^{ns}	97.16 ± 2.08^{ns}
Triglycerides (mg/dL)	58.02 ± 3.54	62.36 ± 3.06^{ns}	55.66 ± 5.64^{ns}	60.05± 4.26^{ns}	61.76 ± 3.23^{ns}
Albumin (g/dL)	4.38 ±0.18	4.14 ± 0.42^{ns}	4.57 ± 0.09^{ns}	4.92 ± 0.12^{ns}	5.02 ± 0.48^{ns}
Creatinine (mg/dL)	0.60 ± 0.14	0.63 ± 0.01 ^{ns}	0.62 ± 0.01^{ns}	0.64 ± 0.12^{ns}	0.66 ± 0.12^{ns}
Urea (mg/dL)	33.2 ± 2.36	37.15 ± 2.78^{ns}	33.63 ± 2.89^{ns}	35.36 ± 3.72^{ns}	38.24 ± 3.22^{ns}
Glucose (mg/dL)	86.32 ± 5.05	89.54 ± 4.05^{ns}	90.32 ± 5.85^{ns}	82.32 ± 4.62^{ns}	92.32 ± 5.22^{ns}

comparison test, ns-no significant difference when the drug treated groups were compared to normal control.

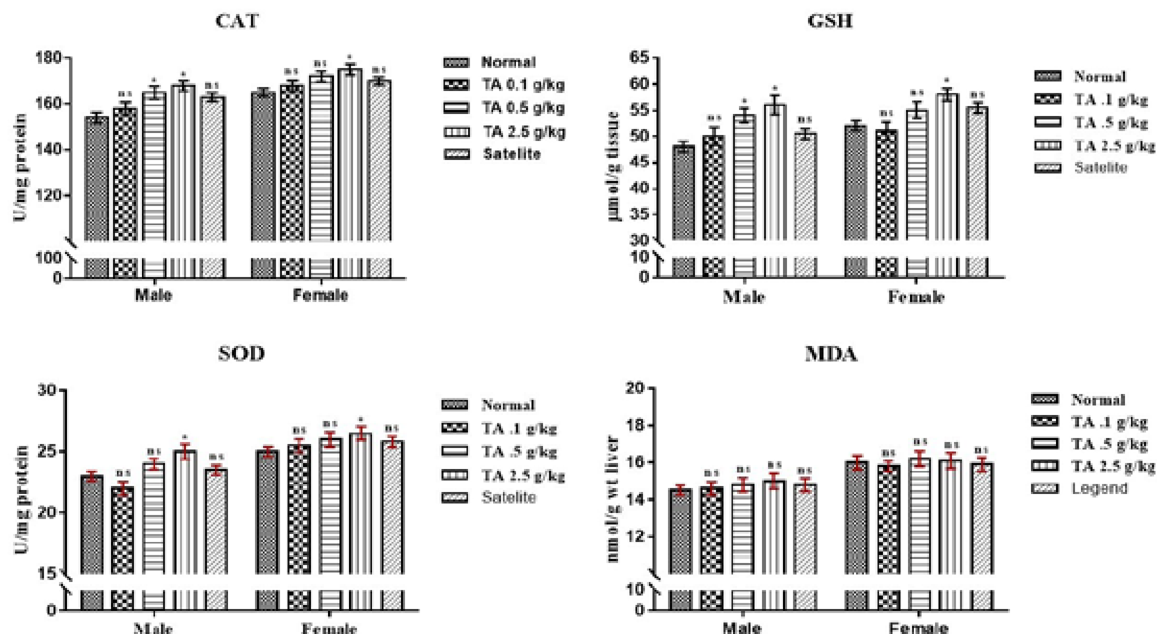

Fig. 3. Effect of *Tetracera akara* ethanolic extract on hepatic CAT, GSH, MDA and SOD after 28 consecutive days of sub-acute toxicity study. Values are expressed as mean ± SD, n = 5, one-way ANOVA followed by Dunnett's multiple comparison test. ns- nosignificant,*$p \leq 0.001$ significant when compared to normal control.

Table 3:

Effects of *Tetracera akara* ethanolic extract on haematological parameters of male Wistar rats treated for 28 consecutive days of sub-acute oral toxicity study.

Hematological parameters	Groups				
	Normal control	TA (0.1 g/kg)	TA (0.5 g/kg)	TA (2.5 g/kg)	Satellite (2.5 g/kg)
WBC ($\times 10^3$/µL)	9.74 ± 2.18	10.18 ± 2.04 ^{ns}	11.02 ± 1.22 ^{ns}	10.24 ± 2.30 ^{ns}	10.15 ± 2.16 ^{ns}
LYM ($\times 10^3$/µL)	6.32 ± 0.53	6.93 ± 0.44 ^{ns}	8.62 ± 0.28 ^{ns}	7.82 ± 0.28 ^{ns}	6.34 ± 0.82 ^{ns}
MONO ($\times 10^3$/µL)	0.34 ± 0.04	0.32 ± 0.07 ^{ns}	0.30 ± 0.04 ^{ns}	0.36 ± 0.22 ^{ns}	0.44 ± 0.16 ^{ns}
GRAN ($\times 10^3$/µL)	2.52 ± 0.54	3.42 ± 0.25 ^{ns}	3.84 ± 0.18 ^{ns}	2.14 ± 0.30 ^{ns}	2.62 ±0.32 ^{ns}
LYM%	65.24 ± 3.14	69.82 ± 2.74 ^{ns}	71.28 ± 2.42 ^{ns}	72.16 ± 3.84 ^{ns}	68.48 ± 2.84^{ns}
MONO%	2.43 ± 0.46	2.16 ± 0.44^{ns}	2.02 ± 0.14^{ns}	2.48 ± 0.46^{ns}	2.10 ± 0.34^{ns}
GRAN%	31.42 ± 1.45	27.52 ± 1.48^{ns}	22.16 ± 2.04^{ns}	24.42 ± 1.88^{ns}	26.14 ± 1.52^{ns}
RBC ($\times 10^6$/µL)	8.24 ± 0.82	7.28 ± 0.73^{ns}	7.68 ± 0.42^{ns}	8.12 ± 0.28^{ns}	8.03 ± 0.45^{ns}
HGB (g/dL)	13.86 ± 1.54	14.34 ± 1.28^{ns}	15.14 ± 0.84^{ns}	13.95 ± 1.72^{ns}	15.22 ± 1.36^{ns}
HCT (%)	40.48 ± 2.82	44.53 ± 2.8^{ns}	45.72 ± 1.64^{ns}	47.25 ± 1.43^{ns}	46.48 ± 2.28^{ns}
MCV (fL)	51.26 ± 1.84	50.22 ± 1.46^{ns}	51.74 ± 2.08^{ns}	53.38 ± 2.42^{ns}	50.47 ± 2.86^{ns}
MCHC (g/dL)	32.46 ± 1.28	35.18 ± 1.42^{ns}	34.48 ± 1.92^{ns}	36.32 ± 2.82^{ns}	31.64 ± 1.29^{ns}

MCH (pg)	17.94 ± 0.62	15.66 ± 0.28^{ns}	18.24 ± 0.62^{ns}	16.63 ± 0.81^{ns}	15.74 ± 0.22^{ns}
RDW (%)	16.24 ± 0.12	17.62 ± 0.22^{ns}	16.52 ± 0.82^{ns}	16.94 ± 0.84^{ns}	16.20 ± 0.28^{ns}
PLT (×10^3/μL)	1084.72 ± 28.42	992.46 ± 20.82^{ns}	1110.44 ± 35.60^{ns}	987.92 ± 22.26^{ns}	1008.84 ± 24.48^{ns}
MPV (fL)	5.12 ± 0.46	5.08 ± 0.92^{ns}	5.82 ± 0.42^{ns}	6.02 ± 0.36^{ns}	5.72 ± 0.14^{ns}
PDW	15.68 ± 1.42	17.22 ± 1.08^{ns}	16.24 ± 0.36^{ns}	17.05 ± 0.21^{ns}	15.44 ± 0.68^{ns}
PCT (%)	0.62 ± 0.10	0.58 ± 0.08^{ns}	0.60 ± 0.24^{ns}	0.57 ± 0.06^{ns}	0.55 ± 0.34^{ns}

Values are expressed as mean ± SD, n = 5, one-way ANOVA followed by Dunnett's multiple comparison test. ns- no significant difference when the drug treated groups were compared to normal control.

Table 4:

Effects of *Tetracera akara* ethanolic extract on haematological parameters of female Wistar rats treated for 28 consecutive days of sub-acute oral toxicity study.

Haematological parameters	Groups				
	Normal control	TA (0.1 g/kg)	TA (0.5 g/kg)	TA (2.5 g/kg)	Satellite (2.5 g/kg)
WBC (×10^3/μL)	**11.86 ± 2.32**	**10.68 ± 2.14^{ns}**	**13.34 ± 1.96^{ns}**	**12.62 ± 2.42^{ns}**	**12.25 ± 2.48^{ns}**
LYM (×10^3/μL)	6.72 ± 0.65	7.13 ± 0.84^{ns}	9.16 ± 0.64^{ns}	8.91 ± 0.32^{ns}	7.94 ± 0.44^{ns}
MONO (×10^3/μL)	0.43 ± 0.02	0.38 ± 0.06^{ns}	0.34 ± 0.03 ^{ns}	0.48 ± 0.12^{ns}	0.52 ± 0.04^{ns}
GRAN (×10^3/μL)	3.52 ± 0.54	2.72 ± 0.22^{ns}	3.22 ± 0.13^{ns}	2.98±0.42^{ns}	3.90±0.54^{ns}
LYM%	68.42 ± 2.34	76.05 ± 2.82^{ns}	72.48 ± 3.48^{ns}	70.92 ± 3.82^{ns}	69.44 ± 2.38^{ns}
MONO%	3.24 ± 0.48	2.85 ± 0.47^{ns}	2.82 ± 0.12^{ns}	3.28 ± 1.65^{ns}	3.18 ± 0.28^{ns}
GRAN%	27.46 ± 1.24	24.52 ± 1.48^{ns}	25.04 ± 2.42^{ns}	26.84 ± 2.50^{ns}	27.27 ± 1.24^{ns}
RBC (×10^6/μL)	9.12 ± 0.46	8.48 ± 0.83^{ns}	8.68 ± 0.52^{ns}	9.52 ± 0.56^{ns}	9.80 ± 0.74^{ns}
HGB (g/dL)	14.24 ± 1.28	15.88 ± 1.26^{ns}	16.02 ± 0.94^{ns}	15.95 ± 1.34^{ns}	16.28 ± 1.86^{ns}
HCT (%)	42.55 ± 2.38	46.48 ± 2.58^{ns}	47.72 ± 1.98^{ns}	51.32 ± 1.84^{ns}	49.52 ± 2.52^{ns}
MCV (fL)	54.06 ± 2.54	53.65 ±1.38^{ns}	50.34 ± 2.49^{ns}	56.27 ± 2.12^{ns}	51.82 ± 2.58^{ns}
MCHC (g/dL)	32.02 ± 1.36	34.98 ± 1.20^{ns}	36.52 ± 2.82^{ns}	37.26 ± 1.92^{ns}	30.86 ± 1.62^{ns}
MCH (pg)	18.44 ± 0.44	16.24 ± 0.38^{ns}	19.17 ± 0.12^{ns}	17.74 ± 0.23^{ns}	16.98 ± 0.62^{ns}
RDW (%)	13.20 ± 0.08	11.14 ± 0.17^{ns}	14.12 ± 0.12^{ns}	12.18 ± 0.14^{ns}	12.80 ± 0.63^{ns}
PLT (×10^3/μL)	1012.70 ± 38.12	1179.24 ± 19.69^{ns}	1105.94 ± 31.46^{ns}	1209.40 ± 42.38^{ns}	1038.10 ± 18.23^{ns}
MPV (fL)	5.62 ± 0.50	5.38 ± 0.29^{ns}	6.22 ± 0.12^{ns}	5.92 ± 0.82^{ns}	5.10 ± 0.48^{ns}
PDW	17.28 ± 1.02	16.62 ± 0.98^{ns}	15.42 ± 0.48^{ns}	16.03 ± 0.1^{ns}	15.28 ± 0.59^{ns}
PCT (%)	0.54 ± 0.02	0.57 ± 0.10^{ns}	0.61 ± 0.19^{ns}	0.51 ± 0.08^{ns}	0.52 ± 0.12^{ns}

Values are expressed as mean ± SD, n = 5, one-way ANOVA followed by Dunnett's multiple comparison test. ns-no significant difference when the drug treated groups were compared to normal control.

3.4. Morphological and Histopathological investigations

The macroscopic analysis of organs of treated animals including satellite group did not showed significant changes in color and texture when compared with the control group. The absolute and relative organ weights were not altered by TA (Tables 5 and 6). Histopathological examination of Liver, Heart, Kidney, Lungs, Spleen, Testis and Ovary of all the drugs treated animals showed normal architecture similar to the normal control group (Fig. 4).

The photomicrograph of Liver (Fig. 4 A1 to A5) showed interlobular septa with portal area consisting of branches of portal veins and hepatic arteries. Radially arranged hepatocytes and clear sinusoidal space, continued from the lobular margins to the centre. Abnormalities like necrosis or fatty changes were completely absent with less number of Kupffer cells infiltration.

Microscopic examination of the kidney showed normal renal architecture with corpuscles and renal tubules which are surrounded by basement membrane. The glomeruli, glomerular epithelium, Bowman's capsule and capsular space appeared normal. The medullary areas exhibited normal papillary ducts and ducts of Loops of Henle (Fig. 4 B1 to B5).

Histopathology of heart showed normal architecture with cardiac muscle fibers. Abnormalities such as disaggregation of connective tissues, interfibrillar haemorrhages and disrupted cardiac muscle fibers were absent in all the drug treated groups when compared to normal. (Fig.4 C1 to C5).

The Spleen histopathology of all the drug treated animals showed normal cell architecture comparable to the normal control. The germinal centers inside white pulp, red pulp, trabecular vein were visible and all appeared normal when compared to control group (Fig. 4 D1 to D5). Histopathology of Lungs showed normal alveoli and the surrounding capillaries which are one cell thick and in very close contact with each other, facilitating gas diffusion (Fig. 4 E1 to E5).

Microscopic examination of Testis of drug treated male rats showed normal histopathology with leydig cells, lamina propria, seminiferous tubules, spermatocytes and spermatids when compared to the normal control. In drug treated female rats, the histopathology of ovary was normal with germinal epithelium, follicle cells, antrum and growing follicles when compared to normal (Fig. 4 F1 to F5 and G1 to G5).

4. Discussion

Traditional medicine has maintained greater popularity all over the developing world and the use is rapidly increasing. Despite the widespread use, few scientific studies have been undertaken to ascertain the safety and efficacy of traditional remedies (Graca *et al*., 2007). Beyond this, the herbal medicines have received greater attention worldwide as alternative to clinical therapy in recent times leading to a subsequent increase in their demand (Sushruta *et al*., 2006). Some plant extract may be dangerous due to the naturally occurring toxins. *T. akara* (Burm. f.) Merr. has been used by the Kani tribes of Kerala for the treatment of liver diseases and inflammatory conditions and there is an urgent need to evaluate the safety of the tribal medicinal plant. In acute toxicity study, *T. akara.* root ethanol extract (up to 5.0 g/kg) did not produce any sign of toxicity or death in mice, suggesting a lethal dose 50% (LD_{50}) above 5.0 g/kg and substances that present

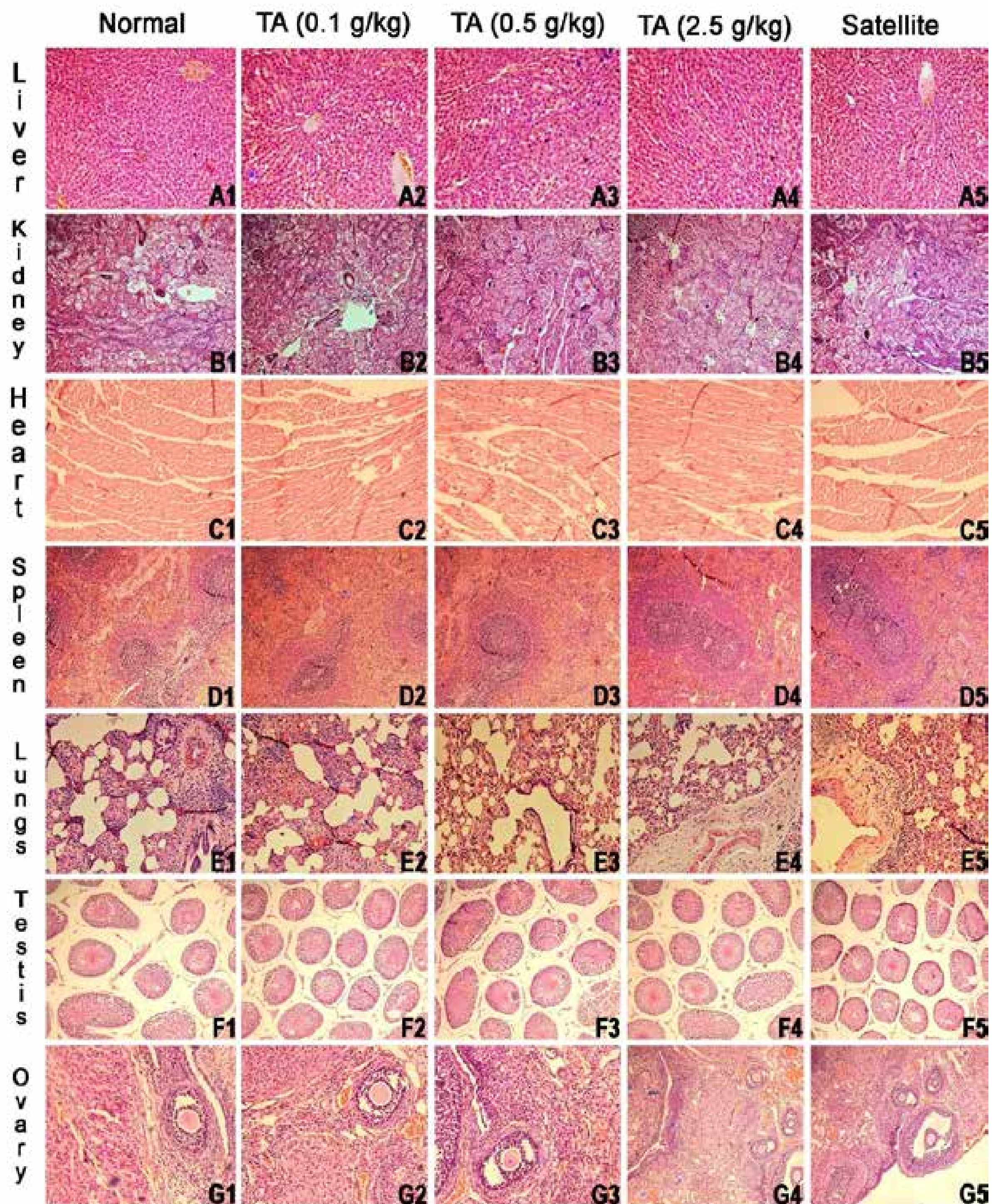

Fig. 4. Effects of the of *Tetracera akara* ethanolic extract on rat vital organs histomorphology 28 consecutive dayssub-acute oral toxicity study. A1 to A5 - Liver, B1 to B5 - Kidney, C1 to C5 – Heart, D1 to D5 – Spleen, E1 to E5 – Lungs, F1 to F5 – Testis, G1 to G5 – Ovary

LD_{50} higher than 5.0 g/kg by oral route may be considered practically non-toxic (Kennedy *et al*., 1986).

In sub-acute toxicity studies, the undesirable effects of repeated exposure of plant extracts or compounds over a portion of the average life span of experimental animals, such as rodents is evaluated and they provide information on target organ toxicity especially in liver and kidneys. In the present study, administration of TA up to 2.5 g/kg for 28 days did not show any clinical signs of toxicity or mortality in either sex. The food and water intake of the drug treated rats were comparable to the normal group showing no alterations in carbohydrate, protein or fat metabolism (Klaassen 2001). Changes in body weight serve as a sensitive indicator of the general health status of animals (Almanca *et al*., 2011). The gradual body weight gain showed by the drug treated animals of either sex is due to normal food and water intake without loss of appetite and nutritive components in TA. The body weight gain is statistically not significant from the normal control values which confirms that TA had no adverse role in the normal metabolism of the Wistar rats. In satellite groups during the recovery period, the increase in body weight, food and water consumption were comparable to the control group. The difference in the growth of body weight between female and male rats may be gender dependent and could not be considered as the toxic effect of TA (Chaotham *et al*., 2013).

Similarly, no significant changes in the weights of the liver, heart, spleen, kidneys, lungs, testis and ovary were observed, suggesting that administration of TA at the sub-acute oral doses had no effect on the normal growth. The morphological analysis and weighing vital organs in toxicity studies helps to predict toxicity, enzyme induction, physiologic perturbations, and acute injury which correlates well with histopathological changes (Michael *et al*., 2007). The relative organ weights observed in toxicity studies act as a relatively sensitive indicator for particular organs, and, thereafter, define toxicity, as significant changes observed in those particular organs (Kluwe, 1981). The results of this study revealed that the essential organs, such as heart, liver, spleen, kidneys, lungs, testis and ovary of the drug treated group have no signs of toxicity throughout the treatment. Since there was no reduction in organ and relative organ weights of the treated animals, it is evident that the extract is nontoxic to the analysed organs.

Haematological and biochemical parameters are the main diagnostic criteria in clinical practice. Evaluation of haematological parameters can be used to determine the extent of the deleterious effect of plant extract on the blood of experimental animal. Such analysis is relevant to risk assessment as changes in the haematological system have higher predictive value for human toxicity when the data are translated from animal studies (Olson *et al*., 2000). After 28 days of treatment, the haematological analysis of drug treated animals when compared with control groups showed no significant difference in all the eighteen haematological parameters. The nonsignificant effect of the extract on RBC count, HGB concentration, HCT %, MCV, MCHC, MCH and RDW indicate that TA does not affect the erythropoiesis, morphology or osmotic fragility of the red blood cells (Hall *et*

al., 2011). White blood cells are the first line of cellular defense that respond to infectious agents, tissue injury, or inflammatory process. No significant changes were observed in the lymphocyte, monocyte and granulocyte count in drug treated groups, which further confirmed the above findings. All these normal haematological profile of TA treated groups justified the non-toxic nature of *Tetracera akara* ethanolic extract.

In living systems, liver is considered to be highly sensitive to toxic agents and its functional analysis is very important in the toxicity evaluation of plant extracts as they are the major organ for toxic metabolism (Olorunnisola *et al*., 2012). The damage of membrane permeability of the liver leads to the leakage of liver enzymes into the blood stream and the quantification of liver marker enzymes in animal system is one of the most frequent approaches adopted to evaluate the toxicity of a plant extract (Ramaiah, 2011). The serum biochemical parameters were evaluated to understand the possible alterations in hepatic function influenced by TA in experimental animals. The insignificant changes in AST, ALT, ALP, GGT, TC, TP and SB values in the drug treated rats suggested that the administration of TA is non-hepatotoxic even at higher doses (up to 2.5 g/kg). The normal levels of AST and ALT also points out that the drugs have no toxic effect on heart tissue (Ozer *et al*., 2008). A decrease in total protein and albumin is a sign of the reduced synthetic function of the liver. Lowered serum albumin content may be due to infection or continuous loss of albumin (Tietz *et al*., 1994). Thus, the insignificant change in serum concentration of total protein and albumin in the *T. akara* extract treated group when compared to control group further confirmed that the extract does not damage the hepatocytes or secretory functions of the liver at any of the doses studied.

Renal dysfunction was evaluated by estimating urea and creatinine levels. Since, almost all drugs, chemicals and xenobiotics are eliminated through renal excretion, a comprehensive study on the effect of the plant extract on kidney function is essential in the toxicity studies (Bhattacharya *et al*., 2012). Increased levels of urea and creatinine are significant indicators of the damaged functional nephrons (Lameire *et al*., 2005). The results of serum biochemical parameters related to kidney functions showed no significant differences with respect to control group animals and the same shows nontoxic effect of TA on kidney function.

Normal glucose level in the blood is maintained by the combined action of two hormones, namely glucagon and insulin, produced by hypothalamus and pancreas respectively. Any type of toxicity in these organs will interfere with the normal secretion of this hormone and alter the normal glucose level in the blood. Glucose level shown by the entire drug treated groups in the present study is normal.

The enzymatic antioxidants like CAT and SOD plays an important role in protecting the liver against lipid peroxidation. They scavenge superoxide ion and hydrogen peroxide. These enzymes prevent generation of hydroxyl radical and protect the cellular constituents from oxidative damage (Scott *et al*., 1991). Lipid peroxidation has been postulated to the destructive process of liver injury and MDA as its end product. The increase in MDA levels in liver suggests enhanced lipid peroxidation leading to tissue damage and failure of antioxidant defense mechanisms (Sapakal*et al*., 2008). Reduced glutathione is an intracellular non-enzymatic antioxidant, which protects cells

against free radicals, hydrogen peroxides, alkoxy radicals, maintenance of membrane protein thiols and as a substrate for glutathione peroxidase and GST (Prakash *et al*., 2001).In the present study, a significant increase ($P \leq 0.001$) in the levels of CAT, SOD, GSH was observed in the extract treated rats when compared to control group. The level of MDA has no significant increase. This suggests that TA has a beneficial effect in increasing the antioxidant defense of the body and may contribute to the management and prevention of degenerative diseases. Early phytochemical study of the plant reported the presence of phenols, flavonoid, tannins, terpenoids and bio active compounds like betulin, betulinic acid, lupeol and β-Sitosterol which is responsible for the antioxidant properties of the *T. akara* (Ragesh *et al.,* 2016). All these findings imply the maintenance of normal hepatic non-enzymatic and enzymatic antioxidant mechanisms during TA administration.

As earlier mentioned, the macroscopic examinations of the organs of rats treated with various doses of TA did not show any changes in colour compared with the normal control group. The microscopic examination revealed that none of the organs of the extract treated rats showed any alteration in cell structure when viewed under the light microscope. No pathologies were recorded in the histological sections of the vital organs (heart, liver, spleen, kidney, lung, testis and ovary). Any damage to the hepatocytes results in elevation of both transaminases in the blood (Slichter *et al*., 2004). The normal levels of hepatic markers and enzymes reveals that the oral administration of TA did not alter the hepatocytes architecture as observed in the histopathology observations of liver tissue. Similarly, there was no significant increase in urea and creatinine in the TA administered animal groups when compared to the control group and is confirmed by the normal histopathology of the kidney tissue in this study. The extract did not cause any cellular constriction or inflammation of the organs which would have resulted in swelling and increase in weight. This finding is well corroborated by the histological findings which did not show any pathological changes in the lungs, heart, liver, spleen, kidney, testis and ovary of the treated animals.

5. Conclusion

Based on the above findings, it is clear that there were no deleterious changes in organ, haematological and biochemical indices in the animals during the course of repeated administration of TA and no mortality was observed during this period. On the basis of findings that emerged from the present investigation, it can be established that *T. akara*h as no toxic effect on sub-acute oral administration in rodents. This is a pioneer study on the species *T. akara* evaluating its acute and sub-acute toxicity which could stand as an assurance for the medicinal use of this plant in traditional medicine thus supporting the traditional claim.

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Conflict of Interest

The authors have no conflict of interest.

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