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ORIGINAL ARTICLE

Sub-chronic oral toxicity assessment (90 days) of ethanolic fraction of leaves of *Neurocalyx calycinus* (R. Br. ex Benn.) Rob. in rodents: A lesser known ethnomedicinal plant from the Cholanaickan tribal community, India

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ABSTRACT

The objective of the present study was to evaluate the safety of long term consumption of ethanolic fraction of *Neurocalyx calycinus* leaves (NCEF) in rodents. The NCEF was subjected to detect the presence of various phytoconstituents. In acute oral toxicity study, graded doses of NCEF was administered in mice and were observed up to 14 days. In sub-chronic oral toxicity study, NCEF was admin- istered to Wistar rats at doses of 50, 500 and 1000 mg/kg b.w. per day for 90 days and after that, observed up to 28 days. NCEF showed the presence of alkaloids, steroids, phenolics and glycosides. In acute toxicity study, there was no mortality and no behavioural signs of toxicity at the highest dose level (6400 mg/kg b.w.). In sub-chronic oral toxicity study, there were no significant difference observed in the consumption of food and water, body weight and relative organ weights. Haematological, serum biochemical, hepatic oxidative stress marker analysis and urine analysis revealed the non-adverse effects of prolonged oral consumption of NCEF. The histopatho- logic examination did not show any differences in vital organs. Based on our findings, NCEF, at dosage levels up to 1000 mg/kg b.w., is non-toxic and safe for long term oral consumption.

KEY WORDS: *Neurocalyx calycinus*; sub-chronic toxicity; histopathology

ABBREVIATIONS

ASL: above sea level; **ABR:** Agasthyamalai Biosphere Reserve; **NCEF:** ethanolic fraction of leaves of *Neurocalyx calycinus*; **b.w.:** body weight; **v/v:** volume/volume; **w/v:** weight/volume; **EDTA:** ethylenediaminetetraacetic acid; **WBC:** white blood cell count; **RBC:** red blood cell count; **HGB:** hemoglobin; **HCT:** hematocrit; **MCV:** mean corpuscular volume; **MCH:** mean corpuscular hemoglobin; **MCHC:** mean corpuscular hemo- globin concentration; **RDW:** red blood cell distribution width; **PLT:** platelet count; **MPV:** mean platelet volume; **PDW:** platelet distribution width; **PCT:** plateletcrit; **LYM:** lymphocyte count; **MONO:** monocyte count; **GRAN:** granulocyte count; **ALT:** alanine transaminase; **AST:** aspartate transaminase; **ALP:** alkaline phosphatase; **GGT:** γ-glutamyltransferase; **LDH:** L-lactate dehydrogenase; **TSP:** total serum protein; **ALB:** albumin; **TBIL:** total bilirubin; **GLU:** glucose; **CK:** creatine kinase; **CRE:** creatinine; **BUN:** urea nitrogen; **UA:** uric acid; **TG:** serum triglyceride; **TC:** total cholesterol; **Ca^{2+}:** calcium; **Na$^+$:** sodium; **K$^+$:** potassium; **Cl$^-$:** chlorine; **PBS:** phosphate buffer saline; **THP:** total hepatic protein; **BSA:** bovine serum albumin; **CAT:** catalase; **GSH:** reduced glutathione; **SOD:** superoxide dismutase; **MDA:** malondialdehyde; **GPx:** glutathione peroxidase; **GLU:** glucose; **BIL:** bilirubin; **KET:** ketone; **SG:** specific gravity; **BLO:** blood; **PRO:** protein; **URO:** urobilinogen; **NIT:** nitrite; **LEU:** leukocytes; **DTNB:** (5,5'-dithio-bis- [2-nitrobenzoic acid]); **NBT:** nitro blue tetrazolium; **NADH:** nicotinamide adenine dinucleotide; **TBA:** thiobarbituric acid; **H$_2$O$_2$:** hydrogen peroxide.

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Introduction

The diverse ecological conditions in India make it a trea- sure house of biodiversity, covering about 8% of global biodiversity. The forests of India are estimated to harbour 90% of India's medicinal plant diversity in a wide range

of forest types (Aneesh *et al.*, 2009). With its rich ethnic diversity and strong traditional knowledge in herbal medicine, India has been using herbal drugs for thousands of years. Due to high prices and harmful side effects of synthetic drugs, people rely more on herbal drugs as evidenced by its rapidly growing trends in international and national markets through exploration of ethnopharmacology and traditional medicine (Fabricant & Farnsworth, 2001). The modern drug discovery screening techniques, mainly based on traditional knowledge, have given clues to the development of valuable drugs (Hashmi & Singh, 2003). Most of the traditional knowledge about medicinal plants was in the form of oral knowledge that has been eroded or distorted due to the persistent invasions and cultural adaptations. Locally available medicinal plants are primary raw material for the development of traditional drug formulations.

Neurocalyx Hook is an endemic taxon in the family Rubiaceae, mainly distributed in South Western Ghats of India and Sri Lanka (Bremer, 1979). *Neurocalyx calycinus* (R. Br. ex Benn.) Rob. is a wild ornamental herbaceous plant classified under the tribe Ophiorrhizeae (Bremer, 1987; Viswanathan *et al.*, 2005; Takhtajan, 2009). It grows up to 20 inches and is dispersed on rocky crevices at the banks of streams in the tropical wet evergreen forests at higher altitudes (1200–1600 m ASL). Cholanaickans, the last remaining hunter-gatherer tribes of South India, are the most primitive and vanishing native tribal communities, living in caves called Alas spread in the Karulai and Chungathara forest ranges in Nilambur, Malappuram district of Kerala, India (Menon, 1996; Mathur, 2013). Cholanaickans use the fresh leaves of *N. calycinus*, locally known as 'Pachachedi', prepared in the form of paste and applied externally to arrest bleeding due to bear bites and to heal fresh wounds, inflammation and pain. Decoction of the leaves (30 mL) is administered orally thrice a day for one week to one month, depending upon the conditions of the symptoms (Saradamma *et al.*, 1994).

From the review of literature, it was evident that a sparse record is available about the usage, safety parameters and medicinal properties of *N. calycinus*. To develop a novel drug with least side effects and multi therapeutic effect, preclinical studies along with toxicity analysis should be conducted *in vivo* with pertinent animal models. The present work was done to evaluate the acute and subchronic (90 days) toxicity effect of the ethanolic fraction of leaves of *Neurocalyx calycinus* (NCEF) in Wistar rats.

Material and methods

Chemicals
Bovine serum albumin (BSA), Catalase, 5,5'-Dithiobis (2-nitrobenzoic acid) (DTNB), L-Glutathione, Superoxide Dismutase (SOD), Nitro Blue Tetrazolium (NBT) and Nicotinamide Adenine Dinucleotide (NADH) were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Biochemical kits were purchased from Coral Clinical System, Goa, India. All other reagents, chemicals, and solvents were of analytical grade and were purchased from HiMedia Laboratories (India). Phosphate Buffered Saline (PBS) and other reagents were prepared according to protocol.

Plant material
The fresh leaves of *Neurocalyx calycinus* (R. Br. ex Benn.) Rob. (vernacular name: Pachachedi) were collected from the evergreen forest streams of the upper hill of Ezhumadakka (Latitude 8°37'28"N, Longitude 77°12'53"E) in Athirumala at the Agasthyamalai Biosphere Reserve (ABR) region, Thiruvananthapuram, Kerala, India during the month of April 2014. The plant material was taxonomically identified by Dr. A.G. Pandurangan, Plant Taxonomist, Plant Systematics and Evolutionary Science Division, Jawaharlal Nehru Tropical Botanic Garden and Research Institute, Thiruvananthapuram, Kerala, India. A voucher specimen (TBGT 86801 dated 14/12/2015) was certified (JNTBGRI/PS/213/2015) and deposited at the institutional herbarium.

Preparation of the plant extract
The fresh leaves of *Neurocalyx calycinus* were thoroughly washed, segmented, shade dried and powdered (Usha Shriram (India), Noida, UMA 29103448). The powder (100 g) was sequentially extracted with petroleum ether, chloroform and ethanol (96% v/v) in a Soxhlet apparatus for 24 h each. Each time before extracting with the next solvent, marc was air dried at below 35±5 °C. The ethanolic extract was concentrated under reduced pressure at 30±10 mbar in a rotary evaporator at 30–60 °C (Rotavapor R-215, Buchi, Switzerland) to a syrupy consistence (6.28±0.47% w/w) named NCEF, finally dried in a desiccator (Auto Secador 401110, USA) and stored in airtight containers in a refrigerator at 4 °C.

Qualitative phytochemical screening
The NCEF was subjected to qualitative phytochemical tests to detect the presence of various phytoconstituents (Sofowora, 1982; Harborne, 1998) such as carbohydrates (Molisch test, Fehling test, Barfoed test and Benedict test), proteins (Millon test, Biuret test and Ninhydrin test), alkaloids (Mayer test, Wagner test, Hager test and Dragendorff test), glycosides (Borntrager test, Legal test, Keller kiliani test and Kedde test), phenolic compounds (ferric chloride test, gelatin test, lead acetate test, alkaline reagent test and Shinoda test), phytosterols (Libermann-Burchard test and Salkowski test), fixed oils and fats (spot test and saponification test), saponins (foam test), gum and mucilage (alcohol 95% test), volatile oils (steam distillation), anthraquinones (chloroform – 10% ammonia test) and iridoids (Trim-Hill reagent test).

Experimental animals
Adult female and male Swiss Albino mouse (*Mus musculus*), 25±2 g, and Wistar Albino rat (*Rattus norvegicus*), 155±3 g, were obtained from the animal house of JNTBGRI, India. All animals were housed in standard polypropylene cages at controlled temperature (25±2 °C),

with light conditions (12 h light and dark cycle), room air changes 15±3 times/h and relative humidity (65±5%). The animals were provided with pellet diet (Lipton India Ltd. Mumbai) and water *ad libitum*. The animals were allowed to acclimatize to the new environment for 7 days before starting the experiment. All experimental protocols described in this study were approved by the Institutional Animal Ethics Committee, JNTBGRI and were in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animal (CPCSEA), Government of India (B-01/03/2015/EM-EP/05).

Acute oral toxicity study

The acute oral toxic effect of NCEF was studied in Swiss albino mice (Lorke, 1983). Animals (54) of both sexes were randomized into 9 groups of 6 each (Group A to Group I). Before starting the experiment, all groups were fasted for 12 h and weighed. The NCEF was freshly prepared by suspending with 1.5% v/v Tween-80 in distilled water. 0.5 mL of the vehicle and test materials were administered orally *via* gavage No. 16 to Group B to I (NCEF at 50, 100, 200, 400, 800, 1600, 3200, 6400 mg/kg b.w. respectively) and Group A, vehicle control, was given 0.5 mL 1.5% v/v Tween-80 in distilled water. Food and drinking water were provided to the mice approximately an hour after treatment. The animals were observed 30 min after dosing, followed by hourly observation for 8 h and once a day for the next 14 days. Cage side observations were systematically recorded for each animal on day 1, 7, 14 and visual observations for mortality, behavioral pattern, body weight, changes in physical appearance, injury, pain and signs of illness were examined daily.

Sub-chronic oral toxicity study

The sub-chronic oral toxicity studies were conducted according to OECD Guidelines No. 408 (OECD, 1998). Wistar rats (48) were randomized into 4 groups and received 1 mL of the test materials orally once a day consecutively for 90 days. Group A (8 rats/sex) served as vehicle-control and they were given 1.5% v/v Tween-80 in distilled water. Group B and C (4 rats/group/sex) received NCEF at 50 and 500 mg/kg b. w. respectively. Group D (8 rats/sex) received NCEF at 1000 mg/kg b.w. After 90 days of treatment, 4 animals of each sex from group A and D were assigned as satellite groups and kept for further observation after the treatment for a period of 28 days, for any reversibility or persistence of toxic effects.

Mortality checks and behavioral observations

All the animals were observed every day in the morning for mortality and signs of morbidity. Any changes in the skin, fur, subcutaneous swellings, eyes, mucous membranes, excretions, autonomic activity, changes in gait, posture and response to handling as well as bizarre behavior were noted during the entire period of the study.

Food intake and water consumption

The quantities of supplied and remaining food and water were measured daily and average weekly consumption was calculated.

Body weight and relative organ weight

The body weight of all animals was recorded before starting oral administration and continued once a week up to the day of sacrifice. Based on the body weight, the quantities of NCEF were calculated again to ensure administration of fixed dose. The overnight fasted animals were sacrificed on the 91st day and 119th day (satellite group) for dissecting internal organs such as liver, kidneys, lungs, spleen, heart, brain, ovaries and testes. The relative organ weight of each animal was then calculated by the following formula:

Relative organ weight = [absolute organ weight (g)/body weight of rat on sacrifice day (g)] × 100

Hematological analysis

The blood samples were collected from overnight fasted animals through cardiac puncture on day 91 and day 119. One part was collected into ethylenediaminetetraacetic acid (EDTA)-coated vials and analysis such as white blood cell count (WBC), 2.9 to $15.3\times10^3/\mu L$; red blood cell count (RBC), 5.60 to $7.89\times10^6/\mu L$; hemoglobin (HGB) concentration, 12 to 15 g/dL; hematocrit (HCT), 36 to 46%; mean corpuscular volume (MCV), 53.0 to 68.8 fL; mean corpuscular hemoglobin (MCH), 16.0 to 23.1 pg; mean corpuscular hemoglobin concentration (MCHC), 30.0 to 34.1 g/dL; red blood cell distribution width (RDW), 11.0 to 15.5%; platelet count (PLT), 100 to $1610\times10^3/\mu L$; mean platelet volume (MPV), 3.8 to 6.2 fL; platelet distribution width (PDW); plateletcrit (PCT); lymphocyte count (LYM), 2.6 to $13.5\times10^3/\mu L$; monocyte count (MONO), 0.0 to $0.5\times10^3/\mu L$; granulocyte count (GRAN), 0.4 to $3.2\times10^3/\mu L$; LYM%, 63.7 to 90.1%; MONO%, 1.5 to 4.5% and GRAN%, 7.3 to 30.1% were performed by auto hematology analyzer (BC-2800Vet, Mindray, Shenzhen, China).

Biochemical analysis

The second part of blood was collected into plain vials and allowed to coagulate for 1 h at room temperature followed by centrifugation at 3 000×*g* for 15 min at 37°C (R8C Remi, Laboratory centrifuge, Remi Industries Pvt. Ltd, India). The serum was separated, transferred into small test tubes for biochemical analysis. Liver function enzymes such as alanine transaminase (ALT; EC 2.6.1.2), aspartate transaminase (AST; EC 2.6.1.1), alkaline phosphatase (ALP; EC 3.1.3.1), γ-glutamyltransferase (GGT; EC 2.3.2.2) and L-lactate dehydrogenase (LDH; EC 1.1.1.27) were determined. Total serum protein (TSP), albumin (ALB), total bilirubin (TBIL) and glucose (GLU) were also assessed. Creatine kinase (CK, EC 2.7.3.2), creatinine (CRE), urea nitrogen (BUN), uric acid (UA) concentrations were determined to evaluate kidney function. The concentrations of serum triglyceride (TG) and total cholesterol (TC) were also determined to give an indication of the influence of any adverse effect of

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NCEF on the lipid profile. Serum electrolyte parameters such as calcium (Ca^{2+}), sodium (Na^+); potassium (K^+) and chlorine (Cl^-) were determined. All the above parameters were evaluated by using the commercial kits.

Hepatic oxidative stress analysis

Fresh liver samples of all groups were parted into two. Each portion was weighted and homogenized separately using a Potter-Elvehjem tissue homogenizer (Universal motor, RQ 127A, Remi Motors Ltd, India). One portion (10% w/v) was homogenized in 50 mM, pH 7.4 (ION 2700, pH meter 2113190, Eutech Instruments, Singapore) phosphate buffer saline (PBS). The tissue suspensions were centrifuged at $6\,000{\times}g$ for 15 min at 3 °C (LAG 412, Remi cooling centrifuge, Remi Industries Pvt. Ltd, India) to remove the cell debris, unbroken cells, nuclei and erythrocytes (Gonzalez-Flecha *et al.*, 1993). The pellet was discarded and the supernatant was used to assess the following oxidative stress markers. Determination of total hepatic protein (THP; mg/g wet tissue) was done by the modified Lowry method using bovine serum albumin

Table 1. Qualitative phytochemical analysis of *Neurocalyx calycinus* leaves ethanolic fraction.

Phytoconstituents		NCEF
Carbohydrates	Molish's test	+++
	Fehling's test	++
	Barfoed's test	++
	Benedict's test	+
Proteins and amino acids	Millon's test	+
	Biuret test	+
	Ninhydrin test	+
Alkaloids	Mayer's reagent	+
	Wagner's reagent	+++
	Hager's reagent	+++
	Dragendorff's reagent	+
Glycosides	Borntrager's test	–
	Legal's test	–
	Keller – Kiliani test	+++
	Kedde test	++
Phenolic compounds	Ferric chloride test	+++
	Gelatin test	+
	Lead acetate test	++
	Alkaline reagent test	+++
	Shinoda's test	+
Phytosterols	Libermann Burchard	–
	Salkowski reaction	+
Fixed oils and fats	Spot test	–
	Saponification test	–
Saponins	Foam test	–
Gum and mucilage	Alcohol 95% test	–
Volatile oils	Steam distillation	–
Iridoids	Trim- Hill reagent test	+++
Anthraquinones	Chloroform - 10% ammonia test	–

+ = slightly present, + + = moderately present, + + + = highly present, – = absent. All the tests were carried out three times. Observations were based on the color intensity and precipitation with appropriate reagents.

(BSA) as standard (Hartree, 1972). Catalase determination (CAT; EC 1.11.1.6; U/mg protein) was performeded by spectrophotometric method (Sinha, 1972). One unit of enzyme activity is defined as the amount of catalase which catalyzed the oxidation of 1 µmol H_2O_2 per min per mg protein under assay conditions. Determination of reduced glutathione (GSH; µmol/g wet tissue) was performed by the reduction of 5, 5'dithio-bis (2-nitro-benzoic acid) (DTNB) method (Tietze, 1969). Superoxide dismutase (SOD; EC 1.15.1.1; U/mg protein) was determined by the inhibition of reduced nicotinamide adenine dinucleotide (NADH)-dependent-nitroblue tetrazolium (NBT) reduction method (Kakkar *et al.*, 1984) using a spectrophotometer at 560 nm (G9821A, Cary 100 UV-Vis Spectrophotometer, Agilent Technologies, United States). The second portion (10% w/v) was homogenized with ice-cold 150 mM KCl-Tris-HCl buffer, (pH 7.2) for the determination of malondialdehyde (MDA; nmol/g wet tissue) during lipid peroxidation using the thiobarbituric acid method (Ohkawa *et al.*, 1979) and glutathione peroxidase (GPx; EC 1.11.1.9; U/ mg protein) was determined by the method of Pinto & Bartley (1969) in which GPx was found as a result of reaction between glutathione and H_2O_2.

Urine analysis

At the 91st and 119th day, 24 h prior to euthanasia, the animals were housed separately in metabolic cages for urine collection. The samples were observed for color, transparency, odor and turbidity. The volume of collected urine from all animals was recorded, centrifuged at $3\,000{\times}g$ for 10 min at 4 °C; supernatant was collected for the estimation of glucose (GLU); bilirubin (BIL); ketone (KET); specific gravity (SG); blood (BLO); pH; protein (PRO); urobilinogen (URO); nitrite (NIT); leukocytes (LEU) by test strips (DIRUI A10) and the sediments were analyzed microscopically for blood cells, casts, crystals, microorganisms and squamous cells.

Histopathological examination

Organs (liver, kidney, lung, spleen, heart, brain, ovary and testis) from different groups were fixed in neutral buffered 10% v/v formalin solution for histopathological assessment. After fixation, the organs were dehydrated in graded alcohol (TP1020, semi-enclosed benchtop tissue processor, Leica Biosystems, Germany), embedded in paraffin (EG1130 and EG1150, cold plate for cooling embedding molds and paraffin blocks and modular tissue embedding center, Leica Biosystems, Germany), sectioned into 4 µm thick (RM2255, fully automated rotary microtome, Leica Biosystems, Germany) and stained with Weigert's Hematoxylin and Eosin (Suvarna *et al.*, 2012). Visualization and microphotographs were captured under a light microscope (Axiostar plus 1169-149, Carl Zeiss, Germany) at magnification 10×.

Statistical analysis

All the data were expressed as mean ± standard error of the mean. The comparisons between normal control and different dose levels of drug samples treated groups were

Table 2. Effect of *Neurocalyx calycinus* leaves ethanolic fraction on body weight and behavioral symptoms in acute oral toxicity study in Swiss albino mice.

	Female			Male			
	1st day	**7th day**	**14th day**	**1st day**	**7th day**	**14th day**	
Control	21.14±0.11	21.71±0.17	22.47±0.24	24.51±0.21	25.26±0.36	26.10±0.34	None
50 mg/kg	20.88±0.29^{ns}	21.42±0.24^{ns}	22.14±0.23^{ns}	25.01±0.34^{ns}	25.76±0.41^{ns}	26.60±0.23^{ns}	None
100 mg/kg	22.17±0.19^{ns}	22.76±0.27^{ns}	23.51±0.23^{ns}	26.56±0.39^{ns}	27.29±0.42^{ns}	28.12±0.27^{ns}	None
200 mg/kg	20.84±0.25^{ns}	21.51±0.18^{ns}	22.35±0.23^{ns}	25.40±0.24^{ns}	26.08±0.22^{ns}	26.91±0.28^{ns}	None
400 mg/kg	21.92±0.31^{ns}	22.57±0.24^{ns}	23.45±0.29^{ns}	25.42±0.23^{ns}	26.07±0.29^{ns}	26.95±0.32^{ns}	None
800 mg/kg	22.94±0.27^{ns}	23.61±0.32^{ns}	24.42±0.29^{ns}	26.56±0.32^{ns}	27.29±0.37^{ns}	28.12±0.35^{ns}	None
1600 mg/kg	21.42±0.39^{ns}	22.03±0.27^{ns}	22.81±0.33^{ns}	23.90±0.13^{ns}	24.56±0.19^{ns}	25.36±0.21^{ns}	None
3200 mg/kg	22.96±0.19^{ns}	23.64±0.24^{ns}	24.42±0.25^{ns}	25.40±0.35^{ns}	26.08±0.31^{ns}	26.91±0.27^{ns}	None
6400 mg/kg	20.17±0.11^{ns}	20.72±0.15^{ns}	21.43±0.20^{ns}	23.90±0.15^{ns}	24.56±0.24^{ns}	25.37±0.17^{ns}	None

Each value in the table is expressed as Mean ± SEM, n=3 ns = no significant differences ($p<0.05$) were observed using two-way ANOVA, followed by Dunnett's multiple comparison test.
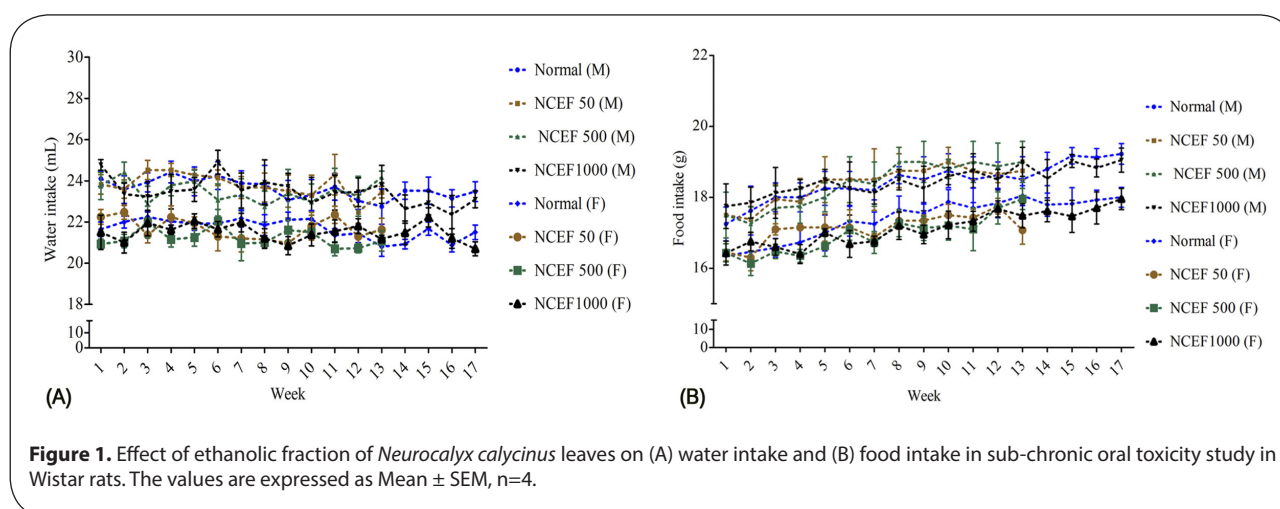
Figure 1. Effect of ethanolic fraction of *Neurocalyx calycinus* leaves on (A) water intake and (B) food intake in sub-chronic oral toxicity study in Wistar rats. The values are expressed as Mean ± SEM, n=4.

performed by two-way analysis of variance (ANOVA) followed by Dunnett's test using GraphPAD Prism 7.01 program (Trial-version) for Windows 10 (GraphPAD, San Diego, California, USA). The $p<0.05$ was considered statistically significant.

Results

Qualitative phytochemical screening
The qualitative study for the phytochemical analysis of the leaf extract of *N. calycinus* (NCEF) showed the presence of carbohydrates, proteins, alkaloids, steroids, phenolic compounds and glycosides, while saponins, anthraquinones, gums, mucilages and volatile oils were not found (Table 1).

Acute oral toxicity study
The control and NCEF treated groups did not show any behavioral changes and mortality during the 14-day post-treatment period after single oral administration. The body weight of the NCEF treated animal group of both

sexes exhibited statistically no significant ($p<0.05$) differences when compared with the control group (Table 2). Hence the median lethal dose (LD_{50}) of NCEF was greater than 6 400 mg/kg b.w.

Sub-chronic oral toxicity

Behavioral observation and mortality
The control and NCEF treated animal groups showed neither mortality and nor clinical signs of toxicity in their skin, fur, eyes, mucous membranes, gait, secretions, excretions, autonomic activity, posture handling, clonic or tonic movements, stereotypes and bizarre behavior during the 90 days of repeated oral administration (dosage: 50, 500, 1000 mg/kg/b.w.) followed by 28 days of recovery period.

Food and water consumption
No changes were noted in the consumption of food and water for the animal groups treated with NCEF orally for 90 consecutive days. Hence the water intake and food consumption (Figure 1) of NCEF treated animal groups

of both sexes showed statistically no significant ($p<0.05$) differences when compared with the control group.

Body weight and relative organ weight

During the 90 days of oral administration of NCEF, both male and female rats in either group did not show statistically significant differences when compared with the control group (Figure 2). The NCEF treated animal groups showed no significant variations ($p<0.05$) in relative organ weights as compared to control animals in both sexes (Table 3).

Hematological analysis

There were no significant ($p<0.05$) variations in hematological parameters in the groups treated with NCEF compared to control animals of each sex (Table 4). However, the platelet count of NCEF treated animals at doses 50, 500, 1000 mg/kg/b.w showed in both sexes statistically significant ($p<0.0001$) increases in dose-dependent manner when compared with the control animals during the 90 days of repeated oral administration followed by 28 days of recovery period.

Biochemical analysis

Analysis of serum biochemical parameters showed no significant ($p<0.05$) variations among the groups treated with NCEF and the control of the both sexes (Table 5).

However, the LDH and CK levels were found significantly decreased, gradually reaching normal level during the recovery period in both sexes as compared with the normal control group.
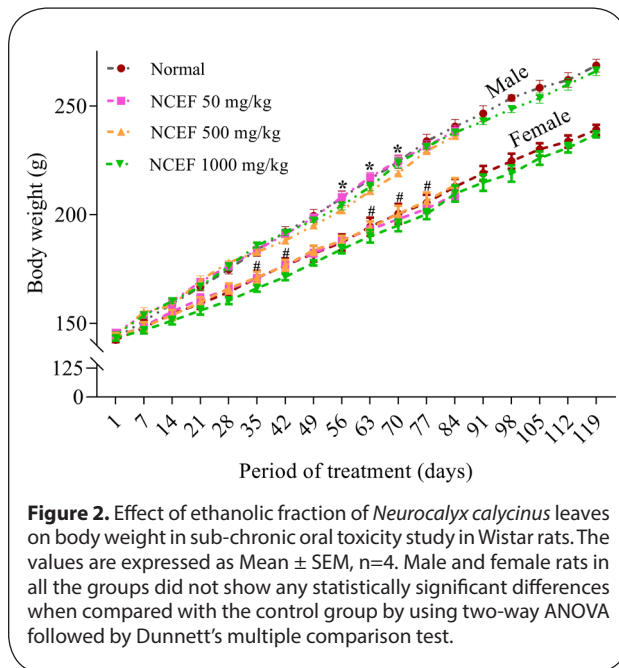
Figure 2. Effect of ethanolic fraction of *Neurocalyx calycinus* leaves on body weight in sub-chronic oral toxicity study in Wistar rats. The values are expressed as Mean ± SEM, n=4. Male and female rats in all the groups did not show any statistically significant differences when compared with the control group by using two-way ANOVA followed by Dunnett's multiple comparison test.

Table 3. Effect of *Neurocalyx calycinus* leaves ethanolic fraction on relative organ weights in sub-chronic oral toxicity study in Wistar rats.

#Organs	Treatment group				Satellite group	
	Control	NCEF 50 mg/kg	NCEF 500 mg/kg	NCEF 1000 mg/kg	Control	NCEF 1000 mg/kg
Female						
Liver	3.14 ± 0.13	3.05 ± 0.08[ns]	2.96 ± 0.12[*]	2.93 ± 0.15[**]	3.02 ± 0.09	3.03 ± 0.14[ns]
Kidney (L)	0.33 ± 0.01	0.32 ± 0.04[ns]	0.32 ± 0.02[ns]	0.34 ± 0.02[ns]	0.32 ± 0.02	0.32 ± 0.02[ns]
Kidney (R)	0.34 ± 0.02	0.29 ± 0.02[ns]	0.32 ± 0.03[ns]	0.35 ± 0.02[ns]	0.31 ± 0.01	0.34 ± 0.01[ns]
Lungs	0.55 ± 0.01	0.59 ± 0.02[ns]	0.63 ± 0.06[ns]	0.55 ± 0.04[ns]	0.55 ± 0.02	0.54 ± 0.01[ns]
Spleen	0.29 ± 0.04	0.32 ± 0.05[ns]	0.37 ± 0.01[ns]	0.31 ± 0.01[ns]	0.35 ± 0.04	0.32 ± 0.04[ns]
Heart	0.36 ± 0.01	0.34 ± 0.01[ns]	0.36 ± 0.02[ns]	0.34 ± 0.02[ns]	0.33 ± 0.02	0.33 ± 0.01[ns]
Brain	0.49 ± 0.01	0.55 ± 0.03[ns]	0.53 ± 0.03[ns]	0.52 ± 0.03[ns]	0.4 ± 0.01	0.51 ± 0.03[ns]
Ovary (L)	0.02 ± 0.01	0.02 ± 0.01[ns]	0.02 ± 0.01[ns]	0.02 ± 0.01[ns]	0.02 ± 0.01	0.02 ± 0.01[ns]
Ovary (R)	0.02 ± 0.01	0.02 ± 0.01[ns]	0.02 ± 0.01[ns]	0.02 ± 0.01[ns]	0.02 ± 0.01	0.02 ± 0.01[ns]
Male						
Liver	2.86 ± 0.05	2.91 ± 0.16[ns]	2.89 ± 0.11[ns]	2.67 ± 0.08[**]	2.82 ± 0.03	2.84 ± 0.14[ns]
Kidney (L)	0.31 ± 0.02	0.30 ± 0.01[ns]	0.32 ± 0.01[ns]	0.32 ± 0.02[ns]	0.30 ± 0.02	0.30 ± 0.01[ns]
Kidney (R)	0.31 ± 0.02	0.32 ± 0.01[ns]	0.33 ± 0.01[ns]	0.35 ± 0.02[ns]	0.32 ± 0.01	0.31 ± 0.02[ns]
Lungs	0.50 ± 0.01	0.51 ± 0.02[ns]	0.52 ± 0.02[ns]	0.48 ± 0.01[ns]	0.54 ± 0.05	0.47 ± 0.01[ns]
Spleen	0.28 ± 0.02	0.29 ± 0.03[ns]	0.23 ± 0.03[ns]	0.29 ± 0.04[ns]	0.27 ± 0.04	0.26 ± 0.03[ns]
Heart	0.35 ± 0.02	0.33 ± 0.02[ns]	0.34 ± 0.01[ns]	0.36 ± 0.01[ns]	0.33 ± 0.01	0.34 ± 0.01[ns]
Brain	0.49 ± 0.03	0.49 ± 0.04[ns]	0.46 ± 0.01[ns]	0.46 ± 0.01[ns]	0.44 ± 0.03	0.42 ± 0.02[ns]
Testis (L)	0.35 ± 0.01	0.34 ± 0.01[ns]	0.35 ± 0.02[ns]	0.36 ± 0.01[ns]	0.33 ± 0.01	0.34 ± 0.01[ns]
Testis (R)	0.35 ± 0.01	0.35 ± 0.01[ns]	0.34 ± 0.01[ns]	0.35 ± 0.01[ns]	0.32 ± 0.01	0.34 ± 0.01[ns]

The values are expressed as Mean ± SEM, n=4. #g/100 g body weight. ns = non-significant, *$p<0.05$, **$p<0.01$ significantly different from the control group by using two-way ANOVA followed by Dunnett's multiple comparison test. L, left; R, right.

Table 4. Effect of *Neurocalyx calycinus* ethanolic fraction of leaves on hematological parameters in sub-chronic oral toxicity study in Wistar rats.

Parameters	Treatment group				Satellite group	
	Control	NCEF 50 mg/kg	NCEF 500 mg/kg	NCEF 1000 mg/kg	Control	NCEF 1000 mg/kg
Female						
WBC ($\times 10^3$/μL)	11.88±0.96	10.98±0.50[ns]	11.23±0.73[ns]	11.45±1.08[ns]	10.58±0.85	10.48±0.77[ns]
RBC ($\times 10^6$/μL)	7.60±0.42	6.71±0.56[ns]	5.96±0.78[ns]	6.71±0.89[ns]	6.16±0.53	7.10±0.50[ns]
HGB (g/dL)	14.11±0.23	13.78±0.35[ns]	12.35±0.40[ns]	13.88±0.34[ns]	13.76±0.36	13.20±0.43[ns]
HCT (%)	40.10±2.00	36.48±3.15[ns]	31.52±4.11[ns]	35.77±4.90[ns]	31.96±2.68	37.16±2.89[ns]
MCV (fL)	52.82±0.31	54.35±0.39[ns]	52.91±0.32[ns]	53.22±0.24[ns]	51.91±0.25	52.25±0.43[ns]
MCH (pg)	18.74±1.13	20.86±1.28[ns]	21.71±2.51[ns]	21.97±3.22[ns]	22.71±1.49	19.01±2.02[ns]
MCHC (g/dL)	35.92±1.69	42.46±5.15[ns]	37.04±6.78[ns]	47.62±6.28[ns]	38.99±1.98	39.37±4.55[ns]
RDW (%)	13.55±1.05	13.15±1.64[ns]	15.24±1.42[ns]	14.28±1.23[ns]	15.60±0.93	13.80±0.96[ns]
PLT ($\times 10^3$/μL)	1088.00±24.62	1125.50±32.95[ns]	1194.25±37.12****	1227.00±34.07****	1069.75±42.29	1209.75±39.22††††
MPV (fL)	5.05±0.18	4.78±0.13[ns]	4.49±0.14[ns]	4.20±0.04[ns]	5.55±0.12	4.65±0.12[ns]
PDW	16.45±0.80	17.05±0.50[ns]	15.90±0.65[ns]	16.75±0.35[ns]	16.73±0.27	16.25±0.36[ns]
PCT (%)	0.55±0.01	0.64±0.01[ns]	0.68±0.02[ns]	0.65±0.02[ns]	0.59±0.02	0.68±0.01[ns]
LYM ($\times 10^3$/μL)	7.63±0.85	7.10±0.58[ns]	6.88±0.60[ns]	7.68±0.67[ns]	6.83±0.63	6.63±0.73[ns]
MONO ($\times 10^3$/μL)	0.45±0.06	0.40±0.04[ns]	0.35±0.03[ns]	0.28±0.05[ns]	0.30±0.04	0.30±0.04[ns]
GRAN ($\times 10^3$/μL)	3.80±0.24	3.48±0.36[ns]	4.00±0.38[ns]	3.50±0.53[ns]	3.45±0.29	3.55±0.36[ns]
LYM%	63.78±2.48	64.58±3.82[ns]	61.13±3.25[ns]	67.23±2.31[ns]	64.37±1.22	62.93±3.91[ns]
MONO%	3.88±0.61	3.65±0.38[ns]	3.11±0.14[ns]	2.48±0.49[ns]	2.99±0.64	2.89±0.41[ns]
GRAN%	32.34±2.03	31.77±3.47[ns]	35.75±3.20[ns]	30.29±2.70[ns]	32.64±1.12	34.17±3.51[ns]
Male						
WBC ($\times 10^3$/μL)	12.20 ± 0.79	12.18 ± 0.95[ns]	11.48±0.40[ns]	10.78±0.98[ns]	10.93±0.99	11.05±0.37[ns]
RBC ($\times 10^6$/μL)	7.85 ± 0.21	7.88 ± 0.49[ns]	7.21±0.47[ns]	6.96±0.56[ns]	7.16±0.66	7.10±0.50[ns]
HGB (g/dL)	13.86 ± 0.38	13.53 ± 0.23[ns]	12.60±0.23[ns]	13.63±0.19[ns]	13.26±0.26	12.95±0.56[ns]
HCT (%)	44.38 ± 0.76	46.08 ± 3.34[ns]	41.22±2.74[ns]	39.87±2.71[ns]	40.19±3.67	41.14±2.72[ns]
MCV (fL)	56.57 ± 0.52	58.35 ± 0.59[ns]	57.16±0.76[ns]	57.47±0.83[ns]	56.16±0.43	58.00±0.45[ns]
MCH (pg)	17.68 ± 0.58	17.39 ± 1.22[ns]	17.76±1.42[ns]	19.96±1.56[ns]	19.04±1.96	18.70±2.20[ns]
MCHC (g/dL)	31.24 ± 0.90	29.87 ± 2.36[ns]	31.09±2.59[ns]	34.64±2.28[ns]	33.92±3.51	32.20±3.67[ns]
RDW (%)	12.55 ± 0.58	11.65 ± 0.42[ns]	12.99±0.55[ns]	13.28±1.10[ns]	13.10±0.86	13.05±0.67[ns]
PLT ($\times 10^3$/μL)	1088.50 ± 24.03	1110.50 ± 23.26[ns]	1206.25±28.14****	1244.00±24.78****	1069.50±43.49	1214.25±18.20††††
MPV (fL)	4.83 ± 0.18	4.65 ± 0.15[ns]	4.35±0.12[ns]	4.10±0.04[ns]	4.95±0.18	4.45±0.13[ns]
PDW	16.20 ± 0.60	16.80 ± 0.23[ns]	15.65±0.70[ns]	16.00±0.75[ns]	16.73±0.24	16.55±0.55[ns]
PCT (%)	0.52 ± 0.03	0.61 ± 0.01[ns]	0.65±0.01[ns]	0.62±0.01[ns]	0.53±0.04	0.63±0.01[ns]
LYM ($\times 10^3$/μL)	7.83 ± 0.76	8.10 ± 0.58[ns]	7.78±0.47[ns]	7.18±0.79[ns]	7.08±0.65	7.38±0.48[ns]
MONO ($\times 10^3$/μL)	0.40 ± 0.07	0.38 ± 0.06[ns]	0.33±0.05[ns]	0.35±0.06[ns]	0.35±0.03	0.28±0.05[ns]
GRAN ($\times 10^3$/μL)	3.98 ± 0.14	3.70 ± 0.36[ns]	3.38±0.29[ns]	3.25±0.36[ns]	3.50±0.33	3.40±0.47[ns]
LYM%	63.72 ± 2.27	66.64 ± 1.14[ns]	67.69±2.72[ns]	66.36±2.12[ns]	64.74±0.79	66.86±4.32[ns]
MONO%	3.35 ± 0.70	3.03 ± 0.37[ns]	2.84±0.40[ns]	3.41±0.86[ns]	3.24±0.22	2.48±0.40[ns]
GRAN%	32.93 ± 0.10	30.33 ± 1.35[ns]	29.47±2.54[ns]	30.23±2.55[ns]	32.02±0.62	30.66±3.92[ns]

The values are expressed as Mean ± SEM, n=4. ns = no significant, ****$p<0.0001$ significantly different from the control group, ††††$p<0.0001$ significantly different from the satellite control group by using two-way ANOVA followed by Dunnett's multiple comparison test. WBC, white blood cell; RBC, red blood cell; HGB, hemoglobin; HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW, red blood cell distribution width; PLT, platelet count; MPV, mean platelet volume; PDW, platelet distribution width; PCT, plateletcrit; LYM, lymphocyte count; MONO, monocyte count; GRAN, granulocyte count. μL, microliter; dL, deciliter; fL, femtoliter; pg, picogram.

Table 5. Effect of *Neurocalyx calycinus* leaves ethanolic fraction on serum biochemical parameters in sub-chronic oral toxicity study in Wistar rats.

Parameters	Treatment group				Satellite group	
	Control	NCEF 50 mg/kg	NCEF 500 mg/kg	NCEF 1000 mg/kg	Control	NCEF 1000 mg/kg
Female						
ALT (U/L)	35.25±1.65	33.25±2.21ns	30.75±1.75ns	32.50±1.32ns	32.25±1.65	31.00±2.58ns
AST (U/L)	64.25±2.32	65.25±1.89ns	61.00±2.65ns	65.00±2.38ns	65.50±2.75	67.25±2.78ns
ALP (U/L)	101.50±3.10	105.50±3.30ns	103.50±2.40ns	104.75±2.81ns	106.25±4.09	102.00±2.38ns
GGT (U/L)	8.25±1.31	6.25±1.11ns	8.00±1.29ns	7.75±1.11ns	9.00±1.47	7.00±1.08ns
LDH (U/L)	533.75±21.54	504.75±12.07ns	523.00±41.94ns	492.75±16.13**	549.25±30.40	522.25±39.64ns
TSP (g/L)	60.25±1.76	61.00±1.58ns	58.50±1.73ns	62.75±1.99ns	59.75±1.99	61.75±1.70ns
ALB (g/L)	49.75±1.49	48.25±1.25ns	47.25±1.93ns	46.25±1.55ns	45.25±1.25	47.75±1.84ns
TBIL (µmol/L)	4.90±0.14	5.03±0.09ns	4.80±0.13ns	4.85±0.16ns	5.00±0.16	4.95±0.14ns
CK (U/L)	360.50±17.49	348.75±26.22ns	380.75±32.02ns	320.75±24.99**	387.00±21.30	344.05±17.08ns
CRE (µmol/L)	44.90±2.27	46.74±3.53ns	45.93±2.42ns	47.08±3.58ns	42.75±3.47	44.25±3.33ns
BUN (mmol/L)	14.85±0.59	14.28±0.84ns	13.68±0.39ns	15.45±0.72ns	13.53±0.49	14.95±0.43ns
UA (µmol/L)	38.50±4.03	34.50±2.47ns	36.00±5.40ns	42.25±3.35ns	36.50±4.21	39.00±4.55ns
GLU (mmol/L)	6.43±0.13	6.28±0.11ns	6.30±0.18ns	6.23±0.13ns	6.18±0.19	6.06±0.12ns
TC (mmol/L)	2.06±0.23	2.19±0.19ns	2.34±0.28ns	1.96±0.12ns	2.16±0.12	2.05±0.12ns
TG (mmol/L)	0.95±0.08	0.96±0.10ns	0.84±0.06ns	0.94±0.08ns	0.91±0.06	0.95±0.09ns
Ca (mmol/L)	0.89±0.11	0.81±0.07ns	0.88±0.05ns	0.83±0.07ns	0.86±0.05	0.86±0.11ns
Na (mmol/L)	133.00±3.76	138.00±3.34ns	133.25±5.62ns	133.75±3.17ns	137.25±4.52	135.75±4.42ns
K (mmol/L)	3.36±0.10	3.56±0.21ns	3.72±0.28ns	3.61±0.25ns	3.66±0.19	3.77±0.15ns
Cl (mmol/L)	97.30±4.27	96.75±4.33ns	99.00±3.03ns	98.75±3.57ns	97.50±3.30	102.50±2.02ns
Male						
ALT (U/L)	39.75±1.11	34.75±0.85ns	33.50±1.85ns	36.75±2.02ns	37.50±1.71	35.50±2.72ns
AST (U/L)	61.50±1.71	63.25±1.11ns	62.50±2.02ns	59.75±3.01ns	62.25±1.65	63.25±3.15ns
ALP (U/L)	96.50±2.50	100.50±2.63ns	95.75±4.37ns	103.00±3.76ns	99.75±2.32	102.75±2.95ns
GGT (U/L)	11.50±1.44	10.25±1.31ns	10.75±1.25ns	13.50±1.71ns	11.75±1.38	9.75±1.55ns
LDH (U/L)	708.75±31.87	729.50±24.84ns	730.50±41.56ns	678.25±49.44*	723.25±31.30	724.50±27.32ns
TSP (g/L)	55.25±1.66	54.80±1.87ns	56.00±1.98ns	55.50±1.46ns	55.12±1.21	54.50±1.54ns
ALB (g/L)	42.00±1.29	41.00±1.22ns	42.50±1.85ns	40.25±1.75ns	41.75±1.38	42.25±1.55ns
TBIL (µmol/L)	4.60±0.13	4.43±0.23ns	4.38±0.17ns	4.40±0.18ns	4.62±0.13	4.48±0.20ns
CK (U/L)	437.25±21.58	429.50±12.69ns	435.25±15.70ns	399.75±14.10*	436.50±16.29	430.25±24.50ns
CRE (µmol/L)	39.55±2.27	42.05±2.80ns	43.13±3.45ns	38.43±3.17ns	40.30±2.49	37.55±2.68ns
BUN (mmol/L)	14.03±0.34	13.90±0.42ns	14.38±0.40ns	14.53±0.39ns	13.63±0.37	14.73±0.89ns
UA (µmol/L)	32.65±2.83	35.00±3.11ns	33.75±3.84ns	36.50±3.62ns	36.50±2.99	38.50±3.88ns
GLU (mmol/L)	6.40±0.12	6.53±0.11ns	6.60±0.13ns	6.45±0.10ns	6.56±0.13	6.43±0.15ns
TC (mmol/L)	1.86±0.20	1.63±0.18ns	1.71±0.25ns	1.83±0.17ns	1.77±0.19	1.80±0.16ns
TG (mmol/L)	1.35±0.18	1.28±0.11ns	1.24±0.10ns	1.35±0.12ns	1.33±0.08	1.39±0.10ns
Ca (mmol/L)	0.77±0.08	0.71±0.06ns	0.78±0.09ns	0.77±0.06ns	0.78±0.04	0.72±0.10ns
Na (mmol/L)	131.00±2.80	132.75±3.77ns	135.25±4.96ns	139.00±3.24ns	135.50±3.80	128.25±4.35ns
K (mmol/L)	3.37±0.11	3.59±0.18ns	3.69±0.27ns	3.50±0.23ns	3.90±0.19	3.44±0.23ns
Cl (mmol/L)	95.35±3.65	89.25±4.33ns	91.00±2.12ns	100.00±4.42ns	97.00±3.42	96.00±3.92ns

The values are expressed as Mean ± SEM, n=4. ns = no significant differences ($p<0.05$) were observed using two-way ANOVA, followed by Dunnett's multiple comparison test. *$p<0.05$, **$p<0.01$ significantly different from the control group by using two-way ANOVA followed by Dunnett's multiple comparison test. ALT, alanine transaminase; AST, aspartate transaminase; ALP, alkaline phosphatase; GGT, γ-glutamyltransferase; LDH, lactate dehydrogenase; TSP, total serum protein; ALB, albumin; TBIL, total bilirubin; GLU, glucose; CK, creatine kinase; CRE, creatinine; BUN, urea nitrogen; UA, uric acid; TG, triglyceride; TC, total cholesterol; Ca, calcium; Na, sodium; K, potassium; Cl, chlorine. U/L, units per litre; g/L, gram per litre; µmol/L, micromoles/litre; mmol/L, millimole per litre.

Hepatic oxidative stress analysis

The hepatic oxidative stress marker index (CAT, GSH, SOD, MDA and GPx) of orally administered NCEF treated groups of female and male Wistar rats is shown in Figure 3. A dose-dependent non-significant increase ($p<0.05$) in total protein content (Figure 3A) and a dose-dependent decrease in CAT activity were observed in both female and male rats (Figure 3B). However, a significant reduction ($p<0.01$) was observed in female rats at NCEF 1000 mg/kg when compared to the control group, which was normalized after the recovery period. A dose-dependent increase in GSH activity was found in both female and male rat (Figure 3C). Further, a significant increase was observed in female rats at NCEF 500 mg/kg ($p<0.05$), 1000 mg/kg ($p<0.01$) and male rats at NCEF 1000 mg/kg

($p<0.05$) as compared to the control group, which was seen normalized during the recovery period. A dose-dependent increase in SOD activity was observed in both female and male rats (Figure 3D). However, a significant increase ($p<0.05$) was observed in female rats at NCEF 1000 mg/kg, as compared to the control group, which became regularized after the recovery period. A dose-dependent non-significant increase ($p<0.05$) in MDA level was observed in both female and male rats (Figure 3E). A dose-dependent increase in GPx activity was observed in both female and male rats (Figure 3F). A significant increase in GPx was observed in female rats at NCEF 500 mg/kg ($p<0.05$), 1000 mg/kg ($p<0.01$) and in male rats at NCEF 1000 mg/kg ($p<0.01$) as compared to the control group, which was normalized after the recovery period.
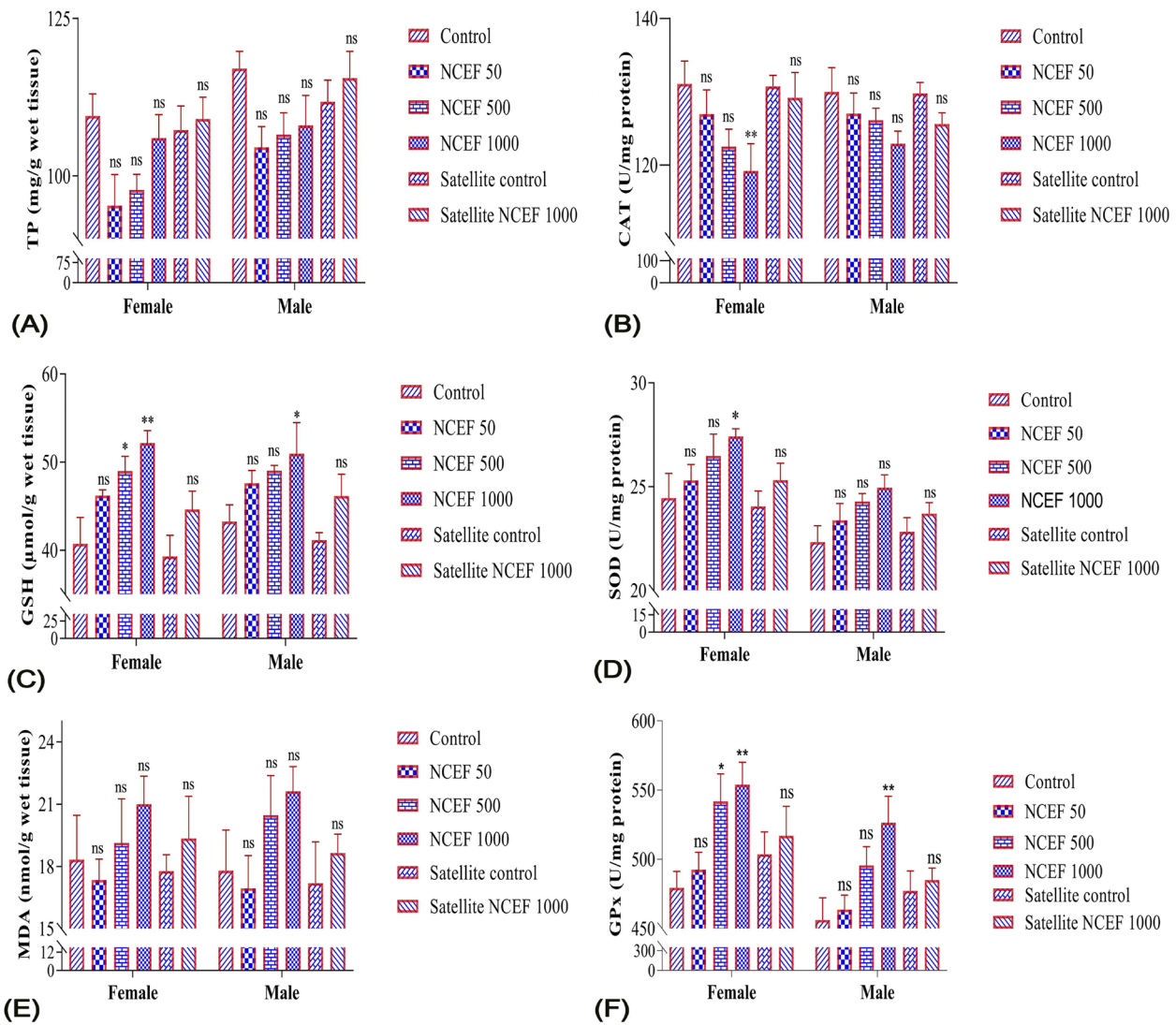
Figure 3. Effect of ethanolic fraction of *Neurocalyx calycinus* leaves on hepatic biochemical markers for oxidative stress in sub-chronic oral toxicity study of both female and male Wistar rats. (A) TP (total protein) expressed as mg/g (milligram/gram) wet tissue; (B) CAT (catalase activity expressed as U/mg (units/milligram) protein; (C) GSH (reduced glutathione) activity expressed as μmol/g (micromole per gram) wet tissue; (D) SOD (superoxide dismutase) activity expressed as U/mg protein; (E) MDA (malondialdehyde) activity expressed as nmol/g (nanomole/gram) wet tissue; (F) GPx (glutathione peroxidase) activity expressed as U/ mg protein). The results are expressed as Mean ± SEM, n=4. ns, no significant differences ($p<0.05$); *$p<0.05$, **$p<0.01$ significantly different from the control group by using two-way ANOVA followed by Dunnett's multiple comparison test.

Urine analysis

There were no significant changes in the urinary parameters observed in the group treated with NCEF compared to controls of both sexes (Table 6). Urine samples showed yellow color and a characteristic odor. Blood cells, casts, crystals, microorganisms and epithelial cells were not seen in the microscopic examination of urine samples collected from the NCEF treated rats.

Histopathological examination

There were no irregularities in the structural integrity of organs observed in the histopathological examination after 90 days of oral administration of NCEF 50, 500 and 1000 mg/kg dose groups. Microphotographs of the liver showed normal sinusoids, portal vein and hepatocytes, which are radially arranged from the central vein. Absence of fatty cells, cytoplasmic vacuolation, necrosis and minimum of Kupffer cell infiltration indicates that

NCEF is safe for oral administration (Figures 4.1, a–d). Cross section of the kidney showed normal architecture. Tubular necrosis and lymphocyctic infiltration were absent in the entire NCEF treated groups (Figures 4.2, a–d). The myocardium layer of the heart showed normal cardiac muscle fibers and capillaries. Infiltration of inflammatory cells, fibrosis and ganglionic abnormalities were not detected (Figures 4.3, a–d). Lung tissues showed normal delicate alveolar septa, uniform distribution of alveoli, bronchioles lined by single ciliated epithelium. No septal breakage, alveoli expansion and bronchiolar inflammation were observed (Figures 4.4, a–d). Brain histologic examination showed normal architecture in the cerebral cortex. No necrosis and vacuolar changes were found (Figures 4.5, a–d). Spleen histology showed no differences in the splenic cords, sinusoids and central arteriole (Figures 4.6, a–d). Histology of the ovary showed normal architecture in cortex and medullary

Table 6. Effect of *Neurocalyx calycinus* ethanolic fraction of leaves on urine analysis in sub-chronic oral toxicity study in Wistar rats.

Parameters	Treatment group				Satellite group	
	Control	NCEF 50 mg/kg	NCEF 500 mg/kg	NCEF 1000 mg/kg	Control	NCEF 1000 mg/kg
Female						
*Volume (mL/24 h)	6.84±1.78	6.45±2.00^{ns}	6.01±2.14^{ns}	5.37±1.04^{ns}	6.92±1.57	6.17±2.64^{ns}
GLU	–	–	–	–	–	–
BIL	–	–	–	+	–	–
KET	–	–	+	+	–	–
SG	1.00	1.00	1.00	1.00	1.00	1.00
BLO	–	–	–	–	–	–
P^H	1$^\#$	1$^\#$	1$^\#$	1$^\#$	1$^\#$	1$^\#$
PRO	++	++	++	+++	++	++
LEU	t	t	t	t	t	t
NIT	–	–	–	–	–	–
URO	–	–	–	–	–	–
*CRE (μmol/L)	124.47±9.35	105.11±14.58^{ns}	112.58±17.43^{ns}	139.84±12.45^{ns}	127.65±11.26	131.72±10.87^{ns}
Male						
*Volume (mL/24 h)	5.29±0.98	5.86±1.76^{ns}	6.25±1.07^{ns}	5.99±2.40^{ns}	5.07±1.88	5.59±2.01^{ns}
GLU	–	–	–	–	–	–
BIL	–	–	+	+	–	–
KET	–	–	–	+	–	–
SG	1.00	1.00	1.00	1.00	1.00	1.00
BLO	–	–	–	–	–	–
pH	1$^\#$	1$^\#$	1$^\#$	1$^\#$	1$^\#$	1$^\#$
PRO	+	++	++	+++	++	++
LEU	t	t	t	t	t	t
NIT	–	–	–	–	–	–
URO	–	–	–	–	–	–
*CRE (μmol/L)	104.36±8.24	111.27±12.35^{ns}	98.75±16.25^{ns}	109.77±11.21^{ns}	96.41±15.64	101.89±13.78^{ns}

*The values are expressed as Mean ± SEM, n=4. ns = no significant differences ($p<0.05$) were observed using two-way ANOVA, followed by Dunnett's multiple comparison test. #pH = between 6 and 9. μmol/L, micromoles/litre; GLU, Glucose; BIL, bilirubin; KET, ketone; SG, specific gravity; BLO, blood; PRO, protein; LEU, leukocytes; NIT, nitrite; URO, urobilinogen (URO); CRE, creatinine. + = slightly present, + + = moderately present, + + + = highly present, – = absent, t = trace.
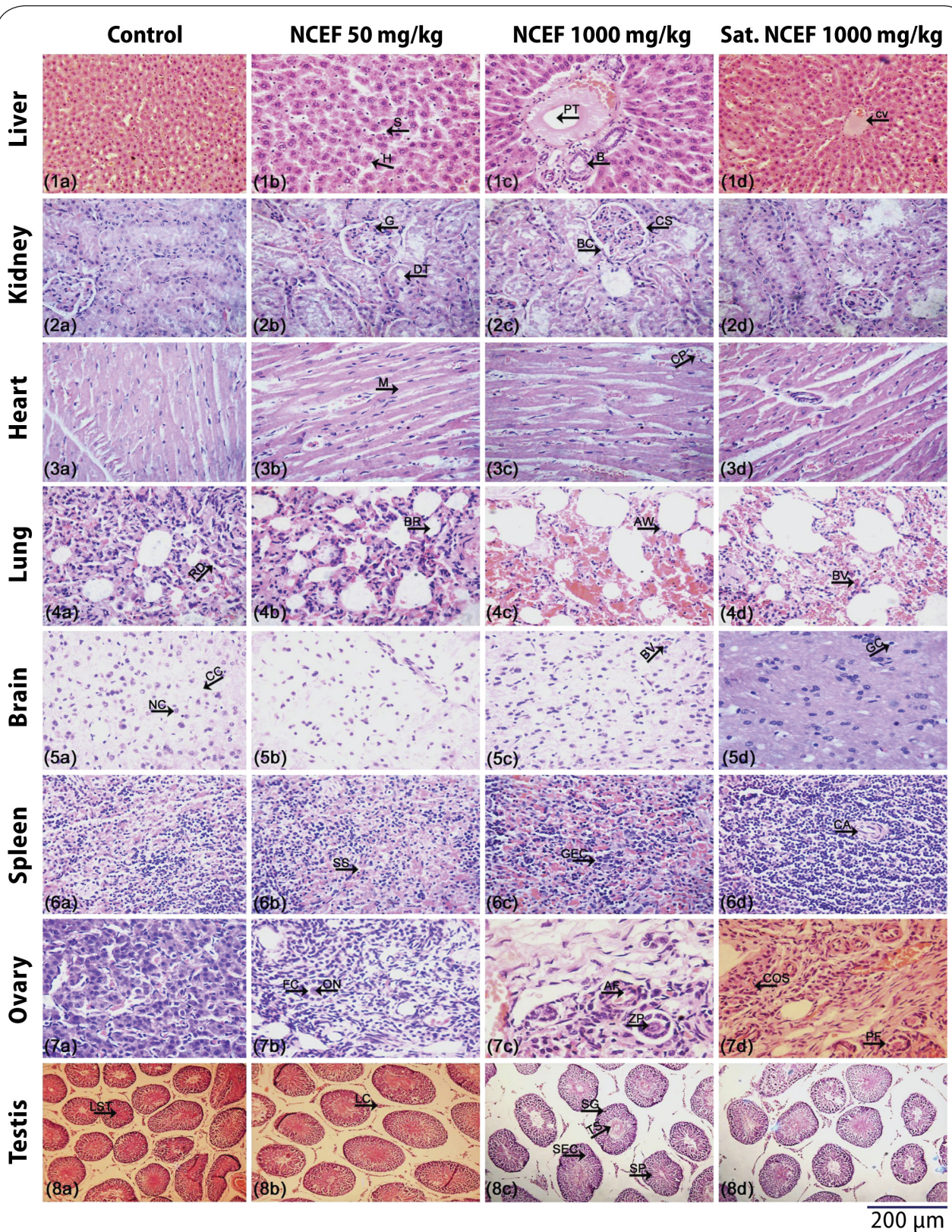
Figure 4. Microphotographs of histopathological evaluation of organs in Wistar rats treated with leaves ethanolic fraction of *Neurocalyx calycinus* in sub-chronic oral toxicity study. Histological sections of selected groups stained with hematoxylin and eosin a. Control, b. NCEF 50 mg/kg, c. NCEF 1000 mg/kg, d. Satellite 1000 mg/kg. (1a-1d) Liver, (2a-2d) Kidney (right), (3a-3d) Heart, (4a-4d) Lung, (5a-5d) Brain, (6a-6d) Spleen, (7a-7d) Ovary (right) and (8a-8d) Testis (right). S = Sinusoid; H = Hepatocyte; PT = Portal vein; B = Bile duct; CV = Central Vein; G = Glomerulus; DT = Distal tubules; BC = Bowman's capsule; CS = Capsular space; M = Myofibrils; CP = Capillaries; RD = Respiratory ducts; BR = Bronchioles; AW = Alveolar walls; BV = Blood vessel; CC = Cerebral cortex; NC = Neuronal cells; GC= Glial cell; SS = Splenic sinuses; GEC = Germinal center; CA = Central arteriole; FC = Follicular cavity; ON = Oocyte nuclei; AF = Atretic follicle; ZP = Zona pellucida; COS = Cortical stroma, PF = Primary follicle; LST = Lumen of seminiferous tubule; LC = Leydig cell; SG = Spermatogonium; TS = Tails of spermatozoa; SEC = Sertoli cell; SP = Spermatozoon.

region. Primordial follicles, thick-walled blood vessels and rete cells were normally functioning (Figures 4.7, a–d). Normal interstitial tissues, Leydig cells, Sertoli cells, seminiferous tubules, primary spermatocytes are seen in the testis (Figures 4.8, a–d).

Discussion

The leaves of *N. calycinus* belonging to the family Rubiaceae were extensively used by the Cholanaickan tribe of Kerala for treating wounds and inflammations. The worldwide acceptance of plant-based medicines is possible only if they fulfil the same efficacy, quality and safety parameters as synthetic products (Rates, 2001). Based on these assumptions, our present study focuses on acute and 90-day sub-chronic toxicity studies for understanding the impacts of long-term consumption of *N. calycinus* leaves as medicine.

In the present study, different solvent extracts of leaves of *N. calycinus* were checked for various *in vivo* and *in vitro* experiments. The ethanolic fraction showed the most promising activity. Preliminary phytochemical screening of NCEF of leaves showed the presence of major phytocompounds like alkaloids, glycosides, iridoids and phenolic compounds like coumarins, flavonoids and tannins. The presence of these compounds depends upon the environmental conditions, the plant part and the extraction solvents used for the study. The presence of phenols is considered to be potentially toxic to the growth and development of pathogens. Although anthraquinones, iridoids and alkaloids are the secondary metabolites in Rubioideae (Martins & Nunez, 2015), anthraquinones were absent in NCEF.

Toxicity studies are the essential criteria for pharmacological evaluation and standardization of unknown substances. Acute oral toxicity study of NCEF showed no mortality even at its highest dose level (6400 mg/kg b.w.). Acute toxicity studies provide information about the therapeutic index and fix the absolute dose for pharmacological tests in rodents (Chinedu *et al.*, 2013). The chemical labeling and classification of acute toxicity based on oral LD_{50} values recommended by the OECD guideline 423 (OECD, 2002) also showed LD_{50} value for ethanolic fraction of *N. calycinus* leaves to be greater than 5000 mg/kg body weight, thus coming under category 5. Body weight is one of the most sensitive indicators of the condition of an animal if it is monitored frequently and carefully during the study. In the toxicity studies no difference in body weight was found at any of the doses throughout the experiment. Body weight is measured at least once a week during toxicity studies. A treatment dose which causes more than 10% reduction in body weight is considered to be toxic (Kushwaha *et al.*, 2013). Our results confirmed that the extract did not possess any acute oral toxicity in mice.

Based on the acute toxicity data, a lower (50 mg/kg bw), middle (500 mg/kg bw) and higher (1000 mg/kg bw) dose of the NCEF were selected for sub-chronic oral toxicity

study in both sexes of Wistar rats. In the sub-chronic oral toxicity study, NCEF did not induce any signs of morbidity and mortality, and that not even in the group treated with the highest dose (1000 mg/kg bw) during the 90 days of treatment and 28 days of recovery period.

Water intake, food consumption, body weight and relative organ weight are interrelated and are the primary indicators of adverse effects of a toxic substance in rodents (Speakman & McQueenie, 1996; Shokryazdan *et al.*, 2016). Any variation in food consumption and water intake will directly affect the normal metabolism of animals (Mukinda & Syce, 2007). Any changes in body weight can be used as a rapid assessment for side effects of a drug (Teo *et al.*, 2002). In the present study, female and male rats of all the NCEF treated groups showed a regular intake of water and food similar to the control rats. Increase in body weight of NCEF treated animals was not significantly different from the control group. This general increase may be due to the quality of food and water. These results confirmed that NCEF did not affect growth retardation and appetite of rats. The mean relative organ weight of kidney, lungs, spleen, heart, brain, testis and ovary did not show any significant differences when compared to the control group. However, the liver of both female and male rats showed a significant decrease in weight at the highest dose of NCEF (1000 mg/kg b.w.). Understanding the organ to body weight ratio on studying herbal products is mandated to identify the target organs, mechanism of action and toxicokinetics (Bailey *et al.*, 2004; Sellers *et al.*, 2007; Adewale *et al.*, 2016). Based on these findings, NCEF is safe and non-toxic to animals for long-term oral administration.

Blood cells, the most sensitive connective tissue in mammals, responsible for the transport of nutrients and foreign bodies, are the target areas of toxic substances. Any damage in these cells will directly indicate the physiological condition, inflammations and infections of the animals (Petterino & Argentino-Storino, 2006; Ezeja *et al.*, 2014). No significant variations were observed in any of the blood parameters of NCEF treated groups and control groups in both sexes, except in the platelet count. A significant dose-dependent increase in platelet count of both female and male rats was observed during the 90 days of treatment , and even after the recovery period. The increases in platelet count might be an indication of the platelet augmentation property of *N. calycinus*. Further studies are required to scientifically validate the platelet augmentation effect of the plant. Increased platelet count plays a significant role in hemostasis (Anitua *et al.*, 2004) and also the release of potent growth factors for repairing various wounds (Knighton *et al.*, 1986).

The liver is the vital organ for drug biotransformation. Abnormalities present in the hepatic cells will affect the normal detoxification mechanism of animals. A wide variety of toxic substances is extensively biotransformed into metabolites with markedly different toxicological properties. Such biotransformation takes variable times in different tissues and different species, convolving the cellular toxicological effects (Nicholson *et al.*, 2002). Serum

biochemical studies will provide a significant inference about the nature of toxic effects on the liver (Wolf, 1999). Oral administration of NCEF did not cause any significant changes in the serum enzyme levels of ALT, AST, ALP and GGT as compared to control rats of both sexes. However, LDH level was significantly reduced in both female and male rats at the highest dose (NCEF 1000 mg/kg bw). The standard level of the LDH was regained after 28 days of the recovery period. An increase in the level of serum markers will give information about the nature and type of chronic liver damage (Sheweita *et al.*, 2001; Ramaiah, 2007). Among these parameters, ALT enzymatic activity is highly sensitive (Ozer *et al.*, 2008). However, NCEF oral consumption did not elevate the enzyme level in all dose groups. Other serum parameters such as TSP, ALB and TBIL showed no significant changes as compared to normal rats of both sexes, indicating that NCEF did not cause any adverse effects in animals. Hepatotoxic substances change the standard level of serum enzymes (ALT, AST, ALP, GGT and LDH), which are vital diagnostic parameters in liver disease and cell injury (Galle *et al.*, 1990; Sutcu *et al.*, 2006). In the present study, no variations were observed in the CRE, BUN, UA and electrolyte levels such as Na, K, Cl and Ca. The CRE levels were also found to be normal during the recovery period of 28 days. These serum biochemical markers are prominent indicators of kidney function and any fluctuations in the normal level of these parameters directly reflect the metabolism (Vasan, 2006). Our study suggests that NCEF has no adverse effect on hepatic and renal cells, indicating its non-toxicity. There were no significant changes observed in the GLU, TC and TG levels of NCEF treated rats compared to control rats. The non-significant difference of hepatic and renal serum marker levels (Hilaly *et al.*, 2004) between the control and treated groups suggests that repeated daily oral consumption of NCEF did not alter the normal biologic process of animals. Furthermore, detailed molecular profiling like genomics, proteomics, and metabolomics analysis (Hellmold *et al.*, 2002) is desirable to understand the effect of oral consumption of NCEF on whole-organism functional integrity overtime after drug exposure.

The liver plays a critical role in the maintenance of body homoeostasis. The imbalance between the excess formation of pro-oxidants and the antioxidant defence mechanism of a cell leads to oxidative stress, which causes tissue damage and cell death (Sanders, 2001; Mates *et al.*, 2008; Weydert & Cullen, 2009). No significant difference was observed in the activity of antioxidant enzymes (CAT, GPx and SOD) together with GSH and the level of oxidation product MDA in any of the groups studied. SOD helps the endogenous antioxidant defence system to scavenge radicals and maintain cellular redox balance. GPx catalyzes the reduction of H_2O_2 and other peroxides. GSH protects cells against lipid peroxidation (Yao *et al.*, 2007). In the present study, we confirmed that repeated 90-day oral consumption of NCEF did not induce any oxidative stress and liver toxicity and normal hepatic non-enzymatic and enzymatic antioxidant status was maintained in rats.

Kidney is one of the primary targets of toxic substances. Urinary parameters, especially BUN and CRE, assist in the detection of renal failure (Hoffmann *et al.*, 2010). Physicochemical and microscopical urine test will give vital information about the abnormalities in renal and urinary tract diseases. But, there was no significant difference in the urinary parameters between the NCEF treated and control animals. However, in the animals of either sex treated with highest dose, the urine color changed to pale yellowish. The microscopic examination of urine sediments did not find blood cells, casts, crystals, squamous cells and microorganisms. A negative nitrite test confirmed the absence of microorganisms.

The vital organs are extremely sensitive to toxic elements, and the resulting hypertrophy of these organs provides a direct indication of toxicity which may be useful in predicting toxicity level, enzyme induction, physiologic perturbations and acute injury (Michael *et al.*, 2007). Histopathological examination of the liver, kidneys, lungs, spleen, heart, brain, ovaries and testes after repeated 90-day oral consumption of NCEF did not show any anatomical disorder and detrimental changes at any dose level compared with normal rats. No signs of inflammations, lesions, color changes, texture and hypertrophy confirmed the non-toxic nature of NCEF. In our biochemical observations slight variations of normal parameters had no effect on the routine function of vital organs, which was corroborated by histological findings. These findings will help to fix dose-dependent safety margins for using NCEF as a drug and its role in targeted visceral organs (Kramer *et al.*, 2007).

Conclusion

The sub-chronic toxicity effect of ethanolic fraction of leaves of *Neurocalyx calycinus* will be a stepping stone for various preclinical and clinical pharmacological studies in future. The acute toxicity study at NCEF 6400 mg/kg/b.w. did not cause any mortality and adverse effects. Repeated oral consumption of NCEF for 90 days among male and female Wistar rats did not show any deleterious changes in food and water consumption, body weight, organ weight, hematological, biochemical, oxidative stress and histopathological parameters. Therefore, based on these findings, it is confirmed for the first time that prolonged oral consumption of NCEF is safe, non-toxic and also not adversely affecting the normal physiological functions of the animal. Our present findings also facilitate the future step for the development of a potent drug candidate in nearby future from the leaves of *N. calycinus*.

Acknowledgements

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