

ORIGINAL CONTRIBUTION

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Acute and sub-acute (30-day) toxicity studies of *Aegialitis rotundifolia* Roxb., leaves extract in Wistar rats: safety assessment of a rare mangrove traditionally utilized as pain antidote

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Abstract

Background: *Aegialitis rotundifolia* Roxb., (Plumbaginaceae) is a small mangrove tree or shrub traditionally used by the local healers of mangroves for curing pain and inflammation. However, there are no scientific reports of its toxicological properties which would guarantee the safety of its folkloric usage as a potent pain reliever. Therefore, the present study deals in acute and sub-acute (30-day) toxicity studies of *Aegialitis rotundifolia* ethanolic leaves extract (ARELE) on male and female Wistar rats administered through oral gavage.

Methods: Acute toxicity study was conducted at a single oral dose of 1000, 1500, and 2000 mg/kg, body weight (b.wt.) for 14 days with a special emphasis on the first four hours after drug administration to find out any mortality and morbidity. In sub-acute toxicity, the extract at the doses of 125, 250 and 500 mg/kg, b.wt., was administered orally for 30 days. Important parameters such as general behaviour, body and organ weight, urinalysis, haematological and biochemical profile, organ macroscopy and microscopy were conducted. Organ sample of liver, kidney, pancreas, heart, lungs, and stomach were taken from both male and female rats, whereas the sample of testis and ovary was taken from male and female rats respectively for organ necropsy and histopathological studies. Neurobehavioral toxicity was conducted by performing a functional observational battery (FOB) and locomotor activity on the initial and final week of the 30-day study period.

Results: No mortality or any major signs of morbidity was recorded for acute toxicity except for the limit dose (2000 mg/kg, b.wt.) which produced a slight short-term sedative effect. In sub-acute toxicity, no major alteration was observed in the evaluated parameters. However, few minor changes were recorded for high dose (500 mg/kg, b.wt.) group.

Conclusion: The results of the present study showed that oral administration of *Aegialitis rotundifolia* ethanolic leaves extract did not produce any severe toxic effects in both acute and sub-acute studies in Wistar rats. Therefore, usage of an appropriate amount of *Aegialitis rotundifolia* leaves extract preferably at low doses for its traditional use should be considered safe.

Keywords: *Aegialitis rotundifolia*, Mangrove, Acute toxicity, Sub-acute toxicity, Neurotoxicity, Locomotor

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Background

A toxicological investigation is considered very essential for the development of new drugs. The US Food and Drug Administration (FDA) have stated that it is important to screen new molecules for toxicity and pharmacological activity in animals [1]. Many developing countries are using herbs and herbal products for their health care needs. In fact, few allopathic/western medicines are also derived from plants which are being used for treatment of several diseases in developing as well as developed countries. Ayurvedic medicine is still practiced in India where approximately 85% of the Indian population uses crude plant extract/formulations for the treatment of various diseases. However, the use of plants by ethnic people for curing diseases without knowing its adverse effects may cause health complications in later stages [2]. Worldwide, especially in developing countries there is a general old age myth that herbal drugs are safe and non-toxic but according to Zhang et al. [3], the traditional uses of plants may cause adverse effects in humans or animals. A number of plants and their constituents traditionally used as medicines are suspected of being carcinogens to rodents and/or humans. Some of the traditionally used medicinal plants such as *Larrea tridentata* (DC.), *Piper methysticum* G. Forst., *Atractylis gummifera* L., *Callilepis laureola* DC. etc., are known to cause human liver injuries [4]. Further, there is a scarcity in scientific evidence on the safety and efficacy of herbal drugs on the increase in a number of its users which has raised concerns regarding toxicity and detrimental effects of these herbal remedies. Thus, there is a need to evaluate the safety and efficacy of these medicinal plants thoroughly to maximize their benefits for mankind [5].

Mangrove plants are a rich source of alkaloids, flavonoids, triterpenes, steroids, saponins, and tannins. Many metabolites belonging to diverse chemical classes and possessing novel chemical structures have been identified from mangroves plants, which are of considerable interest to modern industries and medicine [6]. *Aegialitis rotundifolia* Roxb. (Plumbaginaceae) is a small mangrove tree or shrub which usually grows up to a height of 2–3 m and is available in shorelines of the Andaman Sea and the Bay of Bengal from Orissa to Mergui and on the Andaman Islands and are endemic to the coastal parts of South Asia. In Orissa it is locally known as Banrua [7]. This mangrove species is reported to produce one of the best quality honeys [8]. Traditionally the leaf is used in treatment of sundry cutaneous injuries accompanied by pain and inflammation and is locally utilized as an anti-ache agent [9]. Further, the leaf of the plant is pounded with oil to make a paste which acts as an antidote for insect bites [10]. According to the present literature available for this plant, there have been very few scientific reports of pharmacological screening

conducted such as in-vitro antioxidant [11], antimicrobial [12, 13], antibacterial [14], anticancer [15], analgesic, antipyretic [10], anti-inflammatory [10, 14], and in-vitro thrombolytic activities [14]. Recently, we have reported the presence of gallic acid, chlorogenic acid, caffeic acid, p-coumaric acid, rutin, coumarin, and quercetin by performing quantitative High-performance liquid chromatography (HPLC) analysis and an organosilicon compound, (–)-spiro[1-[(tert-Butyldimethylsiloxy)methyl]-3,5,8-trimethyl-bicyclo[4.3.0]non-2-en-5,7-diol-4,1'-cyclopropane] was detected in GC-MS analysis as the most abundantly found compound [7]. Heavy metal analysis of plant powder was also carried out by us and that showed the presence of arsenic, lead, mercury and cadmium, all of which are known to cause toxic effects to animals [7, 16].

According to current literature *A. rotundifolia* has not been investigated for their toxicological property which is used traditionally as a pain reliever by local healers of mangroves. Thus, the present work deals with the evaluation of acute and sub-acute (30-day) toxicity of *A. rotundifolia* leaves administered on Wistar albino rats through oral gavage.

Methods

Chemicals and reagents

Ethanol 99.9% was procured from Changshu Hongsheng Fine Chemicals Co. Ltd, China. Ethylene di-aminetetraacetic acid (EDTA) was procured from Thermo Fisher Scientific India Pvt. Ltd., (Mumbai, India). All the solvents used were of high purity and HPLC grade. All other chemicals and reagents used in the whole study were of analytical grade.

Collection and authentication of plant materials

The fresh leaves of *Aegialitis rotundifolia* Roxb., was collected from healthy fully-grown plants in the month of May, 2016 from Bichitrapur mangrove located in Kharibil, Orissa, India (21°34'54.0"N - 87°25'25.4"E). The plant materials were then authenticated from Botanical Survey of India (BSI), Central National Herbarium, Botanic Garden, Howrah, West Bengal, India and was assigned with a Voucher no. CNH/Tech.II/2016/11a and specimen no. DG-01.

Preparation of extracts

The collected plant material (500 g) was gently washed in tap water to remove dirt and then they were shade dried in the laboratory under room temperature (24 ± 2 °C) for 3–4 weeks. After complete drying, the dried plant material was pulverized by using a mechanical grinder followed by sieving to obtain a coarse powder. The powdered plant material was then extracted with ethanol (99.9%) using reflux technique. The crude extract solution

obtained was filtered using Whatman No. 42 filter paper after which the excess solvents were evaporated by rotary vacuum evaporator and concentrated on water bath to obtain *Aegialitis rotundifolia* Roxb., ethanolic leaves extract (ARELE). The crude ethanol extract obtained was stored at 4 °C before analysis. The percentage yield of the extract was calculated by the following formula.

$$\text{Percentage yield} = \frac{\text{weight of dry crude extract obtained (g)}}{\text{weight of plant material before extraction (g)}} \times 100$$

Qualitative phytochemical analysis

ARELE was analysed for the presence of various phytochemical groups such as alkaloids, flavonoids, cardiac glycosides, triterpenoids, saponins, tannins, proteins, carbohydrates and sterols using standard procedures [17, 18].

Experimental animals and husbandry

Adult healthy male and female Wistar albino rats (age, 8–12 weeks: body weight (males), 150–200 g; body weight (females), 120–150 g) were used to evaluate acute and sub-acute toxicity studies. Three rats were housed per polypropylene cages. Male and female rats were housed separately, and the selected female rats were nulliparous and non-pregnant. The animals were housed for at least one week in the laboratory animal room prior to testing and maintained in clean polypropylene cages with stainless steel top grill. The cages were kept at standard conditions (Temp, 25 ± 2 °C; relative humidity, 65%; 12/12 h light/dark cycle). Fresh paddy husk was used as the bedding material. The animals were fed with standard rat pellet feed (M/s Hindustan Lever Ltd., Mumbai) and drinking water was provided ad libitum in clean polypropylene bottles with stainless steel sipper tubes. The cage beddings and water bottles were cleaned on daily basis.

All experimental protocols were approved by the Institutional Animal Ethics Committee (IAEC) of GITAM Institute of Pharmacy, Visakhapatnam, Andhra Pradesh, India (CPCSEA regd. no.: 1287/PO/Re/S/09/CPCSEA and Approved proposal no.: IAEC/GIP-1287/DB-S/Approved/11/2017–18). Experiments were performed according to the guide for the care and use of laboratory animals.

Acute toxicity study

The acute toxicity studies were conducted over Wistar albino rats as per Organization for Economic Co-operation and Development (OECD) guidelines 423 [19] with a few minor modifications. Rats of both sexes were used for the study. The selected male and female rats were then assigned to normal control and treatment groups (5/sex/group). The test group rats received ARELE at

the doses of 1000, 1500, and 2000 mg/kg, b.wt., once orally as test sample, which was prepared by suspending ARELE in tween 20 solution (1%, v/v) and mixed thoroughly. The control group rats received only tween 20 solution (1%, v/v) as vehicle. Prior to commencement of the experiment, all rats were weighed, marked for identification, and fasted overnight but were allowed free access to water. After dosing, the rats have further fasted for 4 h and observations were recorded continuously for each individual rat in their respective groups during the first 4 h and then 24 h after drug treatment for any mortality and abnormal changes. They were then observed twice daily for a period of 14 days to find out any toxic effect viz., changes in the eye, hairs and skin colour, food and water intake, tremors, convulsions, salivation, diarrhoea, lethargy, respiration, abnormal behaviour, motor activity, sleep and coma. Acute toxicity was performed to provide information on short-term toxicity level of the test extract which helps in the selection of doses for the repeated oral toxicity study.

Sub-acute toxicity studies

Sub-acute toxicity study of ARELE was conducted on Wistar albino rats according to the protocols described in OECD guidelines 407 [20].

Experimental grouping and dosing regime

Forty-eight healthy Wistar albino rats (24 male and 24 female) having average body weight between 150 and 200 g for males and 120–150 g for females were chosen for the study. They were assigned into four groups (6/sex/group) based on body weight stratification and randomization. Grouping of these animals was done such that the average body weight variation of the rats does not exceed ±25% of the mean body weight of each sex. Rats in Group I (Normal control group) received tween 20 solution (1%, v/v) in distilled water as vehicle, whereas Group II, Group III and Group IV received ARELE (suspended in 1%, v/v, tween 20 solution) at the doses of 125, 250 and 500 mg/kg, b.wt., respectively, for 30 days. The dosing volume was 10 mL/kg and was orally administered once daily through oral gavage. All animals were allowed free access to food and water throughout the course of the study until the day prior to scheduled euthanasia.

Clinical observations and survival

All the animals were monitored twice daily for mortality and morbidity. The clinical observation included changes in fur, skin, eyes, mucus membrane and autonomic activity such as piloerection, changes in pupil size, lacrimation and unusual breathing pattern. Changes in gait and posture were also monitored along with

stereotype activities such as excessive grooming, repetitive circling etc. The period of observation was one week prior to administration of test drug till scheduled necropsy.

Body weight

Individual body weights of all animals were recorded weekly (5 days interval) during the course of the study. The body weights were also recorded prior to testing and terminally (after fasting) prior to necropsy.

Necropsy and organ weight

All animals were fasted overnight prior to necropsy. The animals were euthanized using ketamine hydrochloride (100 mg/kg, i.m.) followed by exsanguination. The macroscopic examination included a study of the external surfaces, all orifices, including the thoracic, abdominal, cranial and pelvic cavity. During necropsy, vital organs such as liver, kidney, pancreas, heart, lungs, and stomach were excised from both male and female rats whereas reproductive organs like testis and ovary were excised from male and female rats respectively. These organs were washed and placed in 10% neutral buffered formalin solution. The macroscopic examination of these organs was also carried out for possible development of lesions or other abnormal signs.

Organ weight (Absolute and relative organ weight) were determined for the following vital organs: liver, kidney, pancreas, heart, lungs, and stomach from both male and female rats whereas testis and ovary from male and female rats respectively. The vital organs were removed and placed on absorbent papers for few minutes and weighed to give their absolute organ weight (g). The relative organ weight (ROW) of each rat was determined using the formula given below

$$\text{ROW} = \left[\frac{\text{Absolute organ weight(g)}}{\text{Body weight of rat on sacrifice day(g)}} \right] \times 100$$

Urinalysis

On the last day of the treatment period all animals from each group were subjected to a urine analysis. Urine from all animals was collected overnight using metabolic cages (Nalgene, USA) and parameters such as pH, specific gravity, leukocytes, nitrites, proteins, blood, ketones, glucose, urobilinogen and bilirubin were examined. Analysis of urine was performed using an automatic urine analyser and test strips (UroColor™ 10, Standard Diagnostic Inc., Korea).

Clinical pathology

On day 31, rats were anesthetized using ketamine hydrochloride (100 mg/kg, i.m.) and the blood samples were

obtained by retro-orbital puncture with the help of capillary tubes and were collected in sterile tubes containing anticoagulant i.e. EDTA (for haematological parameters) and without anticoagulant (for biochemical parameters). All blood samples were collected prior to scheduled necropsy and all rats were fasted overnight prior to collection of blood samples.

Haematological analysis

Haematological profile of the blood samples kept in sterile tubes containing anticoagulant was analysed using automatic haematological analyser (Sysmex XS-1000i). The haematological parameters investigated were as follows: haemoglobin, total white blood cell (WBC) count, total red blood cell (RBC) count, haematocrit (HCT), platelets (PLT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), differential leucocyte count (neutrophils, lymphocytes, eosinophil, monocytes).

Biochemical analysis

Blood serum was obtained by centrifuging the blood samples (without anticoagulant) at 1500×g for 15 min. The serum obtained was stored at -20 °C for later use. The following biochemical parameters were evaluated: glucose, creatinine, urea, sodium, potassium, chloride, total protein, albumin, globulin, bilirubin (bilirubin (T), bilirubin (D)), serum glutamic pyruvic transaminase (SGPT), serum glutamic oxaloacetic transaminase (SGOT), alkaline phosphatase (ALP), triglyceride (TG), cholesterol (total cholesterol, HDL cholesterol, LDL cholesterol, VLDL cholesterol). These parameters were evaluated using an automated biochemistry analyser (ADVIA 2400, Siemens Healthcare) and standard diagnostic test kits (Span Diagnostics Ltd., Surat, India).

Histopathological studies

Histopathological studies were performed on organ samples of liver, kidney, pancreas, heart, lungs, and stomach from both male and female rats whereas testis and ovary from male and female rats respectively. The major organs were surgically taken out and were fixed in 10% buffered formalin (pH 7.4). After fixation the tissue samples were dehydrated in graded series of ethanol (70–99.9%), washed in toluene, and then enclosed in paraffin. Then, thin tissue sections of 5 µm were obtained on a rotary microtome and then the material was stained with hematoxylin-eosin (HE) [21]. The sections were then analysed microscopically for pathological examinations and photomicrographs were recorded.

Neurotoxicological evaluation

Functional observational battery (FOB)

FOB was performed according to standard procedures [22, 23] with few minor modifications. The scoring criteria for neurotoxicological assessment have been given in the Additional file 1: Table S3. The FOB was recorded during an initial week (Day 0) and during final week (Day 30) of the dosing period. The rats were transferred to a sound-attenuated observation room and were allowed to acclimatize for 1 h. After dosing the rats were kept in home cages and observed for 10 mins. The home cage observations were: Posture, presence or absence of tremors and convulsions, spontaneous vocalization and biting. After completing the above observations each rat was held by the observer and the following observations were recorded: lacrimation, salivation, and handling activity, piloerection and ease of removal from the cage. Then, the animals were placed in an open field covered with a clean absorbent paper on the surface and allowed to freely explore for 2 mins. The parameters explored during that time period were rat's arousal activity, gait, mobility, unusual movements and stereotype activities. After the allotted time period the number of faecal boluses and presence/absence of diarrhoea on the absorbent paper were recorded. Sensorial responses were also monitored such as response to various stimuli's (click stimulus using a metal clicker, pinch on the tail using the metal tweezers, constriction of the pupil to a penlight stimulus, touch in the corner of the eye using fine cotton thread).

Locomotor activity

The locomotor activity was performed using actophotometer on Wistar albino rats according to the standard procedures [24] after performing the functional observational battery (FOB) tests. The spontaneous locomotor activity was recorded during an initial week (Day 0) and during final week (Day 30) of the dosing period. Actophotometer consists of a series of photo cells. When the beam of light from these cells is interrupted by rats, reading is observed on a digital counter in the form of counts. The animals were placed in the cage after 30 and 60 mins of the oral administration of the test extracts. Each animal was observed for 10 min after placing in the cage. The counts were recorded when the beam of light falling on the photocell of actophotometer is cut off by rats. The number of obstructions recorded for extracts was compared with the control group.

Statistical analysis

The results were calculated and expressed as Mean \pm Standard deviation. The data obtained in the studies were subjected to one-way of analysis of variance (ANOVA) for determining the significant difference. The

inter group significance was analysed using Dunnet's t-test. A p -value < 0.01 was considered to be significant. All the statistical analysis and data presentation were done using GraphPad InStat Version 3.06 (GraphPad Software, Inc. La Jolla, CA, USA) and Microsoft excel 2013 standard (Microsoft Corp., Redmond, WA, USA).

Results

Preliminary phytochemical screening

ARELE after complete drying, yielded about 17.48% of the extract. Preliminary phytochemical screening of ARELE revealed the presence of major phytochemical groups such as alkaloids, carbohydrates, tannins, steroids and sterols, triterpenoids, saponins and flavonoids as shown in Table 1.

Acute toxicity study

In the acute toxicity studies, no mortality was observed within 4 h of continuous observation and also after 24 h. There was also no lethal effect observed after administration of the extract for the experimental period of 14 days. Morphological characteristics (fur, skin, eyes and nose) appeared normal. No salivation, diarrhoea, lethargy or unusual behaviours were observed. Food and

Table 1 Preliminary phytochemical test of ethanol extract from *A. rotundifolia* Roxb. leaves

| Sl no. | Phytochemicals | Tests performed | Inference |
|--------|--------------------------|------------------------|-----------|
| 1. | Alkaloids | Mayer's test | + |
| | | Dragondorff's test | + |
| | | Wagner's test | + |
| | | Hagers's test | + |
| 2. | Carbohydrates | Molisch's test | + |
| | | Fehling's test | + |
| | | Benedict's test | + |
| 3. | Proteins and amino acids | Biuret test | - |
| | | Ninhydrin test | - |
| | | Xanthoproteic test | - |
| | | Millon's test | - |
| 4. | Tannins | Ferric chloride test | + |
| 5. | Steroids and sterols | Liberman Burchard test | + |
| | | Salkowski's test | + |
| 6. | Triterpenoids | Sulphuric acid test | + |
| 7. | Cardiac glycoside | Keller killiani test | - |
| 8. | Saponins | Foam test | + |
| 9. | Flavonoids | Shinoda test | + |
| | | Ferric chloride test | + |
| | | Lead acetate test | + |
| | | Zn dust test | + |

(-) Absent, (+) Present

water intake, body weight and respiration were normal. However, moderate sedation was observed after 10–15 min of administration of the test extract dose of 2000 mg/kg, b.wt., (Limit dose). The sedative effect observed in the rats stayed for 40–50 min after which the effect started decreasing and the rats returned to their normal activities within few minutes. There were no other toxic effects observed for both male and female rats within the study period. This indicates that ARELE at the doses of 1000, 1500, and 2000 mg/kg, b.wt., were safe. As there was no mortality recorded for all the doses, the LD₅₀ value was assumed to be greater than the limit test dose of 2000 mg/kg, body weight. Hence 125, 250 and 500 mg/kg, oral doses of ARELE were selected to evaluate sub-acute toxicity study. The control group which was administered with normal vehicle (tween 20 solution (1%, v/v)) did not produce any toxic effects or mortality within the study period.

Sub-acute toxicity studies

Clinical observations and survival

No mortalities were recorded in rats over the period of 30 days of treatment with ARELE at the doses of 125, 250 and 500 mg/kg, b.wt., through oral gavage. None of the rats (both male and female) after administration of ARELE at the doses of 125, 250 and 500 mg/kg, b.wt., showed any obvious morbidity or clinical symptoms of toxicity such as changes in the skin and fur, eyes, respiratory rate, autonomic (salivation, perspiration and piloerection), and stereotype activities throughout the experimental period of 30 days. There were no clinical signs of toxicity observed for the normal control group. Any minor changes or activities in rats found in the study period can be considered common findings for Wistar rats.

Body weight

The body weight of Wistar rats recorded at an interval of 5 days over the treatment period of 30 days and statistically significant increase in body weight was compared with the control is presented in Fig. 1. The results showed that in male rats, the body weight in the test extract treated groups increased non-significantly ($P > 0.05$) except in day 20 where the increase in body weight was significant ($P < 0.05$) for ARELE (500 mg/kg, b.wt.) treated group when compared with the control. In female rats, the increase in body weight was non-significant ($P > 0.05$) till day 15 except for the high dose treated group which showed significant ($P < 0.01$) increase on day 15. From day 20 to 30, the extract treated group showed significant increase in body weight when compared with the control. The increase in body weight for all groups was mostly dose dependant as a greater

increase in body weight was observed in high dose group for both male and female rats.

Necropsy and organ weight

The macroscopic observations of the vital organs such as liver, kidney, pancreas, heart, lungs, and stomach from both male and female rats whereas testis and ovary from male and female rats respectively did not produce any major macroscopic changes or developed any lesions after administration of ARELE at different doses. The absolute and relative organ weight of both male and female Wistar rats is given in Fig. 2. In male rats, the absolute weight of vital organs such as liver, kidney, pancreas, heart, lungs, and testis increased significantly in a dose dependant manner, whereas the weight of stomach significantly ($P < 0.01$) decreased in a dose dependant manner. The relative organ weight of male rats showed a significant increase of liver, kidney, pancreas, and heart, mainly in a dose dependant manner whereas non-significant increase in weight of lungs was observed in a dose dependant manner. Weight of stomach significantly ($P < 0.05$) decreased in a dose dependant manner, whereas non-significant decrease in weight of testis was observed when compared with the control. In female rats the absolute weight of liver, kidney, and pancreas increased non-significantly in a dose dependant manner, whereas heart and lungs showed significant weight increase in a dose dependant manner and the decrease in weight of ovary was both non-significant and non-dose dependant. The relative weight of liver, kidney, pancreas, and heart was almost similar to that of control and decrease in relative weight was observed for stomach and ovary which was significant ($P < 0.01$) and non-significant respectively in a dose dependant manner. All the statistical significance calculated was compared with the control. The results showed that administration of ARELE at different doses did not produce any remarkable toxic effect on the macroscopic analysis of vital organs and its absolute and relative organ weight.

Urinalysis

The details of the urine analysis of male and female Wistar rats have been given in the Additional file 1: Tables S1 and S2, respectively. In male rats, the results showed that administration of ARELE at high dose (500 mg/kg, b.wt.) produced minor increase in bilirubin, glucose, ketone bodies, and WBC content, whereas rats in the mid dose (250 mg/kg, b.wt.) also showed minor increase in ketone bodies, and proteins. Rats in the low dose (125 mg/kg, b.wt.) category did not produce any toxic effects. In female rats, minor increase in ketone bodies, protein, and WBC content was observed for all the three doses of ARELE. Minimal RBC content was found in the urine samples of rats treated with high dose (500 mg/kg,

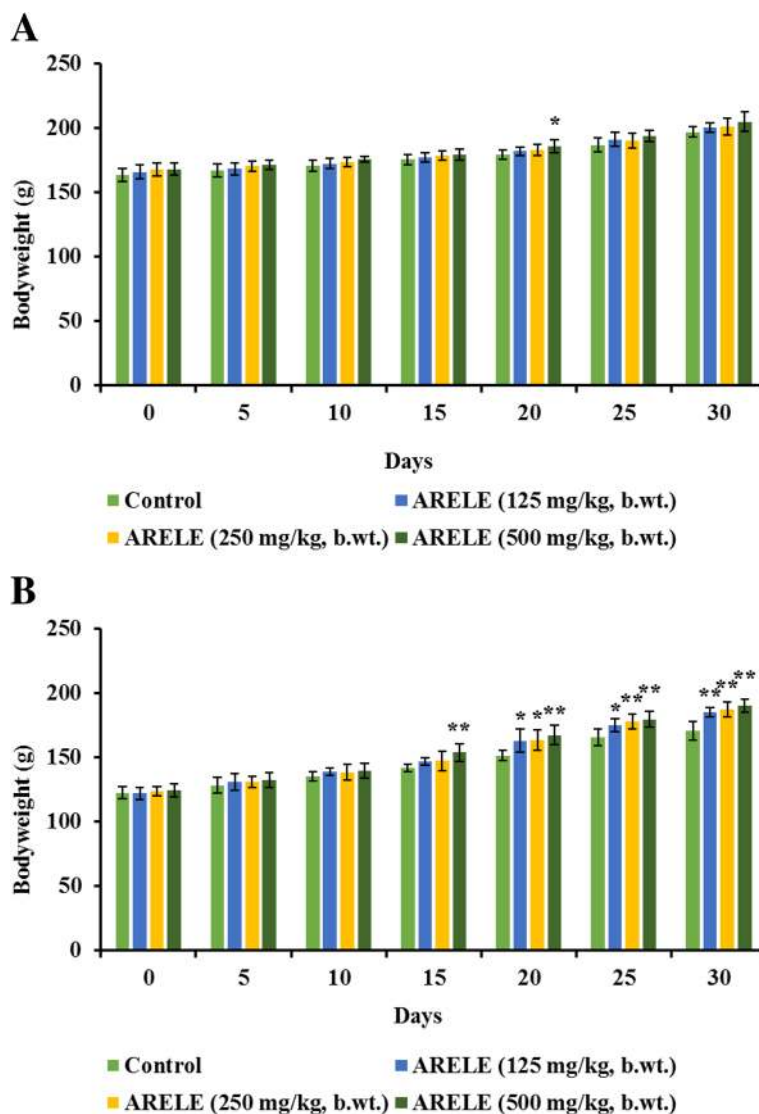


Fig. 1 Effects on body weight of rats after treatment with ARELE at different doses for a period of 30 days. **a** Male rats. **b** Female rats. Values are expressed as mean \pm S.D. ($n = 6$). Statistical analysis done by one-way ANOVA followed by Dunnett's t-test. ** $P < 0.01$ compared to control group. * $P < 0.05$ compared to control group

b.wt.) and mid dose (250 mg/kg, b.wt.). These findings were not considered toxicologically important as the changes were minimal when compared to the normal control group.

Haematological analysis

The effect of ARELE on haematological parameters of both male and female rats is tabulated in Table 2. Some significant changes were observed in the parameters of both male and female rats when compared with the control group. In male rats, increase in WBC count, RBC count, haematocrit (HCT), platelets (PLT), neutrophils and eosinophils were observed which are mainly non-significant and non-dose dependant. Decrease in

haemoglobin and monocytes content were observed except for ARELE at a low dose which showed an increase in haemoglobin when compared with the control. Non-significant and non-dose dependant minor alteration in the values of mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), and lymphocytes were observed in the male rats. In female rats, haemoglobin, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), neutrophils and eosinophils increased mostly in a non-significant manner, whereas WBC content decreased non-significantly along with platelets (PLT) and lymphocytes count which showed significant ($P < 0.01$) decrease in a dose

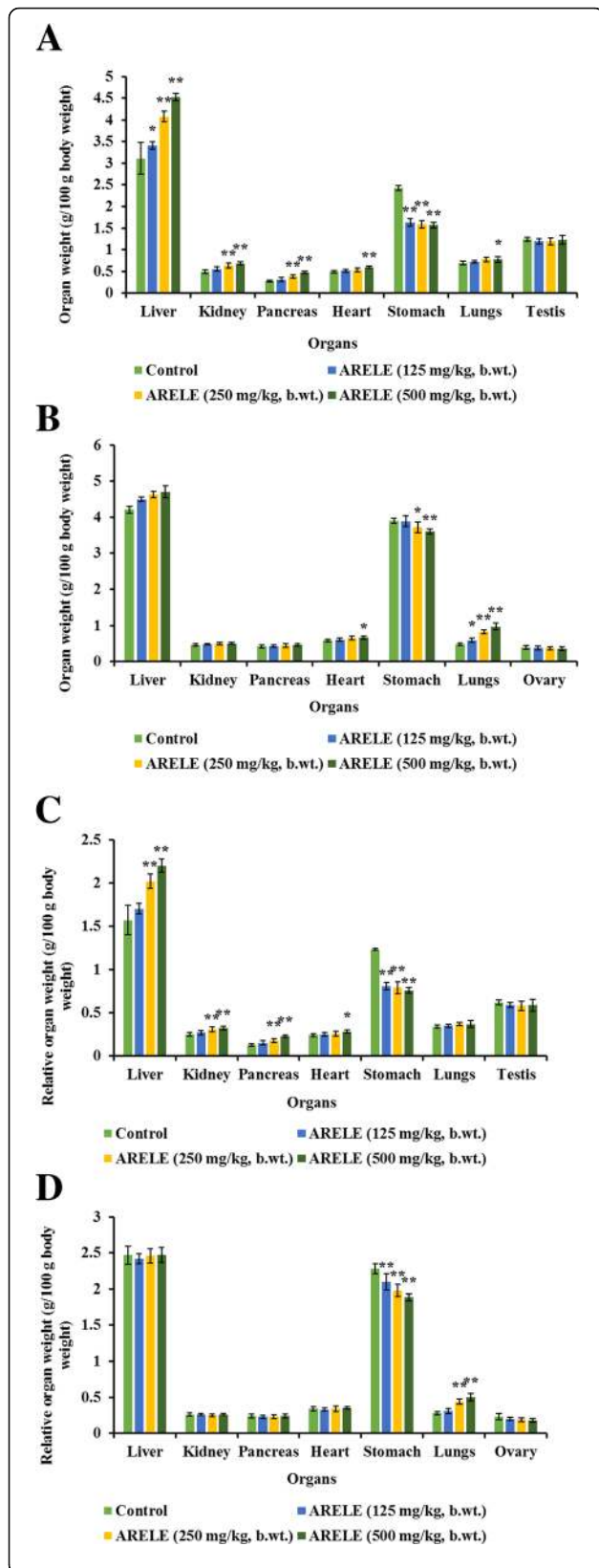


Fig. 2 Effects on organ weight of rats after treatment with ARELE at different doses for a period of 30 days. **a** Absolute organ weight of male rats. **b** Absolute organ weight of female rats. **c** Relative organ weight of male rats. **d** Relative organ weight of female rats. Values are expressed as mean \pm S.D. ($n = 6$). Statistical analysis done by one-way ANOVA followed by Dunnet's t-test. ****** $P < 0.01$ compared to control group. ***** $P < 0.05$ compared to control group

dependant manner when compared with the control. All the other parameters showed non-significant and non-dose dependant changes when compared with the control.

Biochemical analysis

Table 3 portrays the biochemical parameters of both male and female rats. The result in male rats showed a dose dependant increase in glucose and creatinine level which was significant in high dose. Triglycerides and cholesterol levels (Total cholesterol, HDL cholesterol, LDL cholesterol, and VLDL cholesterol) increased mostly significantly in a non-dose dependant manner when compared with the control. Urea and total protein decreased significantly in all doses whereas ALP and SGPT levels decreased significantly only in the high dose group. All the other biochemical parameters showed minor changes which are mostly non-significant and non-dose dependant. In female rats, a decrease in glucose, urea, albumin, SGPT, SGOT, and ALP was observed which were mainly significant when compared to control. Creatinine, bilirubin (both total and direct), triglyceride, and cholesterol levels (Total cholesterol, HDL cholesterol, LDL cholesterol, and VLDL cholesterol) increased mostly non-significantly in a non-dose dependant manner when compared with the control. The rest of the parameters showed minor fluctuations mostly non-significantly when compared with the control.

Histopathological studies

Histopathological analysis of the organ samples of liver, kidney, pancreas, heart, lungs, and stomach from both male and female rats whereas testis and ovary from male and female rats respectively were performed on the final day of the treatment period and the results are tabulated in Figs. 3 and 4. Multiple sections of the male liver showed normal hepatocytes along with normal portal triads, sinusoidal spaces and central venous system in extract treated group. Sections of female rat liver from the extract treated groups showed almost normal cellular architecture with occasional cholestasis. Liver sections of both male and female rats from the control group showed normal liver architecture. Multiple sections taken out from renal biopsy of both male and female rats of the extract treated groups showed almost

Table 2 Effects of ARELE on haematological parameters in both male and female rats treated for 30 days

| Sex | Parameter | Unit | Control | ARELE (mg/kg, b.wt.) | | |
|--------|--|----------------------------------|--------------|----------------------|----------------|----------------|
| | | | | 125 | 250 | 500 |
| Male | | | | | | |
| | Haemoglobin | gms/dL | 13.64 ± 0.53 | 14.45 ± 0.69 | 12.58 ± 1.62 | 11.87 ± 1.04* |
| | WBC | 10 ³ /μL | 8.89 ± 2.22 | 9.46 ± 0.95 | 11.86 ± 1.89* | 11.42 ± 1.94 |
| | RBC | 10 ⁶ /μL | 8.22 ± 0.45 | 8.31 ± 0.61 | 8.33 ± 0.41 | 8.765 ± 0.21 |
| | Haematocrit (HCT) | % | 38.33 ± 4.92 | 40.41 ± 4.17 | 45.83 ± 6.45* | 46.17 ± 4.15* |
| | Platelets (PLT) | 10 ⁵ /mm ³ | 7.87 ± 0.66 | 7.83 ± 0.21 | 8.033 ± 0.36 | 8.27 ± 0.49 |
| | Mean corpuscular volume (MCV) | fL | 52.46 ± 2.71 | 50.38 ± 2.80 | 50.28 ± 3.49 | 52.99 ± 2.53 |
| | Mean corpuscular haemoglobin (MCH) | pg | 17.73 ± 0.91 | 17.95 ± 0.88 | 17.36 ± 0.46 | 17.93 ± 0.74 |
| | Mean corpuscular hemoglobin concentration (MCHC) | % | 29.11 ± 1.81 | 29.01 ± 2.48 | 29.2 ± 2.87 | 26.4 ± 2.51 |
| | Differential leucocyte count | | | | | |
| | Neutrophils | % | 20.5 ± 6.92 | 18.67 ± 5.82 | 24.33 ± 5.82 | 23.83 ± 4.02 |
| | Lymphocytes | % | 73.67 ± 7.09 | 72.5 ± 6.09 | 74 ± 6.13 | 73.16 ± 3.97 |
| | Eosinophils | % | 1.5 ± 0.83 | 2.33 ± 1.03 | 4.67 ± 1.51** | 4.33 ± 0.81** |
| | Monocytes | % | 2.83 ± 1.33 | 2.5 ± 1.05 | 2.17 ± 1.17 | 2.66 ± 1.75 |
| Female | | | | | | |
| | Haemoglobin | gms/dL | 13.66 ± 0.86 | 13.41 ± 0.61 | 13.80 ± 0.71 | 14.13 ± 0.68 |
| | WBC | 10 ³ /μL | 8.19 ± 1.78 | 8.25 ± 1.13 | 7.90 ± 1.35 | 7.17 ± 1.18 |
| | RBC | 10 ⁶ /μL | 7.34 ± 0.70 | 7.39 ± 0.56 | 7.30 ± 0.51 | 7.99 ± 0.44 |
| | Haematocrit (HCT) | % | 41.51 ± 2.74 | 41.19 ± 2.72 | 39.96 ± 2.92 | 43.49 ± 1.76 |
| | Platelets (PLT) | 10 ⁵ /mm ³ | 7.54 ± 0.45 | 6.065 ± 0.52** | 4.34 ± 0.47** | 3.055 ± 0.35** |
| | Mean corpuscular volume (MCV) | fL | 53.18 ± 3.71 | 53.39 ± 3.57 | 54.16 ± 4.11 | 58.64 ± 2.79* |
| | Mean corpuscular haemoglobin (MCH) | pg | 18.08 ± 1.40 | 19.36 ± 1.17 | 18.51 ± 1.88 | 20.61 ± 1.31* |
| | Mean corpuscular hemoglobin concentration (MCHC) | % | 33.72 ± 2.11 | 32.21 ± 2.31 | 32.18 ± 1.70 | 34.15 ± 1.58 |
| | Differential leucocyte count | | | | | |
| | Neutrophils | % | 20.93 ± 4.33 | 32.51 ± 3.04** | 37.93 ± 5.43** | 51.21 ± 4.47** |
| | Lymphocytes | % | 71.82 ± 5.76 | 53.75 ± 5.62** | 49.5 ± 4.31** | 41.46 ± 4.79** |
| | Eosinophils | % | 1.64 ± 0.77 | 3.13 ± 1.15 | 2.65 ± 1.36 | 4.5 ± 0.77** |
| | Monocytes | % | 2.61 ± 0.55 | 2.50 ± 0.66 | 2.76 ± 0.30 | 2.57 ± 0.48 |

Values are expressed as mean ± S.D. (n = 6). Statistical analysis done by one-way ANOVA followed by Dunnet's t-test

**P < 0.01 compared to control group

*P < 0.05 compared to control group

normal size and shape of glomeruli, tubules, intestinum and blood vesicles. However, very mild haemorrhage was observed. There was no strong evidence of acute tubular necrosis and glomerular changes for the extract treated groups. The renal biopsy sections of the control group rats both male and female showed normal findings. Sections of both male and female rat's pancreas showed normal architecture in the control treated group, whereas in extract treated group almost negligible abnormalities were observed in the architecture of both pancreatic acini and islets. Sections of the heart taken from both male and female rats appeared normal in control treated as well as extract treated rats, however in male rats mild haemorrhage was observed. In both male

and female rats, multiple sections of lungs showed normal cellular architecture, alveoli, and lymphatic vessels in the control treated group. In the extract treated groups mild lymphocytic infiltrations were observed for both male and female rats. Sections of the stomach from both sexes of rats showed normal findings in the control treated groups. In the extract treated group, female rats showed normal cellular architecture with normal mucosa, submucosa, muscularis externa and serosa, whereas in male rats mild polyp formation or hyperplastic changes were observed. Sections of the reproductive organs i.e. testis for male and ovary for female showed normal pathology for both the control and extract treated groups.

Table 3 Effects of ARELE on biochemical parameters in both male and female rats treated for 30 days

| Sex | Parameter | Unit | Control | ARELE (mg/kg, b.wt.) | | |
|--------|-------------------|--------|----------------|----------------------|-----------------|-----------------|
| | | | | 125 | 250 | 500 |
| Male | | | | | | |
| | Glucose | mg/dL | 73.82 ± 5.11 | 75.18 ± 4.22 | 75.59 ± 4.24 | 80.99 ± 1.46* |
| | Creatinine | mg/dL | 0.48 ± 0.14 | 0.65 ± 0.11 | 0.70 ± 0.20 | 0.88 ± 0.14** |
| | Urea | mg/dL | 34.78 ± 2.88 | 29.83 ± 2.64* | 28.68 ± 2.51** | 28.09 ± 2.94** |
| | Sodium | mmol/L | 146.97 ± 14.61 | 145.73 ± 12.90 | 144.10 ± 8.38 | 147.23 ± 5.11 |
| | Potassium | mmol/L | 6.27 ± 1.85 | 6.56 ± 1.17 | 6.10 ± 1.18 | 5.64 ± 0.76 |
| | Chloride | mmol/L | 110.82 ± 8.53 | 106.83 ± 5.14 | 111.04 ± 6.11 | 109.69 ± 9.50 |
| | Total protein | g/dL | 6.52 ± 0.41 | 6.01 ± 0.20** | 5.90 ± 0.16** | 5.92 ± 0.16** |
| | Albumin | g/dL | 3.51 ± 0.31 | 3.29 ± 0.35 | 3.54 ± 0.29 | 3.76 ± 0.21 |
| | Globulin | mg/dL | 2.86 ± 0.20 | 2.64 ± 0.42 | 2.87 ± 0.58 | 2.43 ± 0.32 |
| | Bilirubin | | | | | |
| | Bilirubin (T) | mg/dL | 0.76 ± 0.20 | 0.79 ± 0.23 | 0.79 ± 0.15 | 0.75 ± 0.12 |
| | Bilirubin (D) | mg/dL | 0.08 ± 0.02 | 0.103 ± 0.02 | 0.106 ± 0.02 | 0.111 ± 0.04 |
| | SGPT | IU/L | 33.10 ± 5.62 | 32.04 ± 5.21 | 29.06 ± 2.96 | 26.94 ± 4.00 |
| | SGOT | IU/L | 63.95 ± 7.76 | 65.11 ± 4.39 | 59.79 ± 5.99 | 48.51 ± 3.48** |
| | ALP | IU/L | 145.94 ± 11.13 | 143.51 ± 7.16 | 136.51 ± 11.82 | 122.36 ± 6.88** |
| | Triglyceride (TG) | mg/dL | 80.40 ± 10.17 | 94.34 ± 6.97* | 99.20 ± 6.94** | 98.001 ± 5.20** |
| | Cholesterol | | | | | |
| | Total cholesterol | mg/dL | 64.5 ± 5.90 | 108.64 ± 8.44** | 128.3 ± 6.74** | 135.33 ± 6.58** |
| | HDL cholesterol | mg/dL | 31.40 ± 2.39 | 35.86 ± 3.91* | 37.49 ± 3.18** | 38.77 ± 2.06** |
| | LDL cholesterol | mg/dL | 31.59 ± 2.86 | 30.27 ± 1.66 | 31.18 ± 2.05 | 32.69 ± 2.44 |
| | VLDL cholesterol | mg/dL | 15.45 ± 1.49 | 15.75 ± 2.13 | 18.12 ± 1.19* | 20.99 ± 1.38** |
| Female | | | | | | |
| | Glucose | mg/dL | 86.59 ± 7.01 | 80.88 ± 3.81 | 76.23 ± 5.81* | 72.68 ± 7.03** |
| | Creatinine | mg/dL | 0.61 ± 0.19 | 0.78 ± 0.12 | 0.7 ± 0.12 | 0.86 ± 0.06** |
| | Urea | mg/dL | 41.40 ± 7.75 | 28.75 ± 3.00** | 30.45 ± 2.21** | 26.99 ± 3.58** |
| | Sodium | mmol/L | 142.006 ± 4.80 | 140.15 ± 1.51 | 140.05 ± 3.81 | 141.07 ± 2.56 |
| | Potassium | mmol/L | 6.39 ± 1.49 | 6.46 ± 0.51 | 6.27 ± 0.41 | 6.07 ± 0.23 |
| | Chloride | mmol/L | 104.57 ± 6.76 | 107.09 ± 4.80 | 105.96 ± 5.62 | 106.03 ± 5.83 |
| | Total protein | g/dL | 6.30 ± 0.45 | 6.11 ± 0.41 | 6.13 ± 0.39 | 5.96 ± 0.21 |
| | Albumin | g/dL | 3.93 ± 0.39 | 3.81 ± 0.19 | 3.77 ± 0.29 | 3.73 ± 0.16 |
| | Globulin | mg/dL | 3.05 ± 0.39 | 2.97 ± 0.21 | 3.17 ± 0.21 | 2.50 ± 0.35* |
| | Bilirubin | | | | | |
| | Bilirubin (T) | mg/dL | 0.58 ± 0.13 | 0.67 ± 0.12 | 0.69 ± 0.15 | 0.84 ± 0.08** |
| | Bilirubin (D) | mg/dL | 0.14 ± 0.04 | 0.15 ± 0.04 | 0.17 ± 0.04 | 0.16 ± 0.04 |
| | SGPT | IU/L | 51.60 ± 8.66 | 40.92 ± 5.63* | 40.80 ± 5.88* | 37.16 ± 6.29** |
| | SGOT | IU/L | 43.92 ± 5.92 | 42.29 ± 3.61 | 38.22 ± 2.76 | 33.39 ± 3.57** |
| | ALP | IU/L | 129.07 ± 10.45 | 125.25 ± 5.09 | 120.77 ± 4.41 | 119.81 ± 6.26 |
| | Triglyceride (TG) | mg/dL | 73.27 ± 8.03 | 94.09 ± 9.05** | 104.02 ± 9.35** | 112.60 ± 9.99** |
| | Cholesterol | | | | | |
| | Total cholesterol | mg/dL | 91.12 ± 8.67 | 105.89 ± 5.06** | 115.15 ± 6.46** | 114.02 ± 5.75** |
| | HDL cholesterol | mg/dL | 35.65 ± 4.54 | 36.33 ± 2.88 | 36.25 ± 2.87 | 40.72 ± 1.58* |
| | LDL cholesterol | mg/dL | 41.42 ± 4.46 | 43.53 ± 3.76 | 44.47 ± 1.84 | 46.11 ± 5.16 |
| | VLDL cholesterol | mg/dL | 17.11 ± 2.54 | 18.03 ± 2.07 | 20.94 ± 3.04* | 21.63 ± 2.40* |

Values are expressed as mean ± S.D. (n = 6). Statistical analysis done by one-way ANOVA followed by Dunnet's t-test

**P < 0.01 compared to control group

*P < 0.05 compared to control group

Neurotoxicological evaluation

Functional observational battery (FOB) The result of the FOB has been given in the Additional file 1: Tables S4 and S5. In both male and female rats, no abnormalities in home cage observations like posture, presence or absence of tremors and convulsions, spontaneous vocalization and biting were noted for both the control and the extract treated group at different doses. There were also no abnormalities recorded for hand held observations such as lacrimation, salivation, piloerection, and handling activity for rats of both sexes in ARELE treated groups when compared with the control groups. In the open field test, rat's arousal activity, gait, mobility, and stereotype activities were found to be normal and very similar in both treated groups and normal control groups. Responses to various stimuli in the extract treated group were almost normal without any neurotoxicological findings in the extract treated groups when compared with the normal treated groups. After the end of the open field observations, the number of faecal boluses in treated group rats was similar to that of the control treated groups. There were also no signs of diarrhoea found in both treated and control groups.

Locomotor activity The locomotor activity of ARELE is shown in Fig. 5. After the administration of ARELE at doses of 125, 250 and 500 mg/kg, b.wt., to both male and female rats, there was a significant ($P < 0.01$) reduction in the actophotometer reading (no. of photocells counted) after 30 min and 60 min when compared to the control group on the initial day of treatment period. On the final week of the treatment period after the administration of ARELE at different doses to both male and female rats, the actophotometer reading (no. of photocells counted) was significantly reduced ($P < 0.01$) after 30 min and 60 min when compared to the control group. The reduction of photocells count was more on the initial day of the treatment period and after 60 min for all the treated rats (male and female). Maximum reduction in spontaneous motor activity was observed after 60 min in male rats at the dose of 500 mg/kg, b.wt., on initial day of the treatment. The reduction of photocells count was mostly dose dependant in both sexes of rats except for the male rats treated with ARELE at the dose of 250 mg/kg, b.wt., on the initial day.

Discussion

Knowledge, beliefs, and practices which are not based on scientific evidence for treating various illness within a society is termed as traditional or folk medicines, and further when modern societies adopt or practices such long standing traditional concept of medicines these practices are called complementary, non-conventional or alternative medicines [25]. Herbal medicines as well as

their derivatives have been used as an alternative to allopathic medicines in many countries for the treatment of various diseases. Despite the widespread use of herbal medicine, there have been very few scientific studies conducted on herbals to provide knowledge about their efficacy and safety [26]. Plant products can also produce toxic effects and thus to ensure its safety, systematic studies regarding its toxic effects needs to be evaluated as a result providing scientific information for selecting safe doses for animals including humans [27]. According to the current literature the toxicological effects of *A. rotundifolia* has not been studied at all. In fact, there are very few scientific reports of toxicological studies conducted on mangrove plants which are extensively used by local healers of mangroves for treatment of various diseases and such folkloric uses of these mangrove plants without knowing their toxic effects scientifically could be harmful in long term. Hence, the present research will provide the first information report of any toxic effects of this scientifically unexplored mangrove plant which is traditionally used by the local healers for curing pain. Its strong pain-relieving activities has also been studied previously and scientifically proved by Raju et al. [10].

The preliminary phytochemical test of ARELE was conducted and the result revealed the presence of alkaloids, carbohydrates, tannins and phenolic compounds, steroids and sterols, triterpenoids, saponins and flavonoids. Thus, the preliminary phytochemical test helps in determining the class of chemical compound present in the extracts which may lead to their quantitative estimation and also identifying the source of pharmacologically active phyto-constituents [28].

Acute toxicity is considered an initial study which provides us the basis for classification and labelling. It also provides initial information about the mode of toxic action of a substance by which we can fix a dose of a new compound and help in dose determination in animal studies [29, 30]. In our study a single administration of extract with increasing doses did not produce any mortality or any serious abnormalities at all doses in acute toxicity, however, the mild sedative effect was noticed in both male and female rats at a single dose of 2000 mg/kg, b.wt. The limit test dose for the study was 2000 mg/kg, b.wt. which did not show any severe toxic effects and mortality, thus 1/4th, 1/8th, and 1/16th of the limit test dose was chosen as the high dose (500 mg/kg, b.wt), mid dose (250 mg/kg, b.wt) and low dose (125 mg/kg, b.wt), respectively for conducting sub-acute toxicity studies.

General behavioural changes and body weight are preliminary indicators of early signs of toxicity caused by various chemicals and drugs [31]. Sub-acute ingestion of ARELE produced no mortality in rats and produced very minimal behavioural changes in both male and female

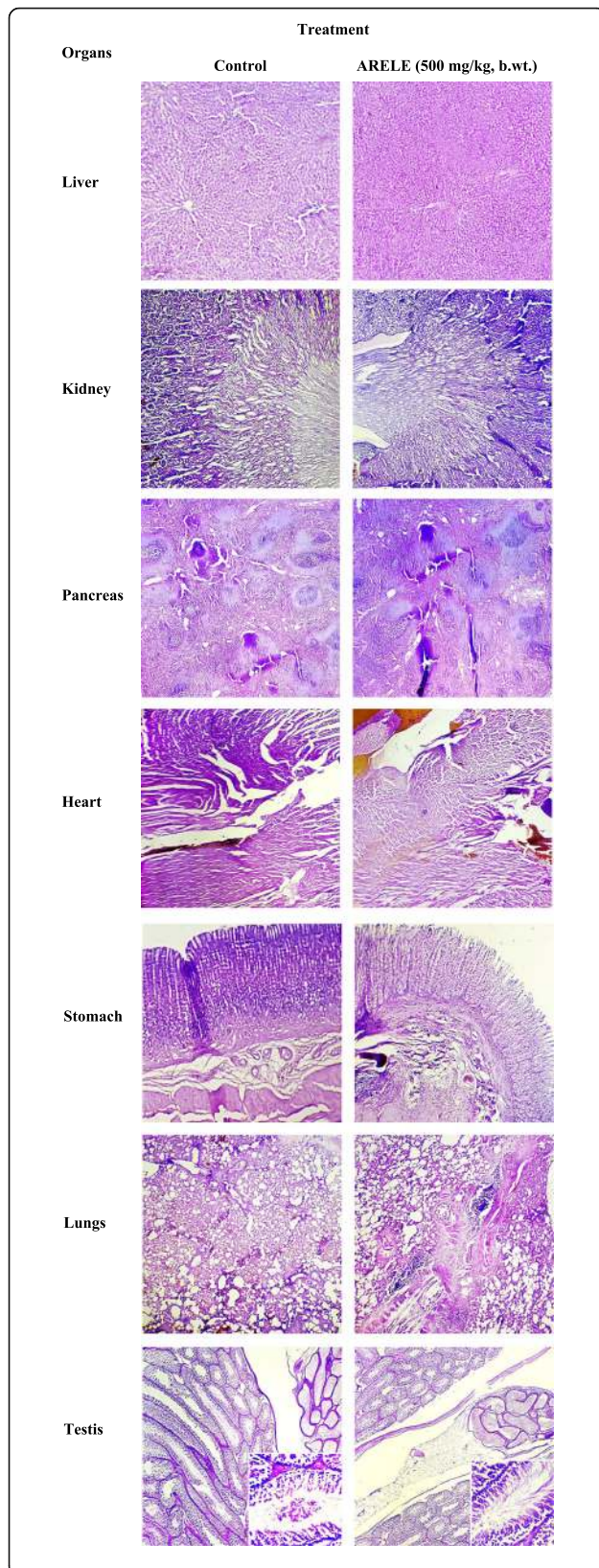


Fig. 3 Histopathological study of vital organs from male rats. Photomicrographs of histological findings of different organs from male rats treated with normal control vehicle and ARELE at high dose (500 mg/kg, b.wt.) for a period of 30 days (H&E staining, 40 × magnification, magnified parts in testis = 100 × magnification)

rats. The body weight increase for both sexes of rats was mostly significant when compared with the control group and was considered normal. Thus, it can be concluded that ARELE oral administration did not produce any major clinical signs and did not affect the normal growth pattern of the rats throughout the treatment period of 30 days.

The haematopoietic system is one of the most sensitive targets for toxic compounds and an important index of physiological and pathological status in human and animals [32]. Evaluation of haematological parameters would be helpful in determining the toxic effects of the extract on animal's blood and thus can be used to explain blood relevant function of extracts [33]. The haematological profile after treatment with extracts showed values which were mainly non-significant when compared with the control and falls within the normal range for both male and female rats. In male rats, when compared with the control group, minor increase in WBC count, RBC count, haematocrit (HCT), platelets (PLT), neutrophils and eosinophils were recorded, whereas decrease in haemoglobin and monocytes content were observed. In female rats, increase in haemoglobin, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), neutrophils and eosinophils were observed, whereas WBC content, platelets (PLT) and lymphocytes count decreased when compared with the control. Thus, ARELE administration at all doses did not produce any major alterations in the haematological profile of both sexes of rats, however few changes which were observed does not carry any toxicological importance and therefore the extract at all the tested doses can be considered safe to the haematological profile. Clinical biochemistry analysis was conducted to investigate any possible influence of the extract on hepatic and renal functions of rats. The biochemical parameters are considered an important marker for toxicity evaluation as both liver and kidney are necessary for the survival of an organism [34]. The liver is a major organ which deals with drug metabolism and elimination and levels of markers such as TP, ALB, SGOT, SGPT, and ALP etc., can help determine whether the drug is hepatotoxic or hepatoprotective in nature. Bilirubin which is produced after breaking down of haemoglobin is also an indicator of liver toxicity and is associated with biliary cirrhosis and hepatic cholestasis [35]. The study revealed that the biochemical parameters of liver showed very little variation mostly non-significant when compared to the

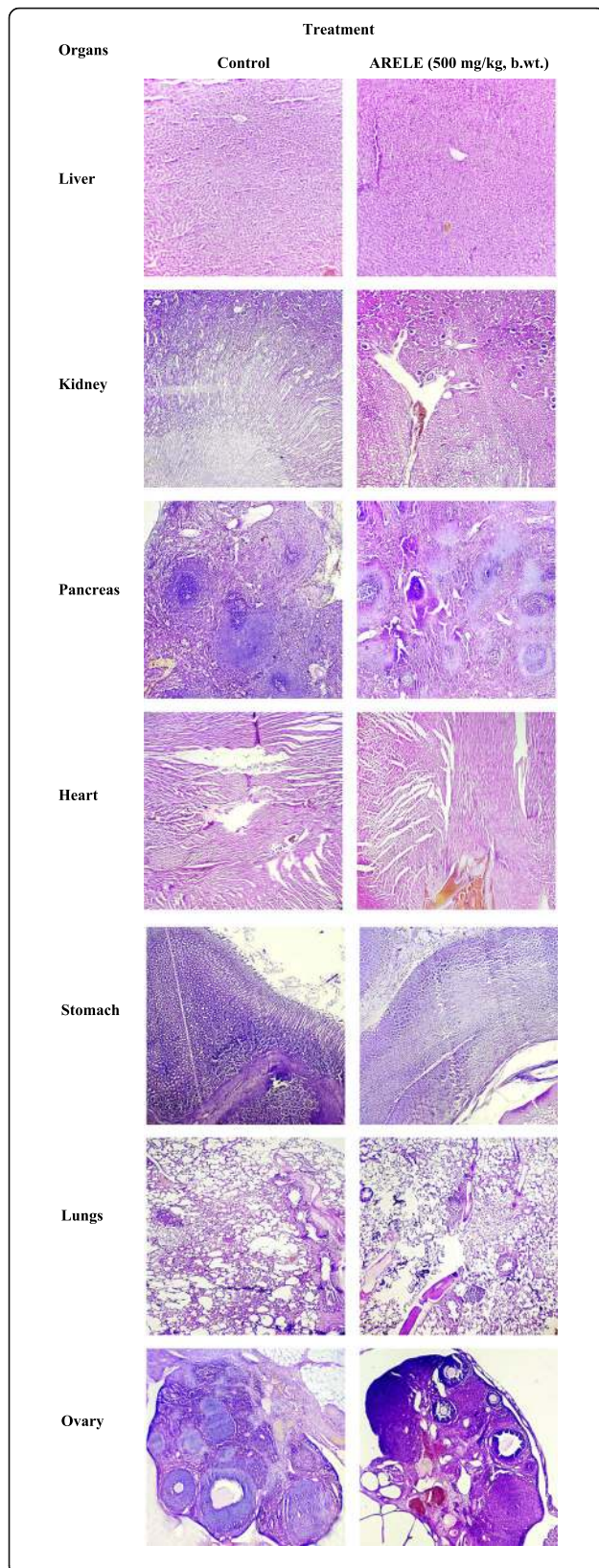


Fig. 4 Histopathological study of vital organs from female rats. Photomicrographs of histological findings of different organs from female rats treated with normal control vehicle and ARELE at high dose (500 mg/kg, b.wt.) for a period of 30 days (H&E staining, 40 × magnification)

control. However, there was a slight increase in the levels of triglyceride and cholesterol levels mostly non-significantly for both male and female rats which might indicate that the extract might induce obesity. Increased level of bilirubin was observed only in female rats when compared with the control. Most of the other hepatic parameters showed minor non-significant changes in the extract treated groups compared to control indicating the extract has no serious toxic effects on hepatic functions. This indicates that the sub-acute administration of ARELE is not able to produce any major toxic effects on the haematological and biochemical profile of rats.

The kidney is known to be hugely susceptible to damage caused by various toxic elements as large volume of blood flows through it and the toxins filtered usually gets concentrated in the kidney tubules [36]. High protein content in urea known as Proteinuria is a urine marker which is a common indicator for various kidney pathologies and dysfunction [37]. In our study, the urine analysis revealed minor increase in bilirubin, glucose, ketone bodies, and WBC content in high dose group for male rats, whereas minor increase in ketone bodies, Protein, and WBC content was observed for female rats. These findings showed minimal changes and thus can be considered toxicologically less important.

Gross morphology was studied to determine the presence of any visible lesion on vital organs such as liver, kidney, pancreas, heart, lungs, and stomach from both male and female rats whereas testis and ovary from male and female rats respectively. The result showed that no lesions or any abnormalities were found on any vital organs from both male and female rats.

Relative organ weight is a reliable indicator which can be used to measure the degree of toxicity of any drugs or chemicals [38]. Our study demonstrated that in male rats, after administration of extract at different doses all organs increased mostly significantly except stomach which showed a decrease in weight, whereas in female rats only stomach and ovary showed decrease in their weight. These findings were considered toxically irrelevant as the alterations were negligible and thus it can be concluded that the administration of ARELE at different doses did not cause any serious complications to the vital organs.

Histopathological slides provide a more in-depth study of any toxic effects or diseases by investigating its effects on tissues as the preparation process preserves the tissue architecture [39]. In histopathological studies, no major

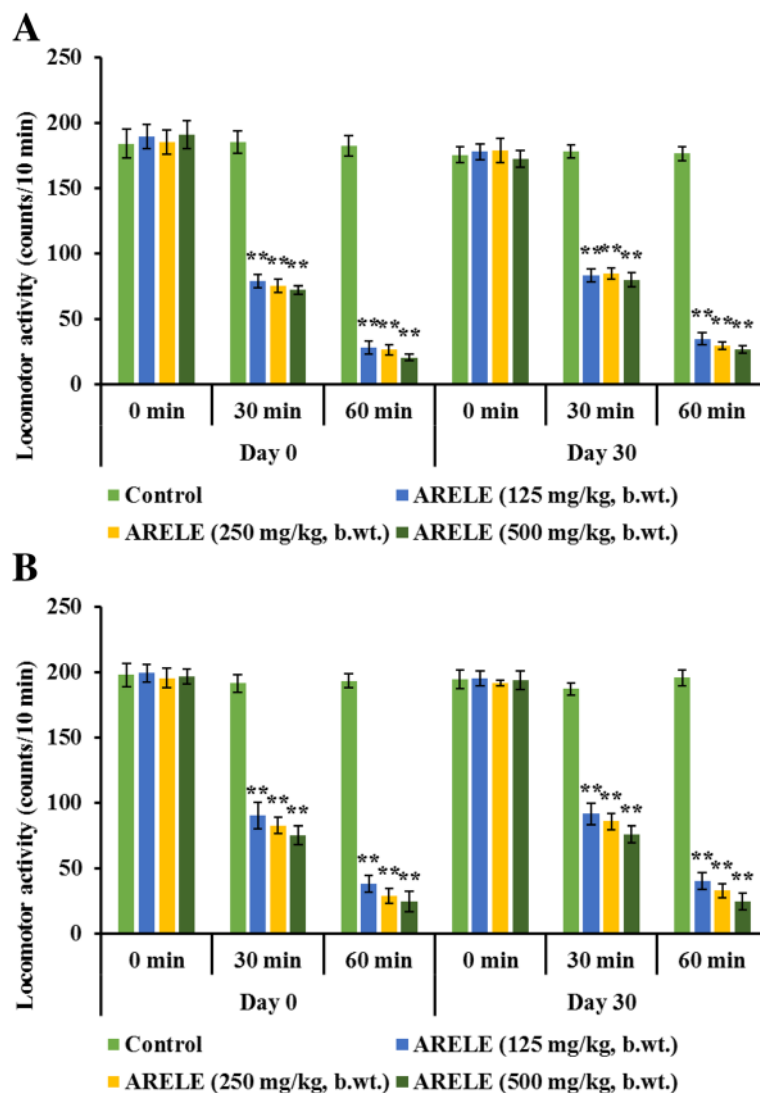


Fig. 5 Locomotor activity of ARELE using Actophotometer method. **a** Male rats. **b** Female rats. Values are expressed as mean \pm S.D. ($n = 6$). Statistical analysis done by one-way ANOVA followed by Dunnett's t-test. ****** $P < 0.01$ compared to control group. ***** $P < 0.05$ compared to control group

alteration in vital organs (liver, kidney, pancreas, heart, lungs, and stomach) and reproductive organs (Ovary and testes) were found. However minor cholestasis, haemorrhage and lymphocytic infiltrations were spotted in liver, kidney and lungs respectively in female rats. In male rats' mild haemorrhage was found in kidney and heart, whereas mild lymphocytic infiltrations and mild hyperplastic changes were found on lungs and stomach respectively. The minor changes observed in the few above mentioned organs could indicate that the extract might be slightly toxic to those organs.

Assessment of neurobehavioral toxicity can be done by the functional observational battery (FOB) describing various behavioural and activity related parameters of a rodent. This tool is a non-invasive procedure developed

to detect gross functional deficits in young adult rats after they have been exposed to drugs or chemicals. Detailed evaluation of neurotoxicity is not provided by this battery of tests but can be used in conjunction with neuropathologic evaluation and/or general toxicity testing [40, 41]. In our FOB tests, no major abnormalities were found in the extract treated rats when compared with the control. Locomotor activity is considered an index of wakefulness and alertness of mental activity. Most of the centrally acting analgesics influence the locomotor activity by reducing the motor activity because of their CNS depressant properties [42]. The decrease in locomotor activity gives an indication of the level of excitability of the central nervous system and this decrease may be due to the sedative effect resulting

from depression in the central nervous system [43]. Our study showed that significant ($P < 0.01$) reduction in locomotor activity when compared to control was found after drug administration which indicates that the test extract might possess sedative properties.

Conclusions

Results from this study provides important information and data on toxicological properties of a rare and scientifically unexplored mangrove plant *Aegialitis rotundifolia* Roxb., leaves, which is traditionally used as a potent pain reliever by the local healers. In the evaluation of acute toxicity, oral administration of ARELE at a dose up to 2000 mg/kg b.wt., did not produce any major toxicological effects except for mild short-term sedation after administration of the limit test dose of 2000 mg/kg b.wt. In the sub-acute study, no severe treatment-related toxicity was observed during the study after the rats were administered with ARELE up to a dose of 500 mg/kg b.wt., for a period of 30 days. Almost all the parameters were normal without any major changes, however minor alterations in few parameters were observed which may or may not be treatment related thus carrying little or no toxicological importance. However, further study is required to investigate and confirm its safety and effectiveness in humans.

Additional file

Additional file 1: Table S1. Urine analysis of male rats treated with ARELE at different doses for a period of 30 days. **Table S2.** Urine analysis of female rats treated with ARELE at different doses for a period of 30 days. **Table S3.** Functional Observational Battery (FOB) scores for neurotoxicological investigation. **Table S4.** Effects of ARELE on behavioural endpoints of the FOB on initial week (Day 0). **Table S5.** Effects of ARELE on behavioural endpoints of the FOB on final week (Day 30). (DOCX 37 kb)

Abbreviations

ALP: Alkaline phosphatase; ANOVA: Analysis of variance; ARELE: *Aegialitis rotundifolia* Roxb., ethanolic leaves extract; EDTA: Ethylene di-aminetetraacetic acid; FOB: Functional observational battery; GC-MS: Gas chromatography-mass spectroscopy; HCT: Haematocrit; HDL: High-density lipoproteins; HPLC: High performance liquid chromatography; LDL: Low-density lipoproteins; MCH: Mean corpuscular haemoglobin; MCHC: Mean corpuscular haemoglobin concentration; MCV: Mean corpuscular volume; OECD: Organization for economic co-operation and development; PLT: Platelets; RBC: Red blood cell; ROW: Relative organ weight; SGOT: Serum glutamic oxaloacetic transaminase; SGPT: Serum glutamic pyruvic transaminase; TG: Triglyceride; VLDL: Very low-density lipoproteins; WBC: White blood cell; WHO: World Health Organization

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Availability of data and materials

All relevant data are given in the manuscript.

Authors' contributions

DG, SM and KR conceived and designed the experiments. DG and SM performed the experiments. DG did the literature survey, calculated and analysed the data and prepared the manuscript. SM and KR checked and analysed the data and edited the manuscript. All authors revised and approved the final manuscript. This research work has been performed in collaboration between all authors.

Ethics approval

All experimental protocols were approved by the Institutional Animal Ethics Committee (IAEC) of GITAM Institute of Pharmacy, Visakhapatnam, Andhra Pradesh, India (CPCSEA regd. no.: 1287/PO/Re/S/09/CPCSEA and Approved proposal no.: IAEC/GIP-1287/DB-S/Approved/11/2017–18). Experiments were performed according to the guide for the care and use of laboratory animals.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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