Acute and sub-chronic toxicity study of Rang Chuet (Thunbergia laurifolia Lindl.) extracts and its antioxidant activities

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ARTICLE INFO
Handling Editor: Dr. L.H. Lash

Keywords:
Rang Chuet extracts
Acute toxicity
Sub-chronic toxicity
Wistar rats

ABSTRACT
Rang Chuet (Thunbergia laurifolia Lindl.) is a Thai medicinal plant with pharmacological properties: it can be used as an antidote, for decreasing body temperature and it is addictive. This study investigated total phenolic contents and antioxidant activities of Rang Chuet extracts, and its acute and sub-chronic toxicities of Rang Chuet extracts. To investigate the acute toxicity of Rang Chuet, male and female Wistar rats were orally gavaged with a single dose of 2000 and 15,000 mg/kg body weight of Rang Chuet leaf extracts prepared by three different extraction solvents, namely water, ethanol, and acetone. The Rang Chuet water extract showed the highest total phenolic contents at 2643 ± 195.05 mg GAE/100 g while the Rang Chuet acetone extract showed the lowest IC50 at 52.91 mg/ml by DPPH assay. The sub-chronic toxicity study was performed using Wistar rats of both sexes which were gavaged with ethanol and water Rang Chuet extracts for 90 days. Rats were gavaged with the equivalent dose of Rang Chuet in a typical consumer drink (to be taken 3 times a day, at dose 1460 mg/kg/day for water extract and 1025 mg/kg/day for ethanol extract), 3000 and 5000 mg/kg. The satellite group was given the same dose of both extracts for 90 days and observed thereafter for 14 days in order to study the reversibility of the adverse effects. The results revealed that none of the Rang Chuet extracts altered the general behavior or mortality or changes in the gross morphology and any histology of the rats’ visceral organs. For sub-chronic toxicity, the result showed that the treatment of ethanol and water Rang Chuet extracts had no significant effect on average body weight, relative organ weights, histopathology of organs, clinical biochemistry, hematological parameters or liver enzymes. This analysis of by-products of a lipid peroxidation study suggested a trend of decreasing malondialdehyde levels in most of the Rang Chuet treated groups. In conclusion, the safety value of Rang Chuet water extract and Rang Chuet ethanol extract in rats is 50 mg/kg body weight which indicate safe dose of Rang Chuet dried powder is 10.27 g/60 kg body weight per day.

1. Introduction
At present, more herbs are being used for health prevention and the treatment of disease instead of synthetic chemical drugs in order to reduce the toxicity of chemicals that may affect the health and the environment. However, we do not have toxicity data for many herbs so it is not known what health risks consumers may be taking. There have been many studies of the toxicity of certain medicinal plants which allow consumers to reduce the risks of using some herbs. Thunbergia laurifolia Lindl., which is known in Thai as Rang Chuet (RC), has been used in Thailand as a natural remedy for centuries. The plant has been reported to contain flavonoids such as apigenin, cosmosdin, delphinidin-3-5-di-O-β-D-glucoside and chlorogenic acid [2]. It has also been reported that extracts of RC leaves have a protective effect on ethanol-induced hepatotoxicity using hepatic lipid peroxidation, blood ethanol concentration as well as hepatic alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) as indicators [3]. The most interesting phytochemical compounds of RC are chlorophyll and phenolic acid [4], which are well-known antioxidants. More recently, in

https://doi.org/10.1016/j.toxrep.2022.11.002
Received 5 August 2022; Received in revised form 19 October 2022; Accepted 5 November 2022
Available online 9 November 2022
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vitro and animal studies have shown that chlorophyll derivatives including chlorophyll a, pheophytin a, and pheophorbide a are potential chemopreventive agents in *Thunbergia laurifolia* Lindl. Family Acanthaceae (Thai name: Rang Chuet). This is a Thai herbal medicinal plant used as an antidote, and for anti-inflammatory and antipyretic treatments. Rang Chuet contains flavonoids such as apigenin, cosmosdin, delphinidin-3-5-di-O-β-D-glucoside and chlorogenic acid. The extract of water leaves contains eight steroids and β-carotene [2]. The extract ethanol and acetone leaves contain chlorophyll a, chlorophyll b, pheophorbide a, pheophytin a and lutein [4]. The pharmacological activity and toxicity of *Thunbergia laurifolia* Lindl. extract using water, ethanol, and acetone have not been reported. This study aimed to evaluate the safety of the water, ethanol, and acetone extract from *Thunbergia laurifolia* Lindl. in rats.

2. Materials and methods

2.1. Plant materials

The medicinal plant *Thunbergia laurifolia* Lindl. (Acanthaceae) was collected from June to December from a local area in Nakhon Ratchasima province in Thailand. Leaves were air dried at 60°C for 6 h, after which they were ground in a blender (Mitsubishi, MX-T1PW, Thailand) to fine powder and stored in a vacuum package at 4°C until use.

2.2. Preparation of Rang Chuet extracts

Rang Chuet extracts were obtained by using different solvents including water (RCW), ethanol (RCE) and acetone (RCA) extracts [5]. Approximately 100 mg of leaf powder was extracted with three 12 ml portions of boiling water, ethanol, or acetone in a shaking water bath at 25°C for 15 min. Centrifugation at 3000 g (Hettich, universal 16R, USA) was applied for 3 min between extractions. The filtrates obtained from vacuum filtration were combined and the volume was adjusted to 50 ml. The water extract was dried in a freeze dryer (GEA, LYOVAC GT2-S, MD) for 48 h but ethanol and acetone extracts were dried in Therbo vap (Caliper, LV, USA). Samples were stored and frozen at −20°C until use.

2.3. Total phenolic

The total phenolic contents of the sample were investigated by the folin ciocalteu procedure [6] and gallic acid (Sigma Aldrich Co.) was used as the standard. Aliquots (0.02 ml) of gallic acid, samples and blanks were transferred into a test tube. After the addition of 0.1 ml folin-ciocalteu reagent, the solution was mixed and allowed to stand for 5 min. Then 0.3 ml of 20 % (w/v) Na₂CO₃ was added and the tubes were shaken and stored in the absence of light for 120 min at room temperature and then compared to the gallic acid standard. Absorbance was measured at 765 nm using a spectrophotometer (Biochrom, Libra S22 S/N 97765, UK). The results were expressed as gallic acid equivalents.

2.4. Antioxidant activities

2.4.1. 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

The DPPH free radical scavenging activity of RC extracts (acetone, ethanol and water), BHT and ascorbic acid were determined using a DU 800 Spectrophotometer (Beckman Coulter, CA) [6], in terms of hydrogen donating or radical scavenging ability. Briefly, 0.1 mm solution of DPPH in methanol was prepared. The initial absorbance of the DPPH in methanol was measured at 515 nm and did not change throughout the period of assay. An aliquot (100 μl) of an extract diluted at a concentration range of 0.01–0.15 mgGAE/ml was mixed with 1.9 ml of methanol DPPH solution. The change in absorbance at 515 nm was measured at 15 min. The percentage of scavenging was calculated as the ratio of the absorption of the sample relative to the control DPPH solution without the extracts. The BHT, ascorbic acid, and trolox in MeOH solution were used as positive controls. The EC₅₀ of the extracts was calculated at the 15 min scavenging value using nonlinear regression of Sigma Plot 9.1 (Systat Software Inc, Illinois). Inhibition of free radical DPPH (%) was calculated according to the formula:

\[
\text{Inhibition} = \left(\frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}}\right) \times 100
\]

where A blank is the absorbance of the control reaction (containing all reagents except the test compound) and A sample is the absorbance of the tested compound. The exact concentration providing 50 % inhibition (IC₅₀) was calculated from a graph plotted with the percentage of inhibition against the extract concentration. Tests were carried out in triplicate. The synthetic antioxidant BHT and ascorbic acid were included in experiments as positive controls [6].

2.4.2. Ferric-reducing antioxidant power (FRAP) assay

This method [6] was utilized. Briefly, the FRAP reagent was prepared from an acetate buffer (pH 3.6), 10 mmol TPTZ solution in 40 mmol HCl and 20 mmol ferrous chloride solution in the proportion of 10:1:1 (v/v), respectively. The FRAP reagent was fresh and prepared daily and was warmed to 37°C in a water bath prior to use. The extract of 50 μl was added to 1.5 ml of the FRAP reagent. The absorbance of the reaction mixture was then recorded at 593 nm after 4 min. A standard curve was constructed using ferric sulfate solution (100–2000 μmol) and the results were expressed as μmol equivalents of ferric per g dry weight of plant materials. All measurements were taken in triplicate and the mean values were calculated.

2.4.3. Scavenging activity of ABTS radical cation

The ABTS radical scavenging activity of extracts [6]. Next, the ABTS + cation radical was produced by mixing 5 ml of 14 mM ABTS solution and 5 ml of 4.9 mM potassium persulfate (K₂S₂O₈) solution, which was stored in the dark at room temperature for 12–16 h. Before use, this solution was diluted with ethanol until it reached an absorbance of 0.700 ± 0.020 at 734 nm. 100 μl of the crude extracts, standards or blanks were added in 2 ml test tubes, after that 1900 μl of ABTS + solution were added then the mixture was thoroughly mixed and incubated for 6 min at room temperature. Absorbance was measured at 734 nm. For the antiradical activity, the ABTS scavenging ability was expressed as IC₅₀ (mg/ml). The inhibition percentage of the ABTS radical was calculated using the following formula:

\[
\text{ABTS scavenging effect} = \left(\frac{A_0 - A_1}{A_0}\right) \times 100
\]

where A₀ and A₁ are the absorbencies of the control and test sample, respectively.

2.5. Animals

Healthy male and female Wistar rats were purchased from the Experimental Animal Department, Suranaree University of Technology, Nakhon Ratchasima province in Thailand. The body weights ranged from 260 to 320 g for males and 170–200 g for females.

The test was carried out following Guideline No. 423: Acute oral toxicity - Acute toxic class method of the OECD guidelines for the testing of chemicals [7] with a modification in terms of increasing doses.

The test was carried out following Guideline No. 408: Repeated doses of a 90-day oral toxicity study in rodents [8].

2.6. Preparation of Preparation of the animals

Four hundred and fifty rats were divided into two treatment groups (ten of each sex per group), ninety rats (five of each sex) were kept as a satellite group. They were acclimatized to the laboratory environment for one week prior to experimentation. Using the simple random sampling method, the rats were randomly selected and marked to indicate individual identification by tail labeling. The rats were tested over night.
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The values are express as mean ± S.D.; superscripts b and c, different from group a, for each solvent extracts and each methods are significantly different (P<0.05).

for 16 h prior to dosing of the test sample while drinking water was available.

2.7. Acute toxicity study

The Wistar rats were randomly divided into 9 groups of 10 animals of each sex and a suspension single dose was administered orally. Control groups 1, 2 and 3 received 1 ml/kg/day of distilled water, 5 % ethanol and 0.09 % acetone, respectively. Groups 4 and 5 were orally administered a suspension of RWF at doses of 2000 and 15,000 mg/kg/day. Groups 6 and 7 were orally administered a suspension of RCE at doses of 2000 and 15,000 mg/body weight/day. Groups 8 and 9 were orally administered a suspension of RCA at doses of 2000 and 15,000 mg/body weight/day. During the period of the experiment, body weight and food consumption were recorded weekly and the rats were closely observed for general appearance, behavior and any sign of abnormality. At the end of the 14-day treatment period, the animals were fasted for 16 h before being humanely sacrificed by CO2 asphyxiation. Larapotomy was performed and blood samples were collected from the inferior vena cava of each animal for analyzing hematological and clinical chemistry values by using an automatic hematological and chemistry analyzer.

2.8. Observation

After dosing, food was withheld for 3–4 h but not water. All treated rats were observed for any toxic signs immediately at 0.5, 1 and 3 h intervals. Special care was taken of those rats that obviously showed toxic signs during the first 4 h after dosing and then they were observed once daily thereafter for 14 days. The specific time at which signs of toxicity appeared and the recovery times were recorded to determine the duration of the recovery period.

2.9. Body weight

The body weight of each rat was recorded on days 1, 8, 15 (at termination) or after death during the experimentation period. The weight changes were recorded and calculated. At the end of the test, all

Table 1

| Parameters | Rang Chue extract (mg/kg) | Percentage relative organ weight (g /100 g) |
|------------|---------------------------|-------------------------------------------|
|            | Control 2000 15000        | Control 2000 15000                        |
| Weight     | 25.0±0.250 33.3±0.166 27.58±0.500 | 11.53±9.23 34.61±4.28 37.93±3.33 |
| Lung       | 0.05±0.02 0.43±0.04 0.45±0.01 | 0.40±0.03 0.47±0.03 0.50±0.02 |
| Heart      | 0.42±0.02 0.31±0.03 0.34±0.02 | 0.40±0.03 0.46±0.02 0.63±0.02 |
| Liver      | 2.95±0.15 3.13±0.15 3.99±0.13 | 2.25±0.16 3.31±0.15 4.60±0.20 |
| Spleen     | 0.21±0.05 0.19±0.03 0.25±0.02 | 0.15±0.02 0.20±0.02 0.34±0.05 |
| Kidney Left| 0.38±0.02 0.27±0.02 0.40±0.01 | 0.29±0.01 0.33±0.01 0.41±0.04 |
| Right      | 0.31±0.01 0.29±0.02 0.34±0.02 | 0.22±0.01 0.37±0.01 0.37±0.01 |
| adrenal    | 0.05±0.00 0.06±0.00 0.08±0.00 | 0.04±0.00 0.08±0.00 0.07±0.00 |
| testis     | 0.00±0.00 0.00±0.00 0.00±0.00 | 0.00±0.00 0.00±0.00 0.00±0.00 |
| ovary      | 0.46±0.04 0.44±0.04 0.40±0.05 | 0.66±0.04 0.60±0.04 0.68±0.02 |

The values are express as mean ± S.D.; *; ** Significantly different from the control group (p<0.05) and (p<0.01).

Table 2

| Parameters | Percentage relative organ weight (g /100 g) |
|------------|-------------------------------------------|
|            | Rang Chue extract (mg/kg) |
|            | Control 2000 15000                        |
| Weight     | 23.52±2.50 29.41±5.00 25.63±2.00 | 29.41±2.00 25.00±3.33 24.32±3.33 |
| Lung       | 0.66±0.03 0.68±0.04 0.70±0.05 | 0.64±0.04 0.54±0.03 0.61±0.04 |
| Heart      | 0.62±0.03 0.74±0.05 0.50±0.03 | 0.42±0.02 0.67±0.05 0.39±0.02 |
| Liver      | 3.78±0.15 3.63±0.13 4.50±0.18 | 3.21±0.20 3.40±0.15 4.66±0.13 |
| Spleen     | 0.27±0.02 0.30±0.01 0.40±0.02 | 0.28±0.01 0.27±0.01 0.33±0.02 |
| Kidney Left| 0.43±0.01 0.45±0.02 0.53±0.04 | 0.34±0.00 0.52±0.04 0.39±0.02 |
| Right      | 0.36±0.01 0.40±0.01 0.49±0.04 | 0.32±0.01 0.46±0.02 0.35±0.01 |
| adrenal    | 0.04±0.00 0.04±0.00 0.06±0.00 | 0.02±0.00 0.05±0.00 0.04±0.00 |
| testis     | 0.00±0.00 0.00±0.00 0.00±0.00 | 0.00±0.00 0.00±0.00 0.00±0.00 |
| ovary      | 0.00±0.00 0.07±0.00 0.07±0.00 | 0.02±0.00 0.05±0.00 0.02±0.00 |
| Right      | 0.05±0.00 0.08±0.00 0.09±0.00 | 0.05±0.00 0.05±0.00 0.06±0.00 |

The values are express as mean ± S.D.; *; ** Significantly different from the control group (p<0.05) and (p<0.01).
Table 4
Hematological values of rats receiving RCW at dose 2000 and 15,000 mg/kg for 14 day.

| Parameters | Control | Male 2000 | Male 15000 | Female 2000 | Female 15000 |
|------------|---------|-----------|------------|-------------|--------------|
| Hematocrit (%) | 49.00±0.00 | 44.50±0.36 | 46.00±0.19 | 46.50±0.12 | 46.00±0.82 | 46.33±0.21 |
| RBC (x10^{12}/cell/mm^{3}) | 8.25±0.18 | 7.32±1.12 | 7.67±1.00 | 8.23±0.76 | 8.22±0.77 | 7.97±0.50 |
| Hemoglobin (g/dl) | 15.60±0.70 | 15.5±2.12 | 15.67±1.52 | 16.50±0.70 | 16.50±0.70 | 16.67±1.15 |
| MCV (mm^{3}/red cell) | 59.00±1.69 | 60.95±1.06 | 59.90±1.96 | 56.30±2.40 | 55.80±1.69 | 57.80±0.72 |
| MCH (pg/red cell) | 19.80±0.70 | 20.75±0.63 | 20.26±0.95 | 19.90±1.13 | 19.75±0.91 | 20.63±0.20 |
| MCHC (g/dl RBC) | 33.45±0.21 | 33.95±0.49 | 33.73±0.51 | 35.30±0.56 | 35.35±0.63 | 35.70±0.10 |
| WBC (x10^{3}/cell/mm^{3}) | 7.10±0.55 | 7.55±0.516 | 8.03±3.74 | 8.05±0.21 | 8.05±0.21 | 8.76±1.50 |
| Lymphocytes (%) | 70.50±13.43 | 78.00±24.04 | 78.67±17.03 | 89.50±0.70 | 90.50±2.12 | 86.33±8.14 |
| Platelets (x10^{3}/cell/mm^{3}) | 390.00±65.05 | 380.00±231.22 | 365.67±220.20 | 317.00±39.32 | 324.50±79.71 | 387.00±240.00 |

The values are expressed as mean ± S.D.

Table 5
Hematological values of rats receiving RCE at dose 2000 and 15,000 mg/kg for 14 day.

| Parameters | Control | Male 2000 | Male 15000 | Female 2000 | Female 15000 |
|------------|---------|-----------|------------|-------------|--------------|
| Hematocrit (%) | 41.50±4.94 | 43.00±4.16 | 43.67±1.15 | 48.00±2.12 | 47.00±0.70 | 47.33±0.57 |
| RBC (x10^{12}/cell/mm^{3}) | 6.65±0.96 | 7.09±0.75 | 7.19±0.26 | 8.31±0.35 | 8.39±0.04 | 8.44±0.10 |
| Hemoglobin (g/dl) | 14.00±1.41 | 14.50±1.52 | 14.67±0.57 | 17.00±1.41 | 16.50±0.70 | 16.67±0.57 |
| MCV (mm^{3}/red cell) | 62.35±1.20 | 60.65±0.15 | 60.56±0.58 | 57.65±2.19 | 56.00±0.14 | 56.03±0.11 |
| MCH (pg/red cell) | 21.15±1.34 | 20.65±0.32 | 20.46±0.26 | 20.10±0.98 | 19.55±0.07 | 19.50±0.10 |
| MCHC (g/dl RBC) | 33.95±1.48 | 34.10±0.52 | 33.80±0.56 | 34.85±0.35 | 34.90±0.28 | 34.80±0.26 |
| WBC (x10^{3}/cell/mm^{3}) | 4.45±3.46 | 6.30±3.76 | 6.70±1.64 | 9.60±3.33 | 7.00±0.14 | 7.03±0.11 |
| Lymphocytes (%) | 85.50±12.02 | 89.00±2.30 | 87.67±6.65 | 72.50±30.40 | 94.50±2.12 | 94.33±1.52 |
| Platelets (x10^{3}/cell/mm^{3}) | 339.50±130.81 | 365.50±171.12 | 332.67±222.33 | 312.50±55.86 | 316.00±42.42 | 361.33±99.32 |

The values are expressed as mean ± S.D.

Table 6
Hematological values of rats receiving RCA at dose 2000 and 15,000 mg/kg for 14 day.

| Parameters | Control | Male 2000 | Male 15000 | Female 2000 | Female 15000 |
|------------|---------|-----------|------------|-------------|--------------|
| Hematocrit (%) | 48.50±2.12 | 45.50±2.12 | 46.00±1.73 | 47.50±3.53 | 45.50±0.70 | 47.75±2.62 |
| RBC (x10^{12}/cell/mm^{3}) | 8.65±0.36 | 7.47±0.31 | 7.58±0.29 | 8.60±0.70 | 8.14±0.09 | 8.56±0.50 |
| Hemoglobin (g/dl) | 17.00±0.00 | 15.50±0.70 | 15.67±0.57 | 16.50±0.70 | 16.50±0.00 | 16.75±0.95 |
| MCV (mm^{3}/red cell) | 55.85±0.21 | 60.20±0.56 | 59.46±1.33 | 55.05±0.77 | 55.95±0.49 | 55.70±0.87 |
| MCH (pg/red cell) | 19.15±0.91 | 16.25±6.29 | 17.50±1.49 | 18.95±0.63 | 19.50±0.28 | 19.40±0.63 |
| MCHC (g/dl RBC) | 34.35±1.48 | 34.30±0.28 | 34.35±0.45 | 34.45±0.77 | 35.05±0.77 | 34.80±0.60 |
| WBC (x10^{3}/cell/mm^{3}) | 7.95±0.21 | 7.00±1.07 | 7.20±0.95 | 6.80±2.26 | 6.80±1.26 | 6.70±1.84 |
| Lymphocytes (%) | 67.00±15.55 | 61.50±48.79 | 70.33±37.74 | 87.50±3.53 | 89.50±0.70 | 88.00±2.16 |
| Platelets (x10^{3}/cell/mm^{3}) | 354.00±76.36 | 356.00±325.26 | 390.00±231.59 | 390.50±60.10 | 376.00±60.81 | 350.25±89.60 |

The values are expressed as mean ± S.D.

Table 7
Clinical chemistry values of male rats receiving RCW, RCE and RCA at dose 2000 and 15,000 mg/kg for 14 day.

| Parameters | RCW | RCE | RCA |
|------------|-----|-----|-----|
| ALP (U/L) | 107.50±19.60 | 77.50±31.18 | 90.00±31.22 |
| ALT (U/L) | 30.00±7.07 | 30.00±0.00 | 28.33±5.77 |
| AST (U/L) | 90.00±7.07 | 102.5±12.50 | 96.67±12.58 |
| BUN (mg/dl) | 23.00±4.24 | 19.25±9.54 | 19.50±6.76 |
| Creatinine (mg/dl) | 0.75±0.10 | 0.72±1.14 | 0.72±0.12 |
| Glucose (mg%) | 100.00±17.07 | 95.00±2.45 | 98.33±1.77 |

The values are expressed as mean ± S.D.
survivors were weighed and humanely killed by CO$_2$ asphyxiation. The means of body weight gains of the rat in the treatment groups were calculated in comparison with the control group.

2.10. Sub chronic toxicity study

The Wistar rats were randomly divided into 14 groups of 10 animals of each sex. Control groups 1 and 2 received 1 ml/kg/day of distilled water and 5 % of tween (80 %), respectively. Groups 2 and 3 were orally

| Parameters          | RCW 2000 | 15000 | RCE 2000 | 15000 | RCA 2000 | 15000 |
|---------------------|----------|-------|----------|-------|----------|-------|
| ALP (U/L)           | 17.50±7.67 | 17.50±3.53 | 17.33±4.16 | 20.00±4.24 | 20.25±5.01 | 20.56±10.00 | 26.05±0.75 | 25.00±0.70 | 25.25±0.50 |
| ALT (U/L)           | 41.00±1.41 | 36.00±8.48 | 33.33±5.70 | 21.00±12.72 | 22.50±3.53 | 22.00±5.00 | 32.50±8.99 | 32.50±10.60 | 36.50±23.50 |
| AST (U/L)           | 143.5±16.26 | 136.0±5.65 | 140.0±15.00 | 107.0±32.52 | 110.0±21.21 | 116.67±18.90 | 165.0±0.05 | 109.5±10.60 | 110.2±21.68 |
| BUN (mg/dl)         | 41.45±8.41 | 45.20±3.11 | 33.66±10.37 | 34.50±2.12 | 37.75±3.18 | 36.67±2.97 | 27.00±5.65 | 29.85±6.57 | 29.07±2.83 |
| Creatinine (mg%)    | 0.93±0.25 | 0.83±0.39 | 0.87±0.10 | 0.80±0.56 | 0.76±0.07 | 0.78±0.12 | 0.72±0.67 | 0.70±0.11 | 0.75±0.44 |
| Glucose (mg%)       | 97.00±2.82 | 99.50±0.70 | 98.00±2.64 | 92.00±16.97 | 95.00±21.21 | 90.00±17.32 | 97.50±3.53 | 102.5±3.53 | 108.0±16.51 |

The values are express as mean ± S.D.

Fig. 1. Gross pathological of liver in male at a single dose of 15,000 mg/kg compared with control. The rats were receiving RCW (A1, B1), RCE (A2, B2) and RCA (A3, B3), respectively for 14 day. hepatocyte (h), central vein (cv), sinusoid (s) (the 50 × magnifications).
administered a suspension of RCW and RCE at doses of 50 mg/body weight/day (quality of consumer drink for three times per day) at doses of 1460 and 1025 mg/kg/day. Low dose groups 4 and 5 and high dose groups 6 and 7 were orally administered RCW and RCE at a low dose of 3000 mg/kg/day and a high dose of 5000 mg/kg/day, respectively, for 3 months. After the three-month period of RCW and RCE treatment, Groups (R) 8–14 (doses of same groups 1–7: satellite groups) were reared for two more weeks without RC extracts in order to observe the recovery or delayed effects of the extracts. During the period of the experiment, body weight and food consumption were recorded weekly and the rats were closely observed for general appearance, behavior and any signs of abnormality. At the end of the 90-day treatment period, the animals were fasted for 16 h before being humanely sacrificed by CO₂ asphyxiation. Laparotomy was performed and blood samples were collected from the inferior vena cava of each animal for analyzing hematological and clinical chemistry values by using an automatic hematological and chemistry analyzer.

2.11. Hematological and biochemical analysis

The hematological parameters examined were hematocrit, erythrocytes (RBC), hemoglobin, mean cell volume (MCV), mean cell hemoglobin (MCH), mean cell hemoglobin concentration (MCHC), white blood cell (WBC), lymphocytes and platelets. The clinical chemistry parameters measured alkaline phosphate (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), blood urea nitrogen (BUN), creatinine and glucose.

2.12. Histopathological analysis

Necropsy was then performed to determine the gross lesions of various visceral organs. Lungs, hearts, livers, spleens, kidneys, adrenal glands, testes or ovaries were weighed. The organ weights were calculated according to relative organ weights (g/100 g body weight). The visceral organs were then preserved in 10 % formalin and subsequently subjected to a histological process for preparing tissue slides stained
with haematoxylin and eosin (H&E) stains. Tissue slides were histo-pathologically examined by a veterinary pathologist.

The rats’ organs were cut and were then fixed in 10 % buffer formalin. The organs were cut into short segments using the paraffin technique. Sections of 5 μm thickness were cut and the routine haematoxylin and eosin (H&E) method was used. Briefly, organs were dehydrated in an ascending ethanol series (75 %, 85 %, 95 % and 100 %, 1 h each) and then the sections were placed in pure xylene for 2 min. The sections were embedded with xylene: paraplast (3:1 and 1:1, 15 min each) and then followed by pure paraplast for 1 h. Post-

Table 9

| Parameters | Percentage relative organ weight (g /100 g) |
|------------|---------------------------------------------|
|            | Control | 5000 | 3000 | 1460 | RCW | Control | 5000R | 3000R | 1460R |
| Weight     | 43.62±4.57 | 41.25±3.60 | 37.55±8.22 | 37.55±4.92 | 43.12±4.60 | 51.13±5.45* | 46.25±7.71 | 43.87±3.45 |
| Lung       | 0.34±0.02 | 0.36±0.03 | 0.29±0.03 | 0.28±0.03* | 0.39±0.04 | 0.37±0.01 | 0.29±0.02** | 0.30±0.03* |
| Heart      | 0.16±0.06 | 0.31±0.03 | 0.26±0.02* | 0.27±0.01* | 0.33±0.03 | 0.33±0.02 | 0.24±0.02* | 0.24±0.02* |
| Liver      | 2.49±0.16 | 2.41±0.15 | 2.31±1.07 | 2.41±0.16 | 2.54±0.15 | 2.57±0.13 | 2.25±0.17 | 2.35±0.19 |
| Spleen     | 0.21±0.05 | 0.20±0.04 | 0.17±0.02 | 0.18±0.02 | 0.22±0.03 | 0.22±0.02 | 0.17±0.02 | 0.17±0.01 |
| Kidney     | 0.30±0.01 | 0.28±0.02 | 0.22±0.02* | 0.24±0.02* | 0.29±0.02 | 0.29±0.01 | 0.21±0.02* | 0.22±0.02* |
| Adrenal    | 0.19±0.00 | 0.01±0.00 | 0.01±0.00 | 0.01±0.00 | 0.19±0.00 | 0.01±0.00 | 0.01±0.00 | 0.00±0.00 |
| Testis     | 0.51±0.04 | 0.52±0.04 | 0.50±0.04 | 0.51±0.08 | 0.51±0.05 | 0.50±0.02 | 0.52±0.04 | 0.53±0.06 |

The values are expressed as mean ± S.D.; * Significant different from the control group (p<0.05) and (p<0.01).

Table 10

| Parameters | Percentage relative organ weight (g /100 g) |
|------------|---------------------------------------------|
|            | Control | 5000 | 3000 | 1025 | RCW | Control | 5000R | 3000R | 1025R |
| Weight     | 57.62±6.07 | 58.84±14.48 | 56.56±7.73 | 58.10±11.72 | 36.41±2.20 | 69.37±16.33* | 60.73±8.27* | 61.35±7.97* |
| Lung       | 0.37±0.03 | 0.47±0.03* | 0.28±0.03 | 0.30±0.04 | 0.44±0.02* | 0.40±0.02 | 0.39±0.02 | 0.38±0.02 |
| Heart      | 0.38±0.03 | 0.35±0.02 | 0.25±0.02* | 0.24±0.02* | 0.34±0.02 | 0.34±0.02 | 0.35±0.02 | 0.35±0.02 |
| Liver      | 2.27±0.04 | 2.44±0.19 | 2.33±0.14 | 2.30±0.20 | 2.34±0.07 | 2.49±0.12 | 2.45±0.17 | 2.47±0.16 |
| Spleen     | 0.20±0.02 | 0.20±0.01 | 0.16±0.02 | 0.17±0.02 | 0.18±0.01 | 0.21±0.02 | 0.17±0.02 | 0.21±0.05 |
| Kidney     | 0.29±0.01 | 0.28±0.01 | 0.20±0.02* | 0.20±0.01* | 0.26±0.00 | 0.29±0.014 | 0.24±0.01 | 0.24±0.01 |
| Adrenal    | 0.02±0.00 | 0.02±0.00 | 0.06±0.00* | 0.02±0.00 | 0.018±0.00 | 0.03±0.00* | 0.017±0.00 | 0.018±0.00 |
| Testis     | 0.52±0.05 | 0.49±0.04 | 0.53±0.07 | 0.54±0.04 | 0.48±0.02 | 0.50±0.02 | 0.52±0.04 | 0.52±0.04 |

The values are expressed as mean ± S.D.; * Significant different from the control group (p<0.05) and (p<0.01).

Table 11

| Parameters | Percentage relative organ weight (g /100 g) |
|------------|---------------------------------------------|
|            | Control | 5000 | 3000 | 1460 | RCW | Control | 5000R | 3000R | 1460R |
| Weight     | 53.62±2.22 | 41.87±8.35 | 45.65±5.24 | 40.78±7.75 | 37.03±7.39 | 28.35±3.69 | 31.05±5.23 | 29.75±1.05 |
| Lung       | 0.49±0.04 | 0.48±0.05 | 0.40±0.04 | 0.41±0.04 | 0.56±0.06 | 0.50±0.03 | 0.41±0.04* | 0.42±0.05* |
| Heart      | 0.37±0.01 | 0.35±0.03 | 0.28±0.02* | 0.29±0.03* | 0.38±0.02 | 0.39±0.03 | 0.29±0.03* | 0.28±0.02* |
| Liver      | 2.50±0.15 | 2.45±0.14 | 2.35±0.17 | 2.49±0.16 | 2.65±0.14 | 2.41±0.17 | 2.33±0.17 | 2.46±0.19 |
| Spleen     | 0.26±0.02 | 0.23±0.01 | 0.22±0.02 | 0.24±0.03 | 0.25±0.01 | 0.25±0.02 | 0.21±0.04 | 0.21±0.02 |
| Kidney     | 0.33±0.03 | 0.32±0.03 | 0.25±0.02* | 0.27±0.02* | 0.33±0.01 | 0.30±0.02 | 0.24±0.02* | 0.25±0.02* |
| Adrenal    | 0.02±0.00 | 0.02±0.00 | 0.01±0.00 | 0.01±0.00 | 0.018±0.00 | 0.02±0.00 | 0.018±0.00 | 0.018±0.00 |
| Testis     | 0.04±0.00 | 0.03±0.00 | 0.03±0.00 | 0.04±0.00 | 0.04±0.00 | 0.04±0.01 | 0.04±0.02 | 0.04±0.02 |

The values are expressed as mean ± S.D.; * Significant different from the control group (p<0.05) and (p<0.01).
Table 12
Percentage body weight and percentage relative organ weight of female rats receiving RCE at dose 1025 (the equivalent dose), 3000 and 5000 mg/kg for 90 day and satellite group.

| Parameters | Percentage relative organ weight (g /100 g) |
|------------|--------------------------------------------|
|            | Control 5000 3000 1025                   | RCE                        |
| Weight     | 19.36±0.34 40.88±16.96* 37.25±7.97*    | 39.75±3.85*                |
| Lung       | 0.47±0.02 0.45±0.06 0.40±0.03           | 0.41±0.06                  |
| Heart      | 0.25±0.02 0.33±0.02 0.30±0.03           | 0.29±0.03                  |
| Liver      | 2.48±0.13 2.31±0.10 2.32±0.14           | 2.28±0.20                  |
| Spleen     | 0.28±0.02 0.21±0.01 0.21±0.02           | 0.21±0.02                  |
| Kidney     | 0.32±0.01 0.33±0.01 0.24±0.02           | 0.23±0.03                  |
| Adrenal    | 0.31±0.01 0.29±0.01 0.25±0.02           | 0.25±0.02                  |
| Testis     | 0.04±0.00 0.03±0.00 0.03±0.00           | 0.04±0.00                  |

The values are express as mean ± S.D.; *, ** Significantly different from the control group (p<0.05) and (p<0.01).

Table 13
Hematological values of male rats receiving RCW at dose 1460 (the equivalent dose), 3000 and 5000 mg/kg for 90 day and satellite group.

| Parameters | Hematological values |
|------------|----------------------|
|            | Control 5000 3000 1460 | RCE                        |
| Hematocrit (%) | 47.28±2.38 46.2±1.09 | 47.30±2.58 47.42±0.35 |
| RBC (x10^12 cell/mm^3) | 8.46±0.58 8.33±0.15 | 8.62±0.40 8.35±0.53 |
| Hemoglobin (g/dl) | 16.28±0.70 16.00±0.00 | 15.42±0.76 15.28±0.87 |
| MCV (xμm³/red cell) | 55.71±1.61 55.44±0.90 | 54.86±1.91 56.84±1.69 |
| MCH (g/red cell) | 19.41±0.57 19.18±0.29 | 17.88±0.56 18.43±0.57 |
| MCHC (g/dl RBC) | 34.85±0.35 34.64±0.37 | 32.61±0.31 32.44±0.48 |
| WBC (x10^9 cell/mm³) | 7.1±1.98 4.52±1.01 | 3.1±0.71 3.54±1.30 |
| Platelets (x10^12 cell/mm³) | 62.7±5.19 90.2±6.64 | 64.4±5.56 70.6±4.95 |

The values are express as mean ± S.D.

Table 14
Hematological values of male rats receiving RCE at dose 1025 (the equivalent dose), 3000 and 5000 mg/kg for 90 day and satellite group.

| Parameters | Hematological values |
|------------|----------------------|
|            | Control 5000 3000 1025 | Satellite group |
| Hematocrit (%) | 48.00±2.44 47.6±3.50 | 47.25±2.10 47.03±1.24 |
| RBC (x10^12 cell/mm³) | 8.46±0.51 8.40±0.54 | 9.01±0.43 9.02±0.44 |
| Hemoglobin (g/dl) | 16.6±0.54 16.44±0.87 | 15.50±0.63 15.47±0.42 |
| MCV (xμm³/red cell) | 58.24±3.23 56.60±0.57 | 52.52±2.18 52.23±2.33 |
| MCH (g/red cell) | 22.32±5.75 19.68±0.21 | 17.23±0.80 17.20±0.81 |
| MCHC (g/dl RBC) | 38.06±8.08 34.76±0.59 | 32.81±0.43 32.93±0.31 |
| WBC (x10^9 cell/mm³) | 14.84±2.86 5.7±0.33* | 3.79±0.58* 2.8±1.00* |
| Lymphocytes (%) | 32.86±6.42 86.8±6.87 | 71.52±10.16 61.33±1.29 |
| Platelets (x10^12 cell/mm³) | 741.2±58.01 633.2±123.28 | 968.93±115.41* 930.14±180.80 |

The values are express as mean ± S.D.; *, ** Significantly different from the control group (p<0.05) and (p<0.01).

embedded tissues were cut into approximately 5 μm with microtome and these sections were moved into a water bath for incubation (60 °C). The tissues were mounted on slides which were warmed at 60 °C and then immersed in pure xylene twice (10 min). Next, the tissues were hydrated in a descending ethanol series (100 %, 90 %, 70 %, and distilled water, 5 min each) and stained with H&E for 45 s. Finally, the slides were immersed in 100 % ethanol (1 min) and then covered with a cover slip after xylene cleaning for light microscopic study. Light microscopic observations were carried out using a standard Olympus-Light Microscope at 20 × magnification.

2.13. A cytotoxicity study measured the level of malondialdehyde (MDA)

Cytotoxicity studies were conducted by testing the level of malondialdehyde (MDA). This process relies on the principle that MDA reacts with thiobarbituric acid (TBA) in the acidic serum when the product shows a MDA-TBA complex of a pinkish-orange color. The level of MDA was measured by absorption at a wavelength of 532 nm [9].
Table 15
Hematological values of male rats receiving RCW at dose 1460 (the equivalent dose), 3000 and 5000 mg/kg for 90 day and satellite group.

| Parameters         | Hematological values | RCW                  | Satellite group |
|--------------------|----------------------|----------------------|-----------------|
|                    | Control              | 5000                | 3000            | 1460          |
| Hematocrit(%)      | 44.60±3.13           | 46.60±1.34          | 46.38±2.08      | 46.10±2.78    |
| RBC (x10^12 cell/mm³) | 7.73±5.69           | 8.22±0.25           | 7.88±0.36       | 7.78±0.43     |
| Hemoglobin (g/dl)  | 15.80±6.16           | 16.00±0.00          | 15.26±0.61      | 15.14±0.92    |
| MCV (mm³ red cell) | 57.62±7.96           | 56.56±1.55          | 58.85±1.55      | 59.24±2.00    |
| MCH (pg/red cell)  | 20.54±13.51          | 19.40±0.51          | 19.36±0.39      | 19.46±0.52    |
| MCHC (g/dl RBC)    | 35.66±3.94           | 34.26±0.48          | 32.91±0.41      | 32.86±0.36    |
| WBC (x10^9 cell/mm³) | 4.54±1.62           | 4.34±1.73           | 2.77±0.54       | 2.46±0.84     |
| Lymphocytes (%)    | 78.00±15.40          | 94.20±1.64          | 69.18±10.82     | 70.23±13.01   |
| Platelets (x10^9 cell/mm³) | 783.00±62.56       | 892.40±48.27        | 916.4±81.77     | 995.4±316.71  |

The values are expressed as mean ± S.D.; *, ** Significantly different from the control group (p<0.05) and (p<0.01).

Table 16
Hematological values of female rats receiving RCE at dose 1025 (the equivalent dose), 3000 and 5000 mg/kg for 90 day and satellite group.

| Parameters         | Hematological values | RCE                  | Satellite group |
|--------------------|----------------------|----------------------|-----------------|
|                    | Control              | 5000                | 3000            | 1025          |
| Hematocrit(%)      | 46.75±2.87           | 46.40±0.89          | 45.57±1.24      | 45.70±1.44    |
| RBC (x10^12 cell/mm³) | 8.32±0.38           | 8.21±0.12           | 7.87±0.45       | 8.02±0.36     |
| Hemoglobin (g/dl)  | 16.25±1.25           | 16.20±0.44          | 15.19±0.75      | 15.18±0.42    |
| MCV (mm³ red cell) | 56.22±1.65           | 56.50±1.23          | 57.89±1.03      | 56.99±1.56    |
| MCH (pg/red cell)  | 19.37±0.45           | 19.84±0.42          | 19.33±0.75      | 19.83±0.53    |
| MCHC (g/dl RBC)    | 34.40±0.35           | 35.14±0.53          | 33.37±0.99      | 33.22±0.41    |
| WBC (x10^9 cell/mm³) | 4.40±2.10           | 6.14±3.12           | 1.71±0.41       | 2.07±0.45     |
| Lymphocytes (%)    | 78.25±15.88          | 85.20±12.91         | 70.45±8.27      | 66.93±9.02    |
| Platelets (x10^9 cell/mm³) | 842.25±32.32       | 625.25±175.91       | 921.33±82.02    | 926.07±73.75  |

The values are expressed as mean ± S.D.; *, ** Significantly different from the control group (p<0.05) and (p<0.01).

Table 17
Clinical chemistry values of male rats receiving RCW at dose 1460 (the equivalent dose), 3000 and 5000 mg/kg for 90 day and satellite group.

| Parameters         | Clinical chemistry values | RCW                  | Satellite group |
|--------------------|---------------------------|----------------------|-----------------|
|                    | Control              | 5000                | 3000            | 1460          |
| ALP (U/L)          | 45.42±15.31           | 60.00±29.23         | 56.47±10.22     | 56.27±7.27    |
| ALT (U/L)          | 31.28±7.09            | 31.40±5.59          | 26.93±9.25      | 24.60±3.60    |
| AST (U/L)          | 94.85±16.32           | 99.60±17.00         | 74.87±11.78     | 86.0±17      |
| BUN (mg/dl)        | 26.01±12.03           | 23.24±2.62          | 19.25±1.95      | 19.54±2.05    |
| Creatinine (mg%)   | 0.60±0.10             | 0.56±0.13           | 0.74±0.07       | 0.75±0.06     |
| Glucose (mg%)      | 142.85±52.59          | 121.40±36.92        | 189.43±48.37    | 185.62±37.08  |

The values are expressed as mean ± S.D.; *, ** Significantly different from the control group (p<0.05) and (p<0.01).

Table 18
Clinical chemistry values of male rats receiving RCE at dose 1025 (the equivalent dose), 3000 and 5000 mg/kg for 90 day and satellite group.

| Parameters         | Clinical chemistry values | RCE                  | Satellite group |
|--------------------|---------------------------|----------------------|-----------------|
|                    | Control              | 5000                | 3000            | 1025          |
| ALP (U/L)          | 54.00±11.44           | 59.20±19.46         | 58.13±8.64      | 57.40±8.86    |
| ALT (U/L)          | 32.40±6.65            | 31.60±2.07          | 33.20±6.7       | 41.53±24.65*  |
| AST (U/L)          | 193.60±25.12          | 129.60±32.33        | 79.73±9.11      | 94.93±34.90   |
| BUN (mg/dl)        | 17.96±3.14            | 22.94±2.85          | 18.09±2.23      | 18.10±2.65    |
| Creatinine (mg%)   | 0.46±0.07             | 0.63±0.05           | 0.75±0.08       | 0.73±0.07     |
| Glucose (mg%)      | 90.00±23.86           | 114.6±26.01         | 172.24±29.35*   | 165.33±23.77* |

The values are expressed as mean ± S.D.; *, ** Significantly different from the control group (p<0.05) and (p<0.01).
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The statistical significance between treated and control groups was assessed using ANOVA. Duncan’s multiple-range test was used as a post hoc test to analyze for differences between means using one-way analysis of variance (ANOVA). The values are expressed as mean ± standard deviation (SE) of mean and standard error (SE) of mean and standard deviation (S.D.). *Significantly different from the control group (p < 0.05) and **Significantly different from the control group (p < 0.01).

### 2.14. Statistical analysis

All values were expressed as mean ± standard error (SE) of mean and standard deviation (S.D.). The data was presented as means ± SE. The data was analyzed for differences between means using one-way analysis of variance (ANOVA). Duncan’s multiple-range test was used as a post hoc test to analyze for differences between means using one-way analysis of variance (ANOVA). The values are expressed as mean ± standard deviation (SE) of mean and standard error (SE) of mean and standard deviation (S.D.). *Significantly different from the control group (p < 0.05) and **Significantly different from the control group (p < 0.01).

### 3. Results

#### 3.1. The phenolic contents and antioxidant activity of Rang Chuet extract

The phenolic contents of the products of the Rang Chuet extracts were tested by folin-ciocalteu reagent. The results showed that water extraction of phenolic compounds was the most efficient (2634 ± 195.05 mg GAE/100 g) compared with ethanol and acetone extracts which had phenolic contents of 305.24 ± 43.43 and 81.58 ± 18.12 mg GAE/100 g, respectively. Furthermore, RCA showed the highest antioxidant activities at a value of IC_{50} at 52.91 ± 8.99 mg/ml using DPPH assay. In addition, the commercial standards of BHT and ascorbic acid showed values of IC_{50} at 135 and 76.51 mg/ml, respectively. Also, the ABTS radical scavenging capacity of RCA showed a value of IC_{50} at 60.64 ± 0.11 mg/ml. Finally, the ferric reducing antioxidant power of RCW showed the value of 0.254 ± 0.0193 mmol Fe^{2+}/g RM (Table 1).

#### 3.2. Acute toxicity

After the rats were given a single dose orally of RCW, RCE and RCA at 2000 and 15,000 mg/kg body weight, there were no significant signs of mortality, general behavior or gross differences of appearance in the internal organs during the 14 days of the testing period. However, male rats treated with RCW at 2000 and 15,000 mg/kg had a significant percentage increase in body weight of 33 % and 10 % (p < 0.05), respectively. Similarly, male rats treated with RCE at 2000 and 15,000 mg/kg body weight had significantly increased by doubling the percentage of their body weight. Moreover, male rats treated with RCA at 2000 and 15,000 mg/kg had significantly increased in the percentage of their body weight of 34 % and 67 %, respectively. Finally, the female rats treated with RCA at 2000 and 15,000 mg/kg had significantly increased by doubling the percentage of their body weight of 34 % and 67 %, respectively. Finally, the female rats treated with RCA at 2000 and 15,000 mg/kg had significantly increased in the percentage of their body weight of 34 % and 67 %, respectively. Finally, the female rats treated with RCA at 2000 and 15,000 mg/kg had significantly increased in the percentage of their body weight of 34 % and 67 %, respectively. Finally, the female rats treated with RCA at 2000 and 15,000 mg/kg had significantly increased in the percentage of their body weight of 34 % and 67 %, respectively. Finally, the female rats treated with RCA at 2000 and 15,000 mg/kg had significantly increased in the percentage of their body weight of 34 % and 67 %, respectively. Finally, the female rats treated with RCA at 2000 and 15,000 mg/kg had significantly increased in the percentage of their body weight of 34 % and 67 %, respectively. Finally, the female rats treated with RCA at 2000 and 15,000 mg/kg had significantly increased in the percentage of their body weight of 34 % and 67 %, respectively. Finally, the female rats treated with RCA at 2000 and 15,000 mg/kg had significantly increased in the percentage of their body weight of 34 % and 67 %, respectively. Finally, the female rats treated with RCA at 2000 and 15,000 mg/kg had significantly increased in the percentage of their body weight of 34 % and 67 %, respectively. Finally, the female rats treated with RCA at 2000 and 15,000 mg/kg had significantly increased in the percentage of their body weight of 34 % and 67 %, respectively. Finally, the female rats treated with RCA at 2000 and 15,000 mg/kg had significantly increased in the percentage of their body weight of 34 % and 67 %, respectively. Finally, the female rats treated with RCA at 2000 and 15,000 mg/kg had significantly increased in the percentage of their body weight of 34 % and 67 %, respectively. Finally, the female rats treated with RCA at 2000 and 15,000 mg/kg had significantly increased in the percentage of their body weight of 34 % and 67 %, respectively. Finally, the female rats treated with RCA at 2000 and 15,000 mg/kg had significantly increased in the percentage of their body weight of 34 % and 67 %, respectively. Finally, the female rats treated with RCA at 2000 and 15,000 mg/kg had significantly increased in the percentage of their body weight of 34 % and 67 %, respectively. Finally, the female rats treated with RCA at 2000 and 15,000 mg/kg had significantly increased in the percentage of their body weight of 34 % and 67 %, respectively. Finally, the female rats treated with RCA at 2000 and 15,000 mg/kg had significantly increased in the percentage of their body weight of 34 % and 67 %, respectively. Finally, the female rats treated with RCA at 2000 and 15,000 mg/kg had significantly increased in the percentage of their body weight of 34 % and 67 %, respectively. Finally, the female rats treated with RCA at 2000 and 15,000 mg/kg had significantly increased in the percentage of their body weight of 34 % and 67 %, respectively. Finally, the female rats treated with RCA at 2000 and 15,000 mg/kg had significantly increased in the percentage of their body weight of 34 % and 67 %, respectively. Finally, the female rats treated with RCA at 2000 and 15,000 mg/kg had significantly increased in the percentage of their body weight of 34 % and 67 %, respectively. Finally, the female rats treated with RCA at 2000 and 15,000 mg/kg had significantly increased in the percentage of their body weight of 34 % and 67 %, respectively. Finally, the female rats treated with RCA at 2000 and 15,000 mg/kg had significantly increased in the percentage of their body weight of 34 % and 67 %, respectively.

#### 3.3. Sub-chronic toxicity

##### 3.3.1. Effects on body weight, food consumption and physical appearance

The average body weight and food consumption of male and female rats which received the equivalent dose of Rang Chuet in a typical consumer drink (to be taken 3 times a day at doses of 1460 mg/kg/day for RCW and 1025 mg/kg/day for RCE), 3000 and 5000 mg/kg and satellite groups were not significantly different when compared with the control groups for 90 days. There was no abnormality in physical appearance and behavior in any of the treatment groups nor in the control groups. The treatment groups did not show any toxic signs or mortality. All groups showed a normal increase in their relative weight gain each month. The RCE group showed a significantly higher weight gain than the control groups. Satellite groups were not significantly different (p < 0.05) when compared with control group for 90 days (Tables 9–12). In addition female rats treated with RCE at 5000, 3000 and 1025 mg/kg significantly increased the percentage of their body weight by 100 %, 92 %, and 100 % respectively. Similar, results also occurred in the satellite group.
3.3.2. Effect on relative weight of organs

The results showed that the group receiving RCW and RCE had a significant decrease in the relative weight of the heart compared to the control group \( (p < 0.05) \), but an increase in the satellite female group which received RCE 5000 mg/kg (5000R). The relative weight of the heart was \( 0.44 \pm 0.03 \) g%, which was significantly higher than the \( 0.35 \pm 0.04 \) g% for the control group \( (p < 0.01) \), (Table 12). The results also showed that the relative weight of the liver was no different from that of the control group. The relative weight of the kidneys decreased in the group receiving RCW and RCE at doses of consumer drink and 3000 mg/kg \( (p < 0.05) \). But there was an increase in the satellite groups of males which received a high dose of 5000 mg/kg of RCW (5000R). The relative weight of the left and right kidneys increased by \( 0.29 \pm 0.01 \) and \( 0.28 \pm 0.01 \) g%, which was higher than the control group with \( 0.26 \pm 0.00 \) and \( 0.25 \pm 0.01 \) g%, respectively \( (p < 0.05) \), (Table 9). The relative weight of the adrenal glands was the same in all groups compared to the control group.

Fig. 3. Gross pathological of liver in male at a single dose of 5000 mg/kg compared with control. Treat group receive RCW (A1, A3), RCE (B1, B3) and satellite group receive RCW (A2, A4), RCE (B2, B4) respectively for 90 day. hepatocyte (h), central vein (cv), sinusoid (s) (the 50× magnifications).
control group (Tables 9–12). With regard to the satellite groups for both males and females treated with RCE and RCW at 5000 mg/kg, there were no significant differences in the relative organ weights when compared to the control group, except for the male rats which received RCW at 5000 mg/kg which showed that their adrenal weight had significantly increased (p < 0.05). In addition, male and female rats treated with RCW and RCE at 3000 mg/kg showed that their the relative organ weight, especially for the lungs and heart, had significantly increased (p < 0.05).

3.3.3. Effects on hematological parameters

The effect of sub-chronic administration of RCW and RCE on the hematological parameters are presented in Tables 13–16. All of the hematological measures: leukocyte (total white blood cell), erythrocyte (red blood cell), hemoglobin, MCV, MCH, MCHC, lymphocyte, thrombocyte (platelet) in treated male and female rats were not significantly

Fig. 4. Gross pathological of liver in female at a single dose of 5000 mg/kg compared with control. Treat group receive RCW (A1, A3), RCE (B1, B3) and satellite group receive RCW (A2, A4), RCE (B2, B4) respectively for 90 day. hepatocyte (h), central vein (cv), sinusoid (s) (the 50 × magnifications).
different from controls, and all results of the treatment were in the range of normal values. Finally, the leukocytes (total white blood cells) showed a decreasing trend in male rats treated with RCE at 5000, 3000, and 1025 mg/kg respectively while similar results were obtained in the satellite groups which showed a significant improvement.

3.3.4. Effects on clinical chemistry parameters

After 90 days, the results showed that all groups receiving RCW and RCE in both males and females did not show any differences in their levels of AST, ALT, and ALP compared to the control group (Tables 17–20). But the female group, which received 5000 mg/kg of RCW, was found to have an AST level higher than in the control group (p < 0.01) (Table 19). The satellite male group which received RCE showed a decrease in AST levels compared with the control group (p < 0.05) (Table 18) when male rats treated with RCE at the equivalent dose of consumer drinks, showed a significant increase in the liver function parameter such as ALT levels (p < 0.05) (Table 18). The male RCE group received doses of consumer drinks, this resulted in an

Fig. 5. Gross pathological of kidney in male at a single dose of 5000 mg/kg compared with control. Treat group receive RCW (A1, A3), RCE (B1, B3) and satellite group receive RCW (A2, A4), RCE (B2, B4) respectively for 90 day. glomerulus (g), bowman’s capsule (b) (the 50 × magnifications).
increase in ALT levels compared with the control group ($p < 0.05$). There were no changes in BUN and creatinine levels in either the male or the female groups treated with RCW and RCE. These results show that there was no effect on renal function parameters, but there was a significant increase in creatinine levels ($p < 0.05$) in the female satellite group treated with RCW at 5000, 3000, and 1460 mg/kg.

3.3.5. Effects on histopathological

The histological examination of the heart did not show any differences in histology or cardiac tissue changes compared to the control group (not shown). Microscopic examination of the vital organs of RCW and RCE in both the male and female treated for 14 days did not reveal any abnormalities in color or texture when compared with the organs of the control group. The light microscopy examinations of the transverse section of the liver and kidney organs of the treated and control group rats are shown in Figs. 1 and 2. There were no changes in the liver when compared with the control group. The hepatocytes were arranged in a radial distribution around the central vein and the hepatocytes did not show any abnormalities (Figs. 3 and 4). Furthermore, no abnormalities were found in the kidneys (the glomerulus and Bowman’s capsule). There were no ruptures in the outer and inner walls. The kidneys remained round or oval shaped (Figs. 5 and 6). In a liver histopathological examination of the control group, the rats treated with RCW and RCE showed normal structures and an absence of any gross pathological lesions in the organs. In a histopathological analysis of the kidney, neither the control group nor the treatment group showed symptoms of cloudy swelling, nuclear necrosis, or fibrosis. No changes were found in the adrenal glands when compared to the control group. Observation of the arrangement of the cells in the cortex layer and the cell characteristics in each layer of the cortex and medulla of the adrenal glands did not show any abnormalities either (Figs. 7 and 8).

Fig. 6. Gross pathological of kidney in female at a single dose of 5000 mg/kg compared with control. Treat group receive RCW (A1, A3), RCE (B1, B3) and satellite group receive RCW (A2, A4), RCE (B2, B4) respectively for 90 day. glomerulus (g), bowman’s capsule (b) (the 50 × magnifications).
3.3.6. A lipid peroxidation study measured the level of malondialdehyde (MDA)

The results showed that the MDA level in female rats treated with RCW at 5000, 3000, and 1460 mg/kg significantly decreased (p < 0.05) when compared to the control group. These results might explain why RCW had affected the antioxidant activity in the female rats treated. In addition, the results showed an increase in the MDA level in the satellite male rats treated with RCE 5000 mg/kg. This could be further evidence of antioxidant activity caused by RCE in male rats (Tables 21 and 22).

4. Discussion

In the preparation stage of producing natural products to be used as a medicine, normally, the first step to evaluating pharmacological activity is the assessment and evaluation of their toxicity. During these assessments and evaluation of toxic characteristics, usually, the first step is to
Fig. 8. Gross pathological of adrenal in female at a single dose of 5000 mg/kg compared with control. Treat group receive RCW (A1, A3), RCE (B1, B3) and satellite group receive RCW (A2, A4), RCE (B2, B4) respectively for 90 day. glomerulus (g), fasciculata (f), reticularis (r) (the 50 × magnifications).

Table 21
MDA level of rats receiving RCW at dose 1460 (the equivalent dose), 3000 and 5000 mg/kg for 90 day and satellite group.

| Rat  | Treat group | MDA level mM | Satellite group |
|------|-------------|--------------|----------------|
|      | Control     | 5000 | 3000 | 1460 | Control     | 5000 | 3000 | 1460 |
| Male | 28.37±1.07  | 24.83±0.75 | 24.75±6.12 | 24.42±6.75 | 53.37±1.57 | 21.31±0.39 | 19.08±2.48 | 27.53±4.76 |
| Female | 47.45±1.70 | 17.23±0.15* | 17.45±0.95* | 17.32±2.52* | 37.12±0.07 | 37.53±0.23 | 17.75±3.25 | 20.12±0.90 |

The values are express as mean ± S.D.; * Significant different from the control group (p<0.05) and ** (p<0.01).
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Table 22

MDA level of rats receiving RCE at dose 1025 (the equivalent dose), 3000 and 5000 mg/kg for 90 day and satellite group.

| Rat     | Treat group | Satellite group |
|---------|-------------|-----------------|
|         | Control     | 5000 | 3000 | 1025 | Control | 5000R | 3000R | 1025R |
| Male    | 42.90±0.96  | 32.47±0.60 | 28.75±4.67 | 28.26±3.50 | 32.25±1.24 | 72.90±0.67** | 29.33±1.11 | 27.16±2.49 |
| Female  | 37.33±0.09  | 15.32±0.10 | 18.19±2.48 | 18.77±0.83 | 35.58±0.19 | 31.31±1.43 | 29.27±3.70 | 27.83±4.25 |

The values are expressed as mean ± S.D.; *, ** Significantly different from the control group (p<0.05) and (p<0.01).

determine the LD50 [10]. There were no significant differences in body weight or food consumption of the rats treated with RCW and RCE, suggesting that the extracts do not adversely affect the general health status of such animals, as RCW at doses of 2 and 5 g/kg for 7 days and at doses of 500 mg/kg for 28 days did not reduce body weight or mortality compared with the control group during the experiment [9]. But, the administration of RCW at doses of 20, 200, 1000 and 2000 g/kg for 6 months showed an increase in the average body weight [11]. But, there were no abnormalities, or differences in behavior or mortality. Similarly, β-sitosterol, a substance contained in Rang Chuet, had no effects after the rats received Rang Chuet at doses of 2.5, 5 and 10 mg/kg for 60 days. The average daily weight gain of both males and females was normal. Although the relative weight of hearts, livers, kidneys, and adrenal glands increased or decreased, there were no gross pathological or histological alterations of these organs [12]. The hearts in the female rats which received high doses of ethanol extract were significantly larger in this study which suggested that flavonoids induced noradrenaline which alters the contraction of the aortic smooth muscles in rats [13]. The extract of Rang Chuet water leaves had an effect on the functioning of the circulatory system including lower blood pressure [14]. It also had an effect on the arteries which improves the relaxation of the veins when used in high doses. Later, the inner heart wall was thin and no blood clots formed on the heart wall when the rats were given 500 mg/kg of Rang Chuet extract for 28 days. The myocardium muscle layer did not show any inflammation or result in death. The epicardium muscle wall was smooth and no necrosis was detected on the fat layer of the outer heart wall [9]. Moreover, Rang Chuet extract has a protective effect against hepatocellular damage in rats exposed to alcohol. At appropriate doses, the rate of normal hepatocytes increased 2–3 times and the AST and ALT enzyme levels decreased. Hepatocellular necrosis levels were normal and hepatic triglyceride (HTg) levels were the same as those in the control group. The extracts of Rang Chuet leaf had the ability to prevent alcohol-induced liver damage [15]. There were no abnormalities in tissues or liver tissue alignment and no hepatocellular deaths of Kupffer’s cells were observed, which would indicate inflammation and congestion of the bile in the liver when given 500 mg/kg of Rang Chuet extract for 28 days [9]. In this research, the male rats treated with RCW at high doses showed significantly increased kidney weight similar to the relative weight of kidneys and adrenal glands which increased at high doses of the water extract when the rats were given water extracted from Rang Chuet leaves for 28 days [9]. When both male and female rats were given 2000 mg/kg of Rang Chuet extract for 6 months, the relative kidney weight increased when compared to that of the control group (p < 0.05) [11]. Despite the statistically significant increase in AST and ALT [16], as the AST and ALT enzymes were released from cells when the cellular tissue in which the enzymes were stored was destroyed and the enzymes increased proportionally to the rate of cell decay. In general, both enzymes can indicate abnormalities in the liver and heart. The National Laboratory Animals Center [17] reported that the normal AST and ALT levels of Wistar rats were approximately 160.83 and 47.53 U/L, respectively, indicating that the levels obtained in this study were normal when compared with the reference levels. The significant increases in BUN and creatinine were also consistent [16]. The BUN in rats was approximately 46–92 mg/dl [18] and the creatinine level in rats was about 0.1–0.8 mg/dl or mg% [17], although the BUN levels in the satellite female group were higher than in the control group (p < 0.05) but there were no changes in the renal tissue. The hematological parameters did not show any significant differences from those of the control group [9]. The National Laboratory Animal Center [17] reported that rats of 4–7 weeks of age had a red blood count of approximately 5.7–8.6 (× 10³ cell/mm³), and a white blood cell count of approximately 5.7–8.6 (× 10³ cell/mm³), with hemoglobin concentrations of approximately 12.3–15.9 g/dL, and MCV, MCH and MCHC in the range of approximately 51.3–64.1 × 10⁻⁶/red cell, 18.6–21.5 pg/red cells and 33.5–36.2 g/dL, respectively. So, the standard levels obtained from the National Laboratory Animal Center support those of this study.

5. Conclusion

A previous study of acute toxicity showed that oral LD50 of three Rang Chuet extracts using different solvents including water (RCW), ethanol (RCE), and acetone (RCA) extraction in Wistar rats of both sexes was higher than 15,000 mg/kg body weight and there was NOAEL (No observed adverse effect level) at 2000 mg/kg. Acute toxicity studies found that RCA had a slight hepatotoxicity but at an acceptable level. However, this study was a sub-chronic toxicity study of only RCW and RCE with doses of consumer drinks at 3000 and 5000 mg/kg. The satellite group was given the same doses of both extracts for 90 days and observed thereafter for 14 days in order to study any adverse effects. The results showed that there was a normal gain in weight. Some vital organs, such as the lungs, hearts, and kidneys decreased in their relative weight while the relative weight of the adrenal glands increased. The hematological and clinical chemistry levels were in the normal range of rats. However, the lipid peroxidation test measuring the level of MDA showed a decrease in all groups compared with the control group, while the satellite male group which received doses of 5000 mg/kg of RCE increased significantly compared with the control group (p < 0.01). From this study, the NOAEL (No observed adverse effect level) of RCW and RCE obtained at 5000 mg/kg body weight shows that the safety levels of toxic substances in humans and NOAEL/100, 100 is derived from difference in individual sensitivity (10) × the difference in sensitivity between humans and animals (10), while the results of the safety levels of RCW and RCE in humans is 50 mg/kg body weight. So, a safe dose of Rang Chuet dried powder is 10.27 g/60 kg body weight/day. Finally, Rang Chuet could be used as a resource for phenolic compound which showed antioxidant activity.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.
Acknowledgements

The author would like to thank Prof. Dr. Banjob Sripa, Department of Parasitology, Khonkaen University for helping with histopathological analysis and Assistant Professor Dr. Benjamart Chitsomboon for giving experimental guidance. This research work was supported by Suranaree University of Technology (SUT), Nakhon Ratchasima and by Thailand Science Research and Innovation (TSRI), and National Science, Research and Innovation Fund (NSRF), Thailand.

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