Food Research 6 (Suppl. 2) : 1 - 8 (2022)
Journal homepage: https://www.myfoodresearch.com

FOOD RESEARCH

FULL PAPER

Acute and sub-acute toxicity studies of roselle leaves

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Article history:
Received: 18 September 2021
Received in revised form: 21 October 2021
Accepted: 21 October 2022
Available Online: 8 December 2022

Keywords:
Acute,
Sub-acute,
Toxicity study,
Roselle leaves,
Sprague Dawley

DOI:
https://doi.org/10.26656/fr.2017.6(S2).011

Abstract

Roselle leaves are widely used in traditional medicine but have not previously been studied for their safety through standard in vivo toxicity studies. This study aimed to evaluate the safety of roselle leaves extract through acute and sub-acute oral toxicity studies in *Sprague Dawley* (SD) rats. An acute toxicity study was carried out using a single high dose of roselle leaves extract at 3000 mg/kg and toxic signs were observed within 14 days. While sub-acute toxicological study was carried out using 28-days repeated doses of 0 mg/kg (control), 1000 mg/kg, 2000 mg/kg, and 5000 mg/kg of roselle leaves extract. Bodyweight changes were recorded throughout the experimental period. On day 29, blood was withdrawn and analysed for haematology and clinical biochemistry values. During the 14-day acute toxicity study, there were no significant changes in body weight increment, adverse clinical signs and mortality. In the sub-acute toxicity study, there were also no toxicology significant changes in haematology and clinical biochemistry except a slight decrease in triglyceride (TG) and glucose level ($p < 0.05$) which is attributed to roselle leaves aqueous extract administration. However, the results were within the normal reference range of SD rats. Therefore, the acute and sub-acute toxicity studies in SD rats showed that roselle leaves aqueous extract is non-toxic and could be classified as a no-observed-adverse-effect level (NOAEL).

1. Introduction

Hibiscus sabdariffa Linne, also known as Roselle, sorrel, mesta and karkade grows in many tropical and sub-tropical countries and is one of the highest volume speciality botanical products in international commerce. Roselle is part of the Malvaceae family and is cultivated in almost all tropical countries such as Malaysia, Southeast Asia, Indonesia and the Philippines (Halimatul *et al.,* 2007; Singh *et al*., 2017). There are three cultivars of roselle in Malaysia known Red Roselle, Wild Red Roselle, and Yellow Roselle (Junus, 2007). Roselle is grown for the fruit calyx which is a plant part of commercial interest (Ansari *et al*., 2013). The fruit is dark red in colour (rich in anthocyanin - phenolic compound) and has a sour taste. The fresh calyx collected can be processed for various food products such as tea, jam, beverages, and food colouring (Qi *et al.,* 2005; Singh *et al*., 2017). In India and China, the roselle leaves and stems are traditionally used for medicinal properties. Roselle is also popular in some countries for health purposes. Roselle leaves and fruits are claimed to reduce menstrual pain in women, treat degenerative diseases such as hypertension, pyrexia, liver damage, and kidney inflammation and control high blood pressure (Lin *et al.,* 2007; Riaz and Chopra, 2018). There are scientific evidence reports that the roselle extract is rich in polyphenols which act as antioxidants with the ability to remove free radicals and protect our body from health problems. Previous studies have also reported that roselle leaves extract has antioxidant, hypoglycaemic, hypolipidemic, and oestrogenic effects. Other scientific studies reported that dried roselle leaves aqueous extract has shown a potent inhibition effect on selected cancerous cells toward human prostate cancer (Lin *et al.,* 2012).

Despite a wide distribution in foods, a broad spectrum of pharmacological activities, and potential health benefits, roselle leaves have not previously been evaluated for their safety using standard in vivo toxicity studies. Therefore, there is a pressing need to clarify the toxicological profile of roselle leaves. The objective of this study was to evaluate the in vivo toxicology study of the roselle leaves extract in *Sprague Dawley* (SD) rats.

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eISSN: 2550-2166
Published by Rynnye Lyan Resources

2. Materials and methods

2.1 Sample preparation

Roselle leaves were collected from a plantation plot in Karak, Pahang, Malaysia. The roselle leaves were cleaned and dried using a commercial oven dryer at a temperature of 40°C until the moisture content reached below 10%. The dried roselle leaves were then ground using a Waring blender (Waring, Connecticut) and passed through a 0.5 mm sieve. The extract of roselle leaves was prepared by infusing 2.0 g ground dried leaves with 250 mL boiling water for 10 mins. The extract was prepared at 3000 mg/kg for acute study and three different concentrations (1000, 2000, and 5000 mg/kg) for sub-acute toxicological study.

2.2 Experimental animals

Male and female *Sprague Dawley* rats weighing 150-250 g each at 5-6 weeks old were used for the acute and sub-acute toxicity study. The animals were housed in a system-controlled environment for the light-dark cycle (12–12 hrs, lights on 7:00–19:00), temperature (24±2°C) and relative humidity (30-70%) during the study. The animals were provided distilled water (ad libitum) with a standard pellet (Specialty Feeds, Australia) and allowed to acclimatize for 7 days to ensure their health status of the animals. All procedures in this study were carried out according to MARDI's Animal Ethics Committee document: 20180810/R/MAEC00035.

2.3 Acute oral toxicity study

A single dose acute oral toxicity study was carried out according to Ryu *et al.* (2004) and OECD 423 (OECD, 2002). A total of ten SD rats (multi-gender equally) weighing 150-200 g were divided into two: a control group and the roselle leaves extract (RE) treatment group. The SD rats were left for overnight fasting (8-10 hrs) and then the treatment group was given a single dose of roselle leaves extract (3000 mg/kg of body weight) by oral administration whereas the control rats received only distilled water. All animals were observed for clinical signs including mortality and any adverse reactions immediately after dosing at 1, 2, 4, and 6 hrs, then once daily until day 14. The visual observation included changes in the skin and fur, eyes, and bizarre behaviour. The body weight was measured once before the commencement of the dosing and then daily until day 14 (Abdullah *et al.,* 2009).

2.4 Sub-acute oral toxicity study

Twenty-eight days of repeated dose or sub-acute toxicological study were carried out according to Ryu *et al.* (2004) and OECD 407 (OECD, 2008). A total of twenty SD rats (female, weighing 200-250 g) were divided into four groups; control and low, medium and high dose treatment groups (5 rats for each group). The control group was given distilled water via ad libitum while the treatment group was dosed with roselle leaves extract of 1000 mg/kg (LD, low dose), 2000 mg/kg (MD, medium dose) and 5000 mg/kg (HD, high dose) of body weight via ad libitum. Each rat will be administered an averagely of 100 ml of water / RE per day. Any remaining sample left was measured. The body weight was recorded weekly and each rat's behaviour/general appearance was observed daily until day 28.

2.5 Haematology and serum biochemistry measurement

On day-29, all the SD rats were anaesthetized after approximately 12 hrs overnight fasting. Blood samples were drawn from the vena cava and approximately 20 µL of blood per animal was treated in a 3 mL ethylene-diamino-tetraacetic-acid (K3-EDTA) tube (Bacton Dickinson, BD Vacutainer) to analyse haematological indexes. The blood sample was analysed for a complete blood profile: red blood cell (RBC), white blood cell (WBC), platelet (PLT), haematocrit (HCT), and haemoglobin (Hb) level. The measurements were performed by Haematology Analyzer (Medonic CA530, Italy).

For serum biochemical blood analysis, one aliquot of blood per animal was placed in a 5 mL Z-serum tube (Bacton Dickinson, BD Vacutainer) and centrifuged at 3,000 rpm for 20 mins. The serum was analyzed for alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) activities, total protein (TP), bilirubin, albumin (Alb), globulin, albumin/globulin (A:G) ratio, urea, creatinine (Cr), glucose (Glu), total cholesterol (TC), low-density lipoprotein (LDL) and high-density lipoprotein (HDL) by using Blood Clinical Analyzer (Vitalab Selectra E, Italy). The reagents for the analyses were from Randox (Randox Laboratories Ltd., Antrim, United Kingdom).

2.6 Statistical analysis

The data for organ weights as well as the results of haematology and serum biochemistry were tested by conducting a one-way analysis of variance (ANOVA) using the SAS System, ver. 9.0 statistical software. When statistically significant differences were indicated, the Duncan New Multiple Range Test (DMRT) was employed for comparisons between control and treated groups. All values are expressed as mean ± standard error mean (SEM) and a difference was considered significant when $p < 0.05$.

https://doi.org/10.26656/fr.2017.6(S2).011

3. Results and discussion

3.1 Acute oral toxicity study

During the 14-day acute toxicity study, there were no significant changes in body weight increment, adverse clinical signs or mortality. The mean weekly body weight increment in the control group and treated group (males and females) are shown in Figure 1 and Figure 2. There were no significant differences in body weight increment between the roselle leaves extract administrated group and the control group in both sexes. This result showed that the roselle leaves extract is safe since there were neither toxic signs nor mortality observed after administration of a single high dose (3000 mg/kg BW) extract and yet, bodyweight increment was noted. Bodyweight increment was a positive indication and suggested no toxicological effect in the treated group.

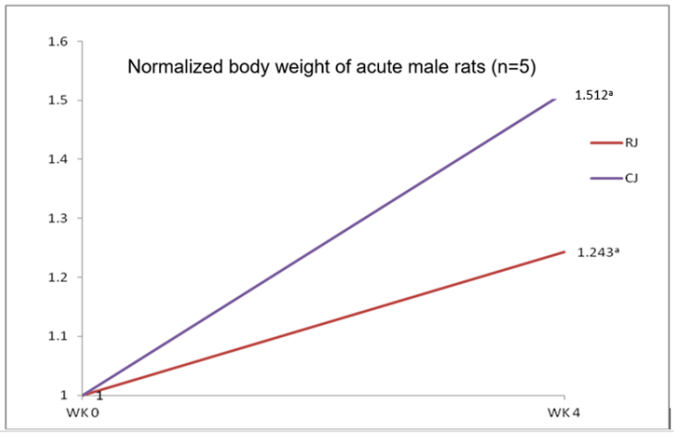

Figure 1. Normalized body weight of male rats in acute toxicity study (n = 5; RJ, single high dose of roselle leaves extract and CJ, control group). Means with different superscript are significantly different (p<0.05).

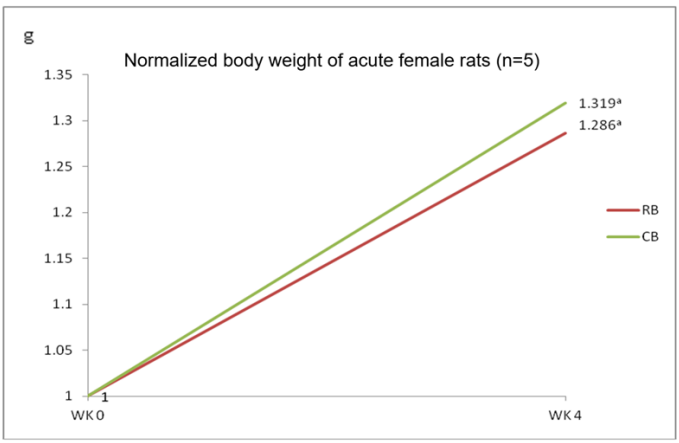

Figure 2. Normalized body weight of female rats in acute toxicity study (n = 5; RB, single high dose of roselle leaves extract and CB, control group). Means with different superscript are significantly different (p<0.05).

3.2 Sub-acute oral toxicity study

3.2.1 Clinical observations and body weight assessment

The mean body weights in control and treated SD rats are shown in Figure 3. The mean body weights of each treated group (1000, 2000 and 5000 mg/kg of body weight) showed that there was no significant difference compared to the control group. According to Teo *et al.* (2002) and Hilaly *et al.* (2004), abnormal body weight changes was used to predict any adverse effects of chemical and drugs. The data showed as the dosage of roselle leaves extract (RE) was increased, no significant differences were detected which indicates non-toxicity effects and good health. During 28-days of observation, there was no death, no toxicological symptoms such as rashes, or skin irritation and no abnormalities in hair coat and eye colour.

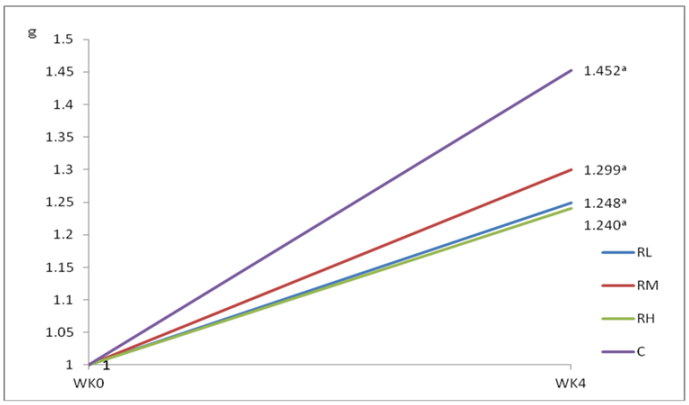

Figure 3. Normalized body weight of female rats for sub-acute toxicity study (n = 5; RL - low dose, RM - medium dose; RH - high dose and C - control group). Means with different superscript are significantly different (p<0.05).

3.2.2 Organ weights assessment

Organ weight changes are one of the most sensitive toxicity indicators for test item-induced changes to organs and are associated with treatment-related effects (Sellers *et al.,* 2007; Piao *et al*., 2013), as significant differences in organ weights between treated and untreated animals may exist if there are no morphological changes (Piao *et al.,* 2013). Organ weights are normally reported as the percentage of body weight or as relative values (Wolfsegger *et al.,* 2009) which is calculated as organ weight per 100 g of body weight during necropsy. The differences in relative organ weights can be used as an indicator of adverse effects and to recognize the affected organs.

In this study, the actual and relative organ weights were weighed while the necropsy was on day 29. The data of the relative organ weights are shown in Table 1. Compared to the control group, there were no significant differences in the relative organ weights of kidneys and

https://doi.org/10.26656/fr.2017.6(S2).011

Table 1. Relative organ weight (percentage) of female *Sprague Dawley* rats for sub-acute toxicity study.

Analysis	*n*	Control (0 mg/kg)	Low Dose (1000 mg/kg)	Medium Dose (2000 mg/kg)	High Dose (5000 mg/kg)
Kidney (%)	5	0.64±0.05[a]	0.66±0.06[a]	0.61±0.06[a]	0.65±0.06[a]
Heart (%)	5	0.36±0.03[b]	0.43±0.04[ab]	0.38±0.03[b]	0.47±0.11[a]
Lung (%)	5	0.64±0.07[a]	0.66±0.05[a]	0.63±0.09[a]	0.70±0.09[a]
Spleen (%)	5	0.18±0.03[b]	0.24±0.03[a]	0.22±0.03[ab]	0.21±0.06[ab]
Liver (%)	5	3.80±0.23[a]	2.82±0.35[b]	2.92±0.41[b]	2.93±0.53[b]

Values are presented as mean±SEM for five female rats in each group. Values with different superscript within the same row are significantly different (p<0.05).

lungs (at dosages of 1000, 2000, and 5000 mg/kg/day). The values are within the normal reference range of 0.58 - 0.85% and 0.45 - 0.77%, respectively (Han *et al.,* 2010). The relative spleen weight was significantly increased in the 1000 mg/kg treated group while the relative heart weight was significantly increased in the 5000 mg/kg treated group when compared to the control rats. But the results are still in the normal reference range of 0.19 - 0.38% and 0.33 - 0.53%, respectively (Han *et al.,* 2010). Furthermore, a gross examination of these internal organs revealed no detectable abnormalities.

In contrast, treated groups of 1000, 2000, and 5000 mg/kg had significantly decreased the relative liver weight (2.82, 2.92, and 2.93%, respectively) at $p < 0.05$ as compared to the control (3.80%). The liver is the most targeted organ as it transforms and clears any toxicants in human blood. According to Gianni *et al.* (2005) and Mumoli *et al.* (2006), hepatotoxicity or chemical-driven liver damage is correlated with the increase in liver weight, alkaline phosphatase enzyme and aminotransferases level in plasma. The reduction of internal organ weight and body weight is usually considered an indicator of a toxic substance (Thanabhorn *et al.,* 2006). The reduction of liver weight shown in Table 2 cannot be assumed as a sign of toxicity since the alanine aminotransferase (ALT) and alkaline phosphatase (ALP) levels significantly decreased but the aspartate aminotransferase (AST) levels increased as compared to control. However, these results still can be acceptable and considered non-toxic since the liver weight values of the treated rats are in the normal range of 2.43 - 3.4% (Han *et al.,* 2010).

Thus, the relative organ weights of *Sprague Dawley* rats were not significantly affected by the roselle leaves extract administration. Meanwhile, the whole observation during the necropsy did not show any experiment-related changes. No lesion, inflammation, bleeding, abnormal enlargement or spots of internal organs have been observed in all treated rats as compared to control.

3.2.3 Serum biochemistry

Serum biochemical data for female *Sprague Dawley* rats treated orally with roselle leaves extract for a 28-day repeated-dose sub-acute study is shown in Table 2. Triglyceride (TG) values were significantly decreased in all treated groups while high-density lipoprotein (HDL) level was decreased ($p < 0.05$) in the medium dose treated group when compared to the control group. There was no significant difference was noted in total cholesterol as well as low-density lipoprotein (LDL) values as compared to the control.

Table 2 also showed that the glucose level decreased significantly in all treated groups as compared to the control and the glucose level stayed in a normal range of 1.9 to 12.0 mmol/L (Petterino and Argentino-Storino, 2006) or 3.5 to 11.2 mmol/L (Delwatta *et al*., 2018). These data suggested that roselle leaves extract possesses a positive effect in reducing glucose levels and lipid serum which can reduce cholesterol and lipid in the blood, thus reducing the risk of heart disease and obesity. The previous study has reported that roselle calyces extract which is rich in anthocyanin, quercetin, and polyphenols have a positive effect on reducing cholesterol level and diabetes management including lowering abnormal elevation in plasma AGE (advanced glycosylation end products) formation, increasing basal insulin level and lower blood glucose level (Patel, 2014). Hopkins *et al.* (2013) also reported that oral administration of roselle calyces extract could reduce triglyceride, total cholesterol and fat tissue accumulation in the hyperlipidemic animal. The extract also could reduce body weight gain and triglyceride levels in high-fat diet animals. Beltran-Debon *et al.* (2010) reported that polyphenol-rich plant extracts reduced triglyceride levels since the major triglyceride containing very-low-density lipoprotein (VLDL) was reduced. These scientific reports supported that roselle leaves extract has the potential in reducing lipids serum as roselle leaves extract is also rich in polyphenols.

Besides, the data showed no significant changes in serum protein (albumin, globulin and total protein level), total bilirubin and A:G ratio in all treated groups when compared to the control group. These data were considered toxicologically irrelevant because they were

https://doi.org/10.26656/fr.2017.6(S2).011

Table 2. Serum biochemical data for female SD rats treated orally with roselle leaves extract (RLE) for 28-days repeated-dose sub-acute study

Analysis	Control (0 mg/kg)	Low Dose (1000 mg/kg)	Medium Dose (2000 mg/kg)	High Dose (5000 mg/kg)
ALT (U/l)	80.77±10.85^{a}	49.33±10.03^{b}	36.17±8.18^{c}	40.17±5.42^{bc}
AST (U/l)	96.72±17.08^{b}	142.21±18.77^{a}	127.44±33.99^{a}	127.56±28.50^{a}
ALP (U/l)	270.33±90.94^{a}	123.00±17.44^{b}	126.50±25.87^{b}	121.17±43.14^{b}
Bilirubin (µmol/l)	2.66±0.53^{a}	2.63±0.60^{a}	2.63±0.37^{a}	3.06±0.39^{a}
Glucose (mmol/L)	6.98±0.42^{a}	5.78±1.01^{b}	5.48±0.33^{b}	5.60±1.06^{b}
Total Protein (g/l)	72.93±3.17^{a}	69.25±7.66^{a}	72.15±5.01^{a}	69.26±4.20^{a}
Albumin (g/l)	41.77±2.60^{a}	41.17±3.53^{a}	41.67±2.93^{a}	41.12±1.96^{a}
Globulin (g/l)	31.11±3.30^{a}	28.17±5.67^{a}	30.33±2.94^{a}	28.00±4.05^{a}
A:G	1.36±0.20^{a}	1.52±0.36^{a}	1.38±0.15^{a}	1.48±0.20^{a}
Urea (mmol/L)	6.27±1.66^{a}	5.48±0.30^{a}	6.23±1.43^{a}	5.92±0.58^{a}
Creatinine (µmol/l)	51.78±6.72^{a}	52.17±5.04^{a}	54.00±8.17^{a}	50.17±3.43^{a}
TC (mmol/L)	1.61±0.22^{a}	1.53±0.30^{a}	1.36±0.28^{a}	1.44±0.36^{a}
HDL (mmol/L)	0.67±0.09^{a}	0.57±0.11^{ab}	0.54±0.13^{b}	0.56±0.10^{ab}
LDL (mmol/L)	0.511±0.28^{a}	0.75±0.14^{a}	0.60±0.11^{a}	0.65±0.24^{a}
TG (mmol/L)	0.97±0.44^{a}	0.46±0.17^{b}	0.54±0.22^{b}	0.49±0.13^{b}

Values are presented as mean±SEM for five female rats in each group. Values with different superscript within the same row are significantly different (p<0.05). ALT: Alanine Aminotransferase, AST: Aspartate Aminotransferase, ALP: Alkaline Phosphatase, A:G: albumin/globulin ratio, TC: total cholesterol, HDL: high density lipoprotein, LDL: low density lipoprotein, TG: triglyceride.

within normal physiological ranges (Alemán *et al.,* 1998; Petterino and Argentino-Storino, 2006; Han *et al*., 2010), and were not dose-related or reflected by any changes in the related parameters. There was also no significant difference noted in the urea level in all treated rats as compared with the control group. Moreover, the results were below the normal reference range of 6.9 to 30.5 mmol/L (Petterino and Argentino-Storino, 2006).

A reduced level of urea is usually caused by malnutrition, insufficient protein, or liver damage. Urea and creatinine levels are used as an indicator to detect any kidney problems. According to Hassan *et al.* (2007) and Rhionani *et al.* (2008), a high level of creatinine and urea in the blood indicates a serious kidney problem in which urea nitrogen in the kidney might not be filtered out of the blood serum into the urine. No kidney malfunction was detected as the data in Table 2 did not show any significant differences in creatinine (range from 50.17 to 54.00 µmol/L) and urea (range from 5.48 to 6.27 mmol/L) levels in all treated groups as compared to the control group.

Ennulat *et al.* (2010) reported that the measurement of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in blood serum is a standard clinical chemistry examination in toxicity studies in animal models. ALT exists in many organ tissues with a high concentration in hepatocytes and it is considered a liver-specific enzyme. While AST presents in other tissues with a high concentration in muscle (Gianni *et al.,* 2005). Alkaline phosphatase (ALP) is a hydrolysed enzyme that is eliminated in the bile and is particularly present in the cells, which line the biliary ducts of the liver. An increased level of ALP is associated with hepatotoxicity (Deepalakshmi and Mirunalini, 2014) and decreased activity is associated with fasting or decreased food consumption (which may result in lower serum ALP activity in rats) (Center, 2007). According to Yakubu *et al.* (2003), Mumoli *et al.* (2006) and Amang *et al.* (2020), liver damage is associated with the increase of ALT, ALP and bilirubin where the increasing level is due to either (i) ALP level higher than the twice upper limit of normal (ULN), (ii) ALT level exceeding three times of ULN, or (iii) total bilirubin level higher than twice ULN. The previous study also showed that an increased level of ALT and/or AST activity in the serum is accepted as an indication of hepatic toxicity in rats (damage to hepatocytes results in leakage of these enzymes into the blood) (Witthawaskul *et al.,* 2003; Ennulat *et al.,* 2010; Amang *et al*., 2020).

Table 2 shows that there were significant changes in ALT, ALP and AST levels in all treated groups when compared to the control group. ALP and ALT levels were decreased (p < 0.05) in all treated groups but within the normal reference range of 90.5 to 769.8 U/L and 13.5 to 52.5 U/L, respectively (Petterino and Argentino-Storino, 2006). These ALP and ALT levels were also within the reference range of 195.0 to 724.2 U/L and 2.1 to 426.9 U/L, respectively (Delwatta *et al*., 2018). While the AST level increased (p < 0.05) in all treated groups as compared to control but the values are in a normal

https://doi.org/10.26656/fr.2017.6(S2).011

Table 3. Data of haematology for 28-days repeated-dose sub-acute study in female *Sprague Dawley* rats.

Analysis	Control (0 mg/kg)	Low Dose (1000 mg/kg)	Medium Dose (2000 mg/kg)	High Dose (5000 mg/kg)
RBC (10^{12}/l)	7.77±0.30[a]	7.86±0.64[a]	7.69±0.62[a]	8.06±0.57[a]
HCT (%)	38.21±2.73[a]	41.35±4.18[a]	40.87±2.90[a]	37.72±3.15[a]
PLT (10^9/l)	1235.44± 220.75[a]	1260.67± 147.16[a]	1270.17± 442.99[a]	1101.33± 104.04[a]
WBC (10^9/l)	4.87±1.30[a]	4.90±2.76[a]	3.77±2.66[a]	4.70±4.92[a]
Hb (g/l)	146.56±7.18[a]	151.17±16.40[a]	144.00±9.76[a]	155.50±11.59[a]

Values are presented as mean±SEM for five female rats in each group. Values with different superscript within the same row are significantly different (p<0.05). RBC: red blood cell, HCT: haematocrit, PLT: platelet, WBC: white blood cell, Hb: haemoglobin.

range of 29.5 to 144.7 U/L (Petterino and Argentino-Storino, 2006) and 20.8 to 470.2 U/L (Delwatta *et al*., 2018). The data also showed non-significant increasing levels of total bilirubin and indicated non-toxicological significant changes in serum biochemistry analyses. These data were supported by Zhang *et al.* (1996) and Amang *et al*. (2020), who reported that a lower level of ALT and ALP could indicate a hepatoprotective effect.

3.2.4 Haematology

In haematology parameters, Table 3 shows that there were no significant differences between the treated and control groups in white blood cell count (WBC), red blood cells (RBC), haematocrit (HCT), haemoglobin (Hb) and platelet (PLT). It can be concluded that there was no toxic effect of roselle leaves extract administration on the haematology value of all treated *Sprague Dawley* rats. In addition, the values are in the normal reference range as reported by Petterino and Argentino-Storino (2006) and Delwatta *et al.* (2018).

4. Conclusion

A high single dose of roselle leaves extract (3000 mg/kg of body weight) did not show any acute toxicological signs in *Sprague Dawley* rats. Thus, the no-observed-adverse-effect level (NOAEL) for *Sprague Dawley* rats is more than 3000 mg/kg. In the 28-day sub-acute oral toxicity study, daily doses of 1000, 2000, and 5000 mg/kg of RLE were well tolerated and did not cause either lethality or toxic clinical symptoms and changes in SD rats. The dose of 5000 mg/kg/day was identified as the NOAEL in this study. These results could be a good reference for roselle leaves extract for further clinical trials as a medication or usage as a food supplement for humans.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

The authors acknowledge with gratitude the financial support given by the Malaysian Ministry of Agricultural and MARDI for the research fund (Project Num: 21003001610001). The authors also thank Mrs Nurul Nabilah Mohd Fiteri, Miss Nazarifah Ibrahim and Miss Nurhafiqa Mohamad Hayadi for their technical supports and assistance with the experiments.

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