

Acute and repeated dose 28-day oral toxicity of *Ruta angustifolia* Pers. leaves ethanolic extract in Wistar rats

[Toxicidad oral aguda y por dosis repetidas durante 28 días del extracto etanólico de hojas de *Ruta angustifolia* Pers. en ratas Wistar]

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Abstract

Context: *Ruta angustifolia* Pers. has been used as a traditional herb among counties. The ethanol extract of *R. angustifolia* has proven active as an anti-hepatitis C virus *in vitro*. Due to the potency of this plant, a safety evaluation is necessary.

Aims: To examine the acute and sub-chronic toxicity of ethanol extract of *R. angustifolia* leaves in rats.

Methods: An acute toxicity study was done by treating 70% ethanol extract of *R. angustifolia* (doses of 5, 50, 300, and 2000 mg/kg of BW), while the control group received 1% Tween-80. Toxicity signs and mortality of rats for 14 days were observed. While sub-chronic (28-days repeated dose) toxicity was evaluated with 4 groups, both males and females. The three groups were treated with 70% ethanol extract of *R. angustifolia* at doses of 50, 500, and 1000 mg/kg BW daily for 28 days, and one group served as a control group. Biochemical, hematological, and histopathological parameters were measured after the period of treatment.

Results: The acute toxicity was no demonstrated toxicity signs or mortality effects up to 14 days of the experiment. Therefore, the LD₅₀ value of the extract was estimated to be higher than 2000 mg/kg BW. The sub-chronic assay revealed no mortality and toxicity effects, including behavior parameters, relative body weight, hematology, and biochemical at 50 and 500 mg/kg BW doses. However, a significant change was seen in the testis of all doses and the liver of the male rats treated at 1000 mg/kg BW. Histopathology assay observed the presence of activated Kupffer cells, sinusoidal dilatation, and infiltration of lymphocytes at the portal vein in the liver with 1000 mg/kg BW. Those may indicate possible toxicity in the liver at the dose of 1000 mg/kg BW. However, no toxic effect at the lower doses tested.

Conclusions: These results suggest that the use of *Ruta angustifolia* leaves ethanolic extract is relatively safe. The application of high doses needs to be evaluated.

Keywords: health; medicine; medicinal plants; *Ruta angustifolia*; toxicity assay.

Resumen

Contexto: *Ruta angustifolia* Pers. se ha utilizado como una hierba tradicional entre los condados. El extracto etanólico de *R. angustifolia* demostró ser activo como virus anti-hepatitis C por *in vitro*. Debido a la potencia de esta planta, es necesaria una evaluación de seguridad.

Objetivos: Examinar la toxicidad aguda y subcrónica del extracto etanólico de hojas de *R. angustifolia* en ratas.

Métodos: Se realizó un estudio de toxicidad aguda tratando extracto etanólico al 70% de *R. angustifolia* (dosis de 5, 50, 300 y 2000 mg/kg de PC), mientras que el grupo control recibió Tween-80 al 1%. Se observaron signos de toxicidad y mortalidad de ratas durante 14 días. Mientras que se realizó una evaluación de la toxicidad subcrónica (dosis repetida de 28 días) con 4 grupos, tanto de machos como de hembras. Los tres grupos fueron tratados con extracto etanólico al 70% de *R. angustifolia* en dosis de 50, 500 y 1000 mg/kg PC diariamente durante 28 días, y un grupo sirvió como grupo de control. Los parámetros bioquímicos, hematológicos e histopatológicos se midieron después del período de tratamiento.

Resultados: La toxicidad aguda no demostró signos de toxicidad ni efectos de mortalidad hasta los 14 días del experimento. Por lo tanto, el valor DL₅₀ del extracto se estimó superior a 2000 mg/kg de peso corporal. El ensayo subcrónico no reveló efectos de mortalidad ni toxicidad, incluidos los parámetros de comportamiento, el peso corporal relativo, la hematología y la bioquímica en dosis de 50 y 500 mg/kg de peso corporal. Sin embargo, se observó un cambio significativo en los testículos de todas las dosis y en el hígado de las ratas macho tratadas con 1000 mg/kg de peso corporal. El ensayo de histopatología observó la presencia de células de Kupffer activadas, dilatación sinusoidal e infiltración de linfocitos en la vena portal hepática con 1000 mg/kg BW. Estos pueden indicar una posible toxicidad en el hígado a la dosis de 1000 mg/kg de peso corporal. Sin embargo, no se observaron efectos tóxicos a las dosis más bajas ensayadas.

Conclusiones: Estos resultados sugieren que el uso del extracto etanólico de hojas de *Ruta angustifolia* es relativamente seguro. La aplicación de dosis altas necesita ser evaluada.

Palabras Clave: ensayo de toxicidad; medicamento; plantas medicinales; *Ruta angustifolia*; salud.

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INTRODUCTION

Medicinal plants have been widely used to protect humans against serious diseases. Around 80% of the global population relies on plant-derived drugs (Ramsay, 2002). The plants possess various secondary metabolites, and their derivatives represent an important source of pharmaceuticals in diverse pharmacological activities, including antiviral (Bachar et al., 2021, Nalawade and Tsay, 2004).

The *Ruta* genus was popularly known as Rue and has been applied as traditional worldwide. The genus of *Ruta* was traditionally used to relieve pain, dermatitis, rheumatoid, and inflammatory diseases in the Mediterranean (Pollio et al., 2008). It is also applicable in Ayurveda Indian traditional medicine for various pharmacology for headaches, muscular chest pain, and respiratory diseases such as bronchitis and arthritis (Parray et al., 2012). While in Indonesia, *R. angustifolia* is traditionally used for cramps, flatulence, fever, and liver disease or jaundice (Richardson et al., 2016; Wahyuni et al., 2014). Jaundice is a yellowing commonly known as a symptom of hepatitis virus infection. This plant also possesses antifungal and antimicrobial activities (Haddouchi et al., 2013; Shuib et al., 2015). Our previous study explored *Ruta angustifolia* Pers. (family Rutaceae) for anti-hepatitis C virus. We reported that the extracts of *R. angustifolia* exhibit anti-hepatitis C virus activities with 50% of inhibitory concentration 50 (IC₅₀) between 1.6 to 15.6 µg/mL (Wahyuni et al., 2014). The ethanol extract showed inhibitory activity dominantly at the post-entry step of virus cycles and decreased the NS3 and NS5A levels of the hepatitis C virus. The extract revealed a synergistic effect with the standard drug for the hepatitis C virus, directly acting as an antiviral (Wahyuni et al., 2019). Phytochemistry screening of 70% ethanol extract of *R. angustifolia* contains alkaloids, flavonoids, terpenoids, and polyphenols (Wahyuni et al., 2021). Isolation of *R. angustifolia* extract obtained chalepin and pseudane IX, which mediated high anti-HCV activity (Wahyuni et al., 2014). Angustifolin, alkaloid graveolin, scoparone, 6,7,8-trimethoxycoumarin, and moskachans A, B, C, and D, have also been isolated from *R. angustifolia* (Del Castillo et al. 1984; 1986). Other studies also determined the quantitative evaluation and standardization of 70% ethanol extracts of *R. angustifolia* (Wahyuni et al., 2021; 2023). Due to the potential activities of *R. angustifolia*, it provides a potential for product development as traditional medicine.

Further studies are needed to guarantee the safety of extract for pharmaceutical products. The present study conducted safety evaluation by acute toxicity

assay and repeated doses for 28 days. The toxicities studies report the lethal dose 50 (LD₅₀) and possible side effects, including the sign of toxicities. Other studies reported no toxic effects of *R. angustifolia* extract derived from another area; however, side effects were shown (Hidayat et al., 2021). The difference in cultivation area may cause a variety of phytochemical properties, which may influence its toxicity. The toxicity assay was conducted in order to ensure the safety for the purpose of product development. Oral acute toxicity assay and repeated dose application for 28 days were performed by *in vivo* studies in the rat model following the Organization for Economic Cooperation and Development (OECD) guideline. Evaluation of toxicities, including hematology and biochemical analysis, examines the effect in various organs and histology profiles, which have not yet been reported in other studies to the best of our knowledge.

MATERIAL AND METHODS

Plant material

The plant of *Ruta angustifolia* was harvested in June 2020 and cultivated in the Jombang area, East Java, Indonesia (7°27'27.8"S, 112°15'49.1"E). Plant material identification and authentication were conducted in Materia Medica Indonesia, and the voucher specimen (RA-01) was deposited at the Natural Product Medicine Research and Development, Institute of Tropical Disease, Universitas Airlangga, Surabaya, Indonesia.

Extraction of plant

The leaves of *R. angustifolia* were dried at 45°C in the oven and pulverized into a powder. The extraction was carried out by macerating the powder plant (1.0 kg) in a flask with 13 L of 70% ethanol. The maceration process was conducted three times with a duration of 24 h. The filtrate was collected and concentrated by evaporation process to obtain the paste consistency of the extract. After that, a drying process in the oven (40°C) was conducted to obtain a constant weight. The final yield of 28% of the dried extract was obtained. The extract was stored in a refrigerator (4°C) until use.

Experimental animal

Wistar male rats (weighing 145-165 g) were used in acute toxicology, while both males and females were used in the study of subchronic toxicity. The rats were obtained from Padia Laboratories, East Java, Indonesia. The rat was housed under standard conditions at 25 ± 3°C, relative humidity of 70%, and at 12 h

light/12 h dark cycle. The rats were allowed to acclimatize for 7 days before commencing the experiments.

Ethical approval

All the procedures followed the animal ethics guidelines of the Faculty of Veterinary Medicine, Universitas Airlangga. The ethical approval was certified by the Animal Care and Use Committee (AUCC), Committee ethic of Universitas Airlangga, and provided the certificate number 2KE.116.03.2021.

Acute toxicity assay

The experiment followed the guideline of OECD 425 for the testing of chemicals (OECD, 2022). All animals fasted overnight before treatment. The rats were weighed and given the doses according to their body weight. Twenty-five male rats were randomly divided into 5 groups ($n = 5/\text{group}$). Group 1 was provided as the control, and the other four groups (G2-G5) were treated with 70% ethanol extract of *R. angustifolia* with 5, 50, 300, and 2000 mg/kg doses body weight. The control group was given 1% Tween-80, while the treatment groups received 70% ethanol extract of *R. angustifolia* suspended in 1% Tween-80 through oral gavage. The observation of animals was done every 30 min, 4 h after treatment, and every 24 h up to 14 days.

Those observations evaluate the mortality case, clinical toxicities signs, abnormalities behavior, and body weight. During this study period, clinical observations were made for mortality, behavior, and any other abnormalities, and their weight was measured every 3 days. In the days of the 15th, the animal was anesthetized with 50 mg/kg body weight of ketamine. The sacrificed organs (liver, heart, lungs, spleen, kidney, stomach, gut, and ovaries) were collected, and the weight was measured.

Subchronic toxicity assay (repeated dose 28 days according to 407 OECD guidelines)

The analysis of subchronic toxicity was performed by the OECD guidelines no 407 (OECD, 2008). Both male and female Wistar rats were divided randomly by separating into 4 groups ($n = 10$; five males and five females). Different doses of extract (50, 500, 1000 mg/kg) were arranged in 1% Tween-80 and orally treated every day for 28 days. The control group received 1% Tween-80, and other groups received 70% ethanol extract of *R. angustifolia* with 50, 500, and 1000 mg/kg body weight doses. The body weight of all rats was evaluated weekly. At the end of 28 days, rats were sacrificed, and the organ of rats (liver, heart, lungs, spleen, kidney, stomach, and sex organs) were

collected for further histopathology analysis. Hematological and biochemical values were also measured.

Calculation of relative organ weight

The collected organs were observed individually for their percentage of relative organ weight (ROW). The calculation was followed by equation [1].

$$\% \text{ ROW index} = \frac{\text{Weight of organ}}{\text{The body weight of rats on the day 28}} \times 100 \quad [1]$$

Hematological and biochemical analysis

Hematology and biochemical evaluation were managed by taking the blood sample from the cardiac puncture at the end of the experiment after being anesthetized with ketamine. Two ml of collected blood was prepared in an EDTA-coated tube, stirred slightly, and kept at room temperature at 3 to 4 h for hematological analyses, while the other 2 mL of blood was kept in non-heparinized, followed by centrifuge at 4000 rpm for 10 min at 4°C for biochemical analysis. Hematological parameters (hemoglobin, erythrocyte, thrombocyte, erythrocyte sedimentation rate (ESR), leucocyte, eosinophils, basophils, neutrophils, lymphocytes, and monocyte) of blood sample were analyzed using an automated hematology analyzer (Sysmex XN450, Hamburg, Germany). The biochemical parameters were examined for serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), blood urea nitrogen (BUN), creatinine, and total bilirubin, which were analyzed with a Tokyo Boeki TMS50i (Japan) (Fan et al., 2022; Yang et al., 2020).

Histopathological studies

The rats were sacrificed, and the organ (liver, kidney, and spleen tissues) of all groups were separated for histological studies. The organ was washed in normal saline, fixed in 10% buffer formalin, and dehydrated with alcohol. The organs were fixed and cut into 5 μm thick sections, followed by hematoxylin and eosin stain staining. The histopathology profile was observed under a light microscope. The microscope feature of the organs was analyzed compared to the control for further statistical evaluation (Yi-Chen et al., 2018).

Statistical analysis

The results of all parameters tested were evaluated by statistical analysis using the GraphPad Prism 9.0 software (GraphPad Co. Ltd., San Diego, CA, USA). The data were analyzed using a one-way ANOVA. The significant differences among the groups were

Table 1. The behavioral pattern of rats in all groups.

Parameter	Observation of control and treatment groups																			
	4 h					24 h					72 h					14 days				
	G1	G2	G3	G4	G5	G1	G2	G3	G4	G5	G1	G2	G3	G4	G5	G1	G2	G3	G4	G5
Fur and skin	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Eye color	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Convulsion	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Sedation	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Skin color	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Drownise	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Diarrhea	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Urination	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Hypersensitivity	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Erection of fur	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Behavior	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Breathing	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Saliva	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Mortality	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil

G: Group; G1: 1% Tween-80 (negative control); G2-G5: 70% ethanol extract of *R. angustifolia* at doses of 5, 50, 300, and 2000 mg/kg body weight; N: Normal; Nil: No mortality observed.

examined by the Dunnett-comparison test. $P < 0.05$ expressed as significant.

RESULTS

Acute toxicity assay

The oral administration of 70% ethanol extract of *R. angustifolia* leaves had not recorded the toxic sign of behavioral responses up to 14 days of observation. During the monitoring period, there were no changes in the fur and skin, eye color, tremors, salivation, diarrhea, or behavior patterns. There was no observed lethality case over the 14 days of observation (Table 1). Those indicated that the LD₅₀ was estimated at more than 2000 mg/kg. The body weight increase of the rat throughout the experiment was within the normal range. The average weight among the groups had no significant difference on all days (Fig. 1). The statistical evaluation of relative organ weights showed no significant differences among groups (Table 2).

Subchronic toxicity study

General conditions

Daily oral administration of 70% ethanol extract of *R. angustifolia* leaves was administered for 28 days and did not induce toxicity in both male and female

rats in all tested doses. No death was observed among all groups throughout the experiment. Physical observations indicated no significant abnormalities in fur and skin, eye color, tremors, salivation, diarrhea, breathing, behavior patterns, sleep, and coma. The observation of body weight showed no significant differences between the treated and control groups (Fig. 2).

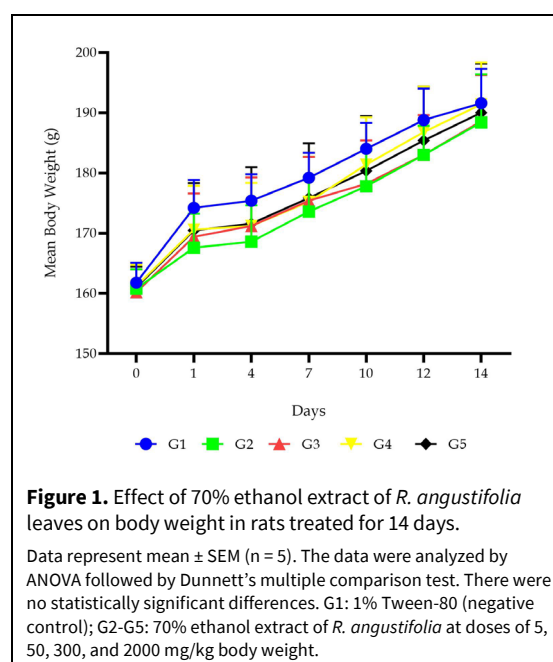
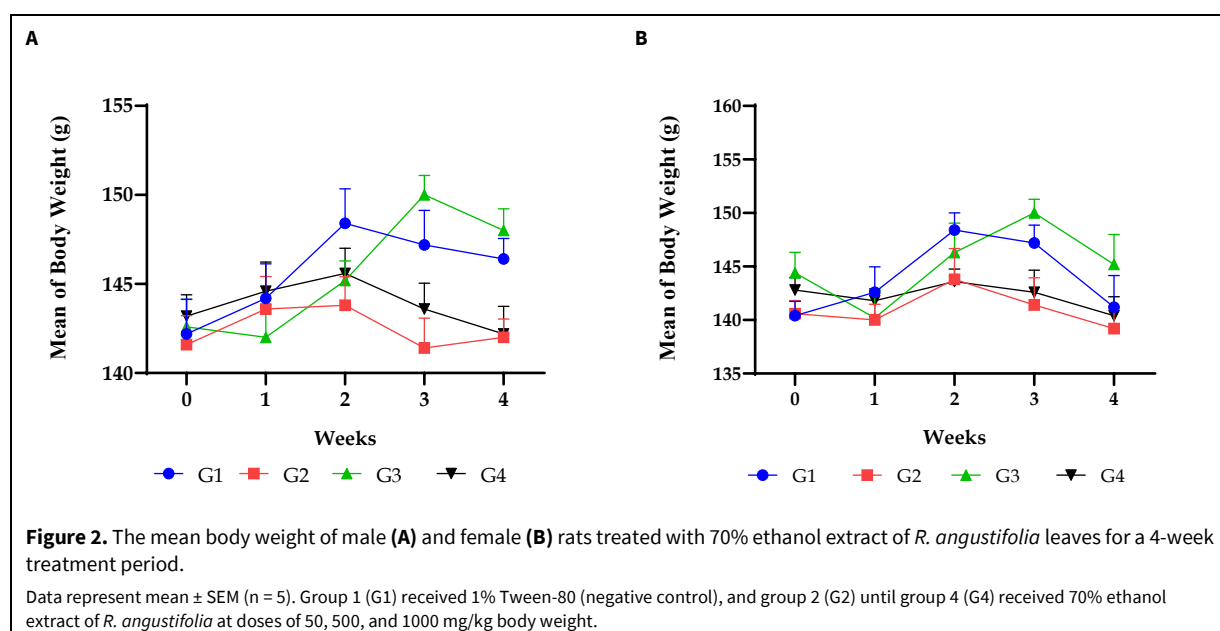


Table 2. Effects of 70% ethanol extract of *R. angustifolia* on relative organ weight (g) of rats during acute oral toxicity study.

Organs	G1	Extract of <i>R. angustifolia</i>			
		G2	G3	G4	G5
Liver	4.28 ± 0.14	4.45 ± 0.12	4.43 ± 0.25	4.02 ± 0.02	4.32 ± 0.18
Heart	0.36 ± 0.02	0.37 ± 0.01	0.36 ± 0.01	0.35 ± 0.01	0.43 ± 0.03
Lungs	0.83 ± 0.06	0.74 ± 0.04	0.87 ± 0.09	0.75 ± 0.04	0.81 ± 0.08
Spleen	0.39 ± 0.04	0.31 ± 0.01	0.33 ± 0.02	0.34 ± 0.02	0.40 ± 0.06
Kidney left	0.42 ± 0.02	0.43 ± 0.02	0.42 ± 0.01	0.39 ± 0.01	0.43 ± 0.02
Kidney right	0.41 ± 0.02	0.42 ± 0.02	0.44 ± 0.02	0.35 ± 0.01	0.41 ± 0.01
Stomach	3.13 ± 0.53	4.43 ± 0.41	4.98 ± 0.63	3.35 ± 0.33	3.65 ± 0.39
Gut	12.17 ± 0.32	11.26 ± 0.69	11.71 ± 0.41	11.87 ± 0.46	12.07 ± 0.37
Ovaries	0.67 ± 0.02	0.96 ± 0.08	0.82 ± 0.06	0.70 ± 0.04	0.68 ± 0.01

Values were represented as mean ± SEM (n = 5). The data were evaluated by ANOVA followed by Dunnett's multiple comparison test. G1: 1% Tween-80 (negative control); G2-G5: 70% ethanol extract of *R. angustifolia* at 5, 50, 300, and 2000 mg/kg body weight, respectively.



Relative organ weight of male and female rats

The relative organs weight of the rats of either sex treated with 70% ethanol extract of *R. angustifolia* for 28 days was observed and generally revealed no significant differences (Table 3). However, in this study, a significant difference was observed in the liver organ of the male rats treated with 1000 mg/kg BW doses (G4). The testis weight among all doses tested was also found to have a significant difference compared to the negative control.

Hematology and biochemical analysis

The effect of 70% ethanol extract of *R. angustifolia* on hematological and biochemical parameters did not

mediate any significant difference between the treated and the control groups (Tables 4 and 5).

Histopathology

Macroscopic examination of organs, including the liver, kidney, and spleen no abnormalities in the texture and color of the treated groups compared with the control group. The histopathological studies of the kidney and spleen of 70% ethanol extract treated the male and female rats showed no alteration or toxicity. However, a significant difference was shown in the organ liver of the male rat-treated group at a dose of 1000 mg/kg BW (G4). The histopathology results are shown in Figs. 3, 4, and 5.

Table 3. Relative organ weight of male and female rats orally treated daily with 70% ethanol extract of *R. angustifolia* for 28 days.

Organs	Sex	G1	Extract of <i>R. angustifolia</i>		
			G2	G3	G4
Liver	Male	2.80 ± 0.13	2.87 ± 0.09	3.13 ± 0.08	3.37 ± 0.09**
	Female	3.04 ± 0.08	3.18 ± 0.17	3.44 ± 0.12	3.42 ± 0.09
Heart	Male	0.35 ± 0.01	0.34 ± 0.02	0.34 ± 0.02	0.33 ± 0.01
	Female	0.39 ± 0.05	0.42 ± 0.03	0.40 ± 0.02	0.33 ± 0.01
Lungs	Male	0.88 ± 0.04	0.91 ± 0.10	0.92 ± 0.06	0.98 ± 0.09
	Female	1.09 ± 0.05	0.87 ± 0.04	0.99 ± 0.11	1.03 ± 0.01
Spleen	Male	0.31 ± 0.03	0.28 ± 0.03	0.22 ± 0.03	0.27 ± 0.04
	Female	0.35 ± 0.03	0.34 ± 0.03	0.32 ± 0.03	0.34 ± 0.04
Kidney left	Male	0.32 ± 0.01	0.35 ± 0.02	0.34 ± 0.02	0.36 ± 0.02
	Female	0.37 ± 0.01	0.37 ± 0.01	0.35 ± 0.01	0.36 ± 0.02
Kidney right	Male	0.31 ± 0.02	0.35 ± 0.02	0.32 ± 0.03	0.37 ± 0.02
	Female	0.35 ± 0.01	0.33 ± 0.02	0.36 ± 0.01	0.33 ± 0.02
Stomach	Male	1.12 ± 0.15	1.37 ± 0.14	1.16 ± 0.11	1.26 ± 0.13
	Female	1.19 ± 0.02	1.41 ± 0.14	1.32 ± 0.11	1.55 ± 0.16
Gut	Male	7.42 ± 0.43	7.86 ± 0.16	8.76 ± 0.91	8.40 ± 0.53
	Female	7.81 ± 0.27	8.58 ± 0.27	7.92 ± 0.21	8.22 ± 0.23
Testis/Ovaries	Male	0.82 ± 0.01	1.32 ± 0.06*	1.51 ± 0.06**	1.53 ± 0.04**
	Female	0.84 ± 0.14	0.72 ± 0.13	0.58 ± 0.08	0.67 ± 0.10

The data are expressed as mean ± SEM (n = 5 for each group). *P<0.05 and **p<0.005 were considered significant using one-way ANOVA followed by Dunnett's multiple comparison test compared to the negative control. Group 1 (G1) received 1% Tween-80 (negative control), and group 2 (G2) until group 4 (G4) received 70% ethanol extract of *R. angustifolia* at doses of 50, 500, and 1000 mg/kg body weight.

Table 4. The effects of 70% ethanol extract of *R. angustifolia* on hematology parameters.

Parameters	Sex	G1	Extract of <i>R. angustifolia</i>		
			G2	G3	G4
Hemoglobin (g/dL)	Male	13.26 ± 0.23	13.58 ± 0.45	13.49 ± 0.37	13.23 ± 0.72
	Female	13.04 ± 0.19	13.00 ± 0.42	12.98 ± 0.35	12.38 ± 0.31
Leukocytes (10 ³ /mm ³)	Male	10.94 ± 1.44	11.22 ± 1.51	10.58 ± 1.84	15.65 ± 2.94
	Female	9.62 ± 0.91	10.60 ± 1.62	9.46 ± 1.62	12.64 ± 1.98
Thrombocyte (10 ³ /mm ³)	Male	838.40 ± 46.74	996.60 ± 108.05	882.00 ± 54.25	927.75 ± 40.52
	Female	896.40 ± 91.30	826.40 ± 27.07	902.60 ± 51.55	841.00 ± 68.97
Erythrocyte (10 ³ /mm ³)	Male	8.04 ± 0.09	8.22 ± 0.33	8.08 ± 0.16	8.10 ± 0.40
	Female	7.56 ± 0.13	7.60 ± 0.25	7.58 ± 0.23	7.22 ± 0.20
ESR (mm/h)	Male	2.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00
	Female	2.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00
Eosinophils (%)	Male	2.60 ± 1.20	1.20 ± 0.73	1.50 ± 0.64	0.25 ± 0.25
	Female	2.60 ± 1.03	2.60 ± 0.98	3.40 ± 1.40	1.80 ± 0.58
Basophils (%)	Male	0.00 ± 0.00	0.00 ± 0.00	0.25 ± 0.25	0.50 ± 0.28
	Female	0.00 ± 0.00	0.40 ± 0.24	0.00 ± 0.00	0.20 ± 0.20
Neutrophils (%)	Male	10.80 ± 2.15	7.00 ± 1.45	10.75 ± 3.54	8.25 ± 3.19
	Female	12.00 ± 2.76	8.00 ± 1.18	9.20 ± 1.16	8.40 ± 0.81
Lymphocyte (%)	Male	74.20 ± 3.03	75.80 ± 4.10	80.00 ± 2.48	81.00 ± 3.37
	Female	73.60 ± 1.81	75.60 ± 2.25	78.80 ± 2.40	77.20 ± 2.76
Monocytes (%)	Male	12.40 ± 1.74	16.00 ± 5.32	7.50 ± 2.36	10.00 ± 3.70
	Female	11.80 ± 3.61	13.40 ± 3.14	8.60 ± 1.91	12.40 ± 2.11

Values are represented as mean ± SEM (n = 5 for each group). The data were analyzed by ANOVA and followed by Dunnett's multiple comparison test. Group 1 (G1) received 1% Tween-80 (negative control), and group 2 (G2) until group 4 (G4) received 70% ethanol extract of *R. angustifolia* at doses of 50, 500, and 1000 mg/kg BW. ESR: erythrocyte sedimentation rate.

Table 5. Effects of the 70% ethanol extract of *R. angustifolia* on biochemical parameters.

Parameters	Sex	G1	Extract of <i>R. angustifolia</i>		
			G2	G3	G4
SGOT (U/L)	Male	160.20 ± 13.78	163.80 ± 10.94	152.50 ± 11.32	145.75 ± 15.34
	Female	148.80 ± 7.53	135.40 ± 6.17	148.20 ± 5.62	149.80 ± 5.25
SGPT (U/L)	Male	69.00 ± 6.49	54.80 ± 2.15	63.00 ± 5.98	55.75 ± 7.56
	Female	51.60 ± 3.40	54.00 ± 2.05	54.80 ± 3.09	44.80 ± 0.97
BUN (mg/dL)	Male	19.64 ± 1.22	18.30 ± 1.44	17.97 ± 0.89	17.28 ± 1.62
	Female	20.52 ± 2.44	21.74 ± 1.43	17.94 ± 1.19	18.62 ± 1.17
Creatinine (mg/dL)	Male	0.32 ± 0.02	0.24 ± 0.01	0.28 ± 0.01	0.28 ± 0.03
	Female	0.29 ± 0.01	0.29 ± 0.01	0.30 ± 0.01	0.31 ± 0.01
Glucose (mg/dL)	Male	135.20 ± 11.50	103.40 ± 13.99	117.50 ± 10.90	121.00 ± 12.78
	Female	111.40 ± 1.78	126.40 ± 5.26	116.00 ± 11.19	115.00 ± 4.84
Bilirubin total (mg/dL)	Male	0.13 ± 0.01	0.11 ± 0.01	0.14 ± 0.01	0.14 ± 0.005
	Female	0.17 ± 0.01	0.17 ± 0.02	0.21 ± 0.01	0.22 ± 0.01

Values are represented as mean ± SEM (n = 5 for each group). The data were determined by ANOVA followed by Dunnett’s multiple comparison test. Group 1 (G1) received 1% Tween-80 (negative control), and group 2 (G2) until group 4 (G4) received 70% ethanol extract of *R. angustifolia* at doses of 50, 500, and 1000 mg/kg body weight. SGOT: serum glutamic oxaloacetic transaminase; SGPT: serum glutamic pyruvic transaminase; BUN: blood urea nitrogen.

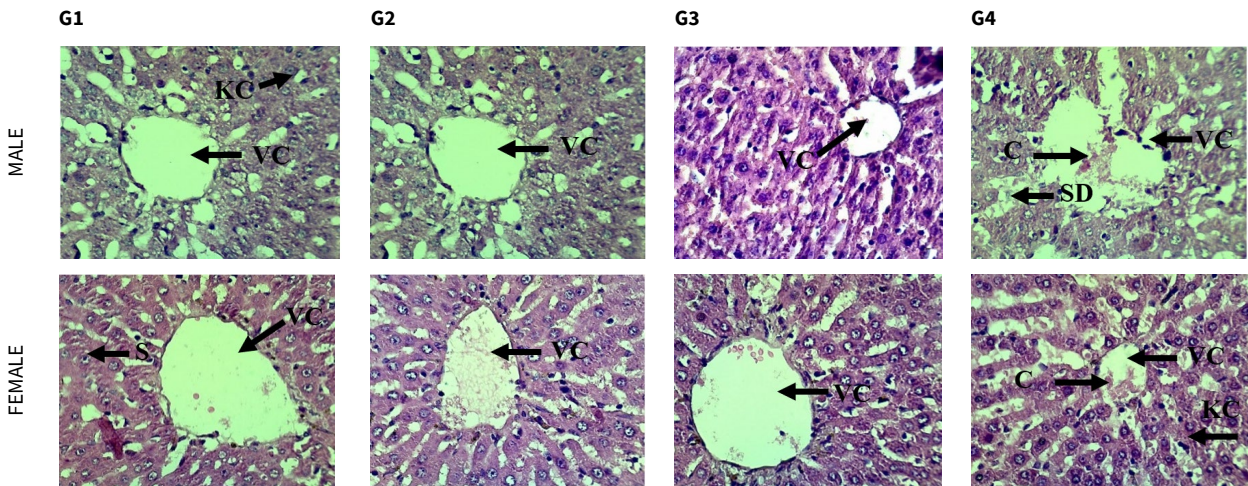


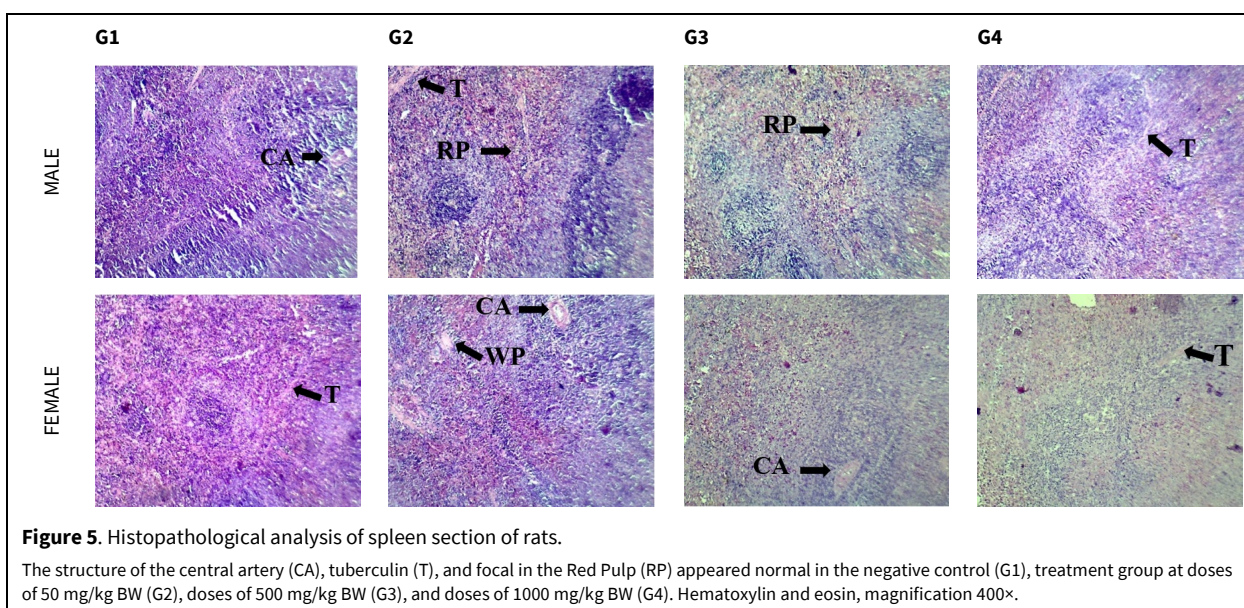
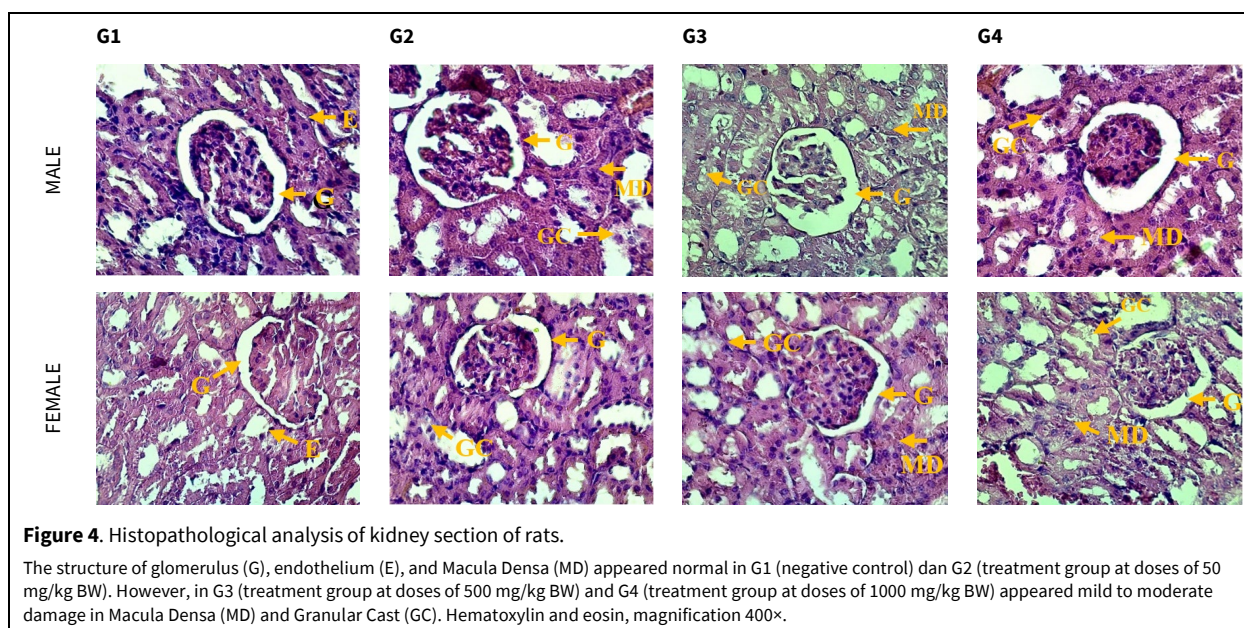
Figure 3. Histopathological analysis of liver section of rats. The structure of the vena centralis (VC) and sinusoids (S) appeared normal in the G1 (negative control), G2 (treatment group at doses of 50 mg/kg BW), and G3 (treatment group at doses of 500 mg/kg BW). However, activated Kupffer cells and sinusoidal dilatation were detected in the liver tissue in the G4 (treatment group at doses of 1000 mg/kg BW). Hematoxylin and eosin, magnification 400×.

DISCUSSION

Medicinal plants have been used to prevent and cure various diseases since 60.000 years ago (Kifayatullah et al., 2015). It was believed to be less toxic and have less side effects. However, many medicinal plants have insufficient scientific information, especially on their safety and toxicology data. Moreover, using medicinal plants as self-medication was often run without medical supervision, and this case has raised concerns (Vaghasiya et al., 2011). Hence, proper scientific knowledge of medicinal plant toxicity and safe administration is essential. The toxicity and safe-

ty evaluation of the medicinal plant can be done in various experiments in an animal model before human consumption (Jothy et al., 2011; Singh et al., 2013).

R. angustifolia has been proven in many communities to possess various pharmacological effects. However, scientific studies are necessary for developing *R. angustifolia* as an alternative and complementary medicine. The current study determined the acute and subchronic toxicity of *R. angustifolia* extract in an animal model.



The acute toxicity study showed that 70% ethanol extract of *R. angustifolia* did not mediate toxicity effects during the monitoring period of 14 days experiment. All animals were healthy without changes in their skin, eye color, or behavioral patterns. The statistical analysis did not demonstrate any significant difference in the body weight and relative organs between the treated group and the negative control. No mortality cases were observed. The LD₅₀ value of the extract was considered to be more than 2000 mg/kg BW.

In the sub-chronic toxicity assay, body weight in all groups tended to decrease. However, statistical analysis revealed no significant difference compared to the control group in males and females (Fig. 2).

There was no sign of toxicity, no death, abnormalities in fur and skin, eye color, tremors, salivation, diarrhea, breathing, behavior patterns, sleep, and coma. The reductions in the body weights of rats during toxicity studies are possible due to the physiological adaptation responses, which may lead to low appetite and reduce the caloric intake by the animal (Rhiouani et al., 2008; Vysakh et al., 2020). The administration of a high dose of plant extract might also induce stress in animals, which could decrease their food intake and interfere with body weight (Harris et al., 1998).

In toxicology studies, relative organ weight is another basic indicator to measure the harmful effects of plant metabolites. Changes in the organ weight of the treatment group compared to the control give a pre-

liminary sign for any damage or swelling of the organ caused by extract (Yam et al., 2013). The relative organ weight of the liver, heart, lungs, spleen, kidney, stomach, gut, and sex organs (ovaries and testes) was monitored. There were no significant differences in the relative organ weight compared to the control group. However, significant differences were found in the liver of the male rats treated with 1000 mg/kg BW and testis in all doses compared with the control. The significant changes in the testes' weights in rats treated with 70% ethanol extract of *R. angustifolia* may reflect changes in seminiferous tubules or interstitial edema (Sellers et al., 2007). The seminiferous tubules are the basic units of the testicles to generate spermatozoa (Schlatt and Ehmcke, 2014). Administration of 70% ethanol extract of *R. angustifolia* might be toxic to the physiological function of the male reproduction system. Further research needs to be done to measure the effect of the extract on sperm production and reproductive disorders in male rats.

Hematology and biochemical parameters were measured to assess the systemic effects caused by the extract administration (Pariyani et al., 2015). The hematology parameters were measured to express the quality of blood cells. Blood is the transport medium of metabolites which could indicate any toxic effect. The toxic plants revealed an alteration in the hematological profile. Our results indicated that normal values and all hematology parameters in the treated groups were not statistically significant compared with the control group (Table 4). It indicates that the extract may not mediate a toxic substance that causes abnormal conditions.

The liver and kidney are essential in metabolism and indicate health status among other organs (Kaur, 2019). Moreover, the biochemical parameters are important to express the organs' function. Evaluation biochemistry parameters in liver function such as SGOT and SGPT showed no significant difference compared with control groups.

A histopathology assessment was conducted to further evaluate the cellular figure due to the drug intervention. Generally, it did not observe any abnormalities in textures in the liver, kidney, and spleen of the tested groups. However, activated Kupffer cells, sinusoidal dilatation, and infiltration of lymphocytes at portal pain were detected in the liver tissue in the treated group at 1000 mg/kg BW. The liver has the ability to regenerate damaged tissue. Those are why liver function may not be impaired early following an insult from a toxicant (Salawu et al., 2009). Therefore, we suppose that the extract exerted an effect on the liver in the highest dose tested.

CONCLUSION

Administration of 70% ethanol extract of *R. angustifolia* did not produce acute toxicity in rat animal models with an LD₅₀ value of more than 2000 mg/kg BW. At the same time, repeated dose 28-day oral toxicity demonstrated no toxic effects at 50 and 500 mg/kg BW, no mortality or adverse effects concerning body weight, general behavior, relative organ weights, and hematological and biochemical parameters. Histopathological evaluation of vital organs demonstrated a normal profile suggesting no morphological alterations. However, significant differences in the relative organ weight of the liver and testis in male rats treated with 1000 mg/kg BW doses need further assay.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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AUTHOR CONTRIBUTION:

Contribution	Wahyuni TS	Ilmi H	Kristiaji AS	Dewi MC	Nisa HK	Hafid AF	Widyawaruyanti A
Concepts or ideas	x					x	x
Design	x					x	x
Definition of intellectual content	x						
Literature search	x	x	x	x	x		
Experimental studies		x	x	x	x		
Data acquisition	x	x	x	x	x		x
Data analysis	x	x	x	x	x	x	x
Statistical analysis		x	x	x	x		
Manuscript preparation	x	x			x		
Manuscript editing	x	x				x	x
Manuscript review	x	x	x	x	x	x	x

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