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Subacute toxicity study of *Jatropha curcas* leaves on hematology parameters and renal function in Wistar rats

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ABSTRACT: The anti-inflammatory properties of *Jatropha curcas* leaves have been established. The Acute toxicity test showed that *J. curcas* leaves were safe. This study aimed to assess the safety of the ethanol extract of *J. curcas* (EJC) leaves on hematological indicators and renal function. In the present study, using 40 male and 40 female Wistar rats were conducted to evaluate subacute toxicity. The EJC leaves with doses of 150, 300, and 600 mg/kg BW, were given orally and repeated for 30 days. The result showed no mortality and no changes in red blood cells (RBC) and white blood cells after administration of EJC leaves, but it caused toxic gastrointestinal symptoms such as a dark and watery stool. The use of the EJC at a dose of 150 mg/kg BW was safe because there was no change in the serum creatinine level and only the tiniest number of dark-stools toxic symptoms was experienced. Histopathologically, the EJC caused necrosis and congestion at doses of 300 and 600 mg/kgBW, which suggests that renal toxicity has already occurred. In conclusion, the EJC leaves with a dose of 150 mg/kg BW were found to be safe on hematology parameters and renal function. In conclusion, the administration of EJC leaves at a dose of 150 mg/kg BW was proven to be safe for renal function and hematological parameters.

KEYWORDS: *Jatropha curcas*, toxicity, subacute, renal, hematology

1. INTRODUCTION

Jatropha curcas, a plant belonging to the Euphorbiaceae family, is a common sight in various parts of Indonesia. Traditional Asian medicine treats a variety of ailments with various components of the *J. curcas* plant. The anti-inflammatory properties of *J. curcas* leaves have been demonstrated, and they can be used to treat arthritis. The results of previous studies showed that *J. curcas* leaves ethanolic extract (EJC) at doses of 150, 300, and 600 mg/kg BW effectively decreased the arthritic score in CFA (Complete Freund Adjuvant)-induced arthritic rats [1]. The extract of *J. curcas* leaves that given orally also shown anti-inflammatory activity [2,3]. In addition, Ogunnaike *et al.* [4] showed that a methanolic extract of *J. curcas* leaves (200 mg/kg) can lessen the thickness of the paws in mice with arthritis brought on by formaldehyde. The essential oil of *J. curcas* leaves had antioxidant and antimicrobial [5,6].

J. curcas leaves extract has anti-inflammatory effects, according to previous studies. Yet, in terms of the creation of medicinal plants, the potential toxicity assessment of herbal medicine should be established in addition to the efficacy from the pharmacological standpoint. Hence, it is possible to obtain the safe herbal medicine dosage range for therapeutic purposes. Mice were used in a test to determine the acute toxicity of *J. curcas* leaves. Baroroh and Rachmani [7] reported that LD_{50} of ethanolic extract from *J. curcas* leaves using Balb/C male mice is 5734 mg/kg BW and it is categorized as practically not toxic. The ethanolic extract of *J. curcas* leaves is confirmed to be safe for use in the short term by the acute toxicity test, which also revealed that the extract had no effect on animal behavior. The subacute toxicity test should also be carried out to assess the long-term safety of the ethanolic extract of *J. curcas* leaves. The purpose of this study was to ascertain the subacute toxicity of *J. curcas* (EJC) leaves ethanolic extract on Wistar rat hematological parameters and renal function.

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2. RESULTS

2.1. Clinical Toxic Symptoms Observation

The result of the clinical toxic symptoms observation showed that there was no mortality observed from all animal tests (80 Wistar rats) in each group during the 30-days subacute toxicity study. During the 30-days morbidity observation in this study, there was no toxicity signs in the animal test's SSP-somatomotor system in both the control and extract groups (Table 1). The Wistar rat was known to have a light-brown, smooth feces from the pre-observation test, and there were no convulsions or changes in body posture. It can be assumed that there was no pain, particularly abdominal pain, because no changes in body posture were noticed after the extract was administered to the rats. According to the observations, neither male nor female rats experienced convulsions at any of the extract dose levels, hence it can be said that the EJC leaves did not elicit muscle spasms in animal experiments.

Table 1. The result of clinical toxic symptoms observation during 30-days administration of ethanolic extract *of J. curcas* (EJC) leaves

Groups	Sex	Number of Dead (Mortality) /Number Of Treated Rats	Clinical Toxic Symptoms (Morbidity)															
			A				B				C*				D*			
			Week				Week				Week				Week			
			1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
Na-CMC	Male	0/5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1 %	Female	0/5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Dose of	Male	0/5	-	-	-	-	-	-	-	-	-	√	√	-	√	√	-	-
150 mg/kgBB	Female	0/5	-	-	-	-	-	-	-	-	-	√	√	√	√	√	-	-
Dose of	Male	0/5	-	-	-	-	-	-	-	-	-	√	√	√	√	√	-	-
300 mg/kgBB	Female	0/5	-	-	-	-	-	-	-	-	√	√	√	√	√	√	√	-
Dose of	Male	0/5	-	-	-	-	-	-	-	-	√	√	√	√	√	√	-	-
600 mg/kgBB	Female	0/5	-	-	-	-	-	-	-	-	√	√	√	√	√	√	-	-

(-) : No toxic symptoms occured during the observation
A : Change of body posture
B : Convulsion
* : Assessmeny by using *Bristol Stool Chart*
(√) : Toxic symptoms recorded
C : Dark stool
D : Watery stool

According to the observations, neither male nor female rats experienced convulsions at any of the extract dose levels, hence it can be said that the EJC leaves did not elicit muscle spasms in animal experiments. According to Table 1, the control group (Na-CMC 1%) did not experience any symptoms of gastrointestinal system toxicity, such as black and watery stools. However, in the extract groups at the dose of 150, 300 and 600 mg/kg BW, clinical toxic symptoms such as black and watery stools were actually observed in all male and female rats.

2.2. Examination of Serum Creatinine Level

On day 31 of this study, serum creatinine levels were examined. The result indicated that neither male nor female rats in the control or treatment groups had significantly different levels of creatinine (Table 2). According to the study's findings, all groups had serum creatinine levels within the normal range, with the exception of the male rats given a dosage of 300 mg/kg BW and the control Na-CMC female rats group. Rat creatinine serum is typically between 0.2 and 0.8 mg/dl [8].

The male rats at the dose of 300 mg/kg BW and female rats Na-CMC control group in this study were experienced an increment in the serum creatinine levels exceeding the normal range (0,2-0,8 mg/dl), they were 0,840 mg/dl and 1,010 mg/dl, respectively (Table 2). However, the increment which is occurred cannot indicate an impairment of renal function.

Table 2. Serum creatinine level in both male and female wistar rats after 30 days administration ethanolic extract of *J. curcas* (EJC) leaves

Groups	Treatment	Serum Creatinine level (mg/dl) (Mean ± SD; n=5)	
		Male rat	Female rat
I	Control Na-CMC 1 %	0,760 ± 0,188	1,010 ± 0,188
II	EJC 150 mg/kg BW	0,786 ± 0,125	0,766 ± 0,151
III	EJC 300 mg/kg BW	0,840 ± 0,154	0,760 ± 0,144
IV	EJC 600 mg/kg BW	0,784 ± 0,169	0,746 ± 0,188

Serum creatinine level in all extract groups did not differ significantly from the control
One way ANOVA test (p > 0,05)

2.3. Examination of Renal Histopathology

The result of the renal histopathological examination in 3 fields of view/organs in this study showed that there was a change in the renal structure after 30-day of administration of EJC leaves. Those changes were the occurrence of renal damage in both male and female rats which was shown as parenchymatous degeneration, congestion, and necrosis. The microscopic appearance of a normal and damaged renal in both male and female Wistar rats in this study was presented in Figures 1 and 2. The extract group at a dose of 150 mg/kg BW, in both male and female rats, had renal damage scores which did not significantly differ from the control group. It can be explained by the result of histopathological observation from the control group and extract group at 150 mg/kg BW.

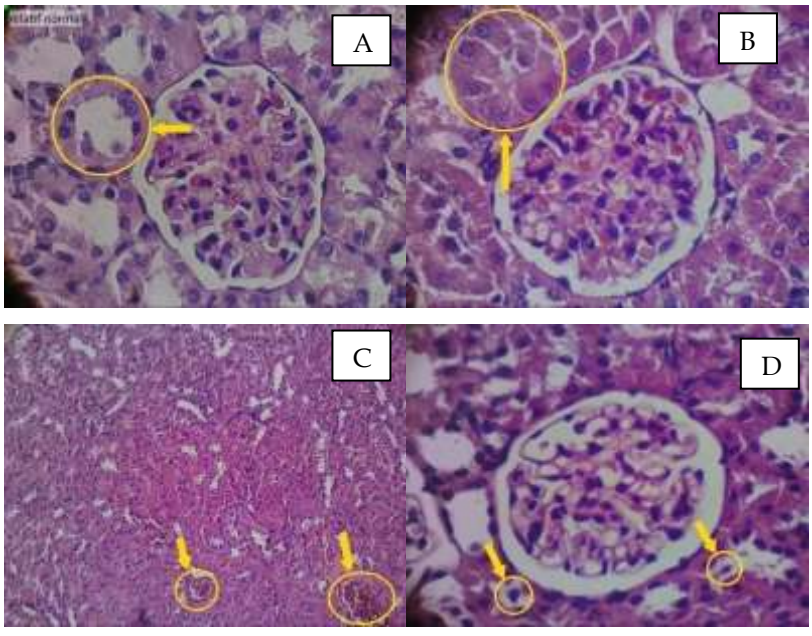

Figure 1 : Histopathological representation of male Wistar rat with H&E staining after 30 days administration of The ethanolic extract of *J. curcas* (EJC) leaves. The arrow (→)showed :(A) Control (Na-CMC 1%) : normal renal- 10 x 40 magnification, (B) EJC 150 mg/kgBW: parenkimatosa, degeneration-10 x 40 magnification(C) EJC 300 mg/kgBW: congestion-10 x 10 magnification, (D), EJC 600 mg/kgBW: Necrosis-10 x 40 magnification

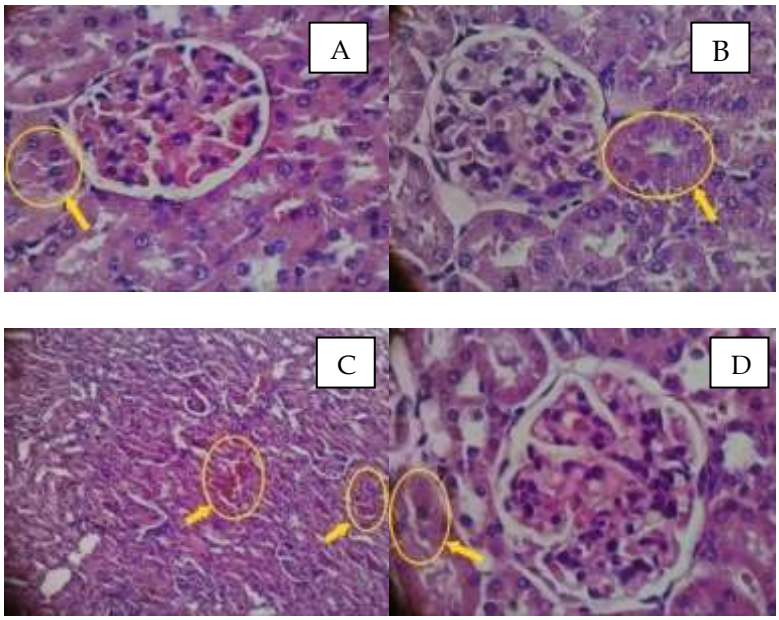

Figure 2 : Histopathological representation of female Wistar rat with H&E staining after 30 days administration of The ethanolic extractof *J. curcas* (EJC) leaves.The arrow (→) showed :(A) control (Na-CMC 1%) : parenkimatosa degeneration -10 x 40 magnification, (B) EJC 150 mg/kgBW : parenkimatosa degeneration -10 x 40 magnification, (C) EJC 300 mg/kgBW : congestion -10 x 10 magnification, (D) EJC 600 mg/kgBW : parenkimatosa degeneration -10 x 40 magnification

The result of histopathological scoring analysis in both male and female Wistar rats were presented in Tables 3 and 4. The scoring system is according to the score of renal cell damage based on its severity. Normal cells were given a '0' score, parenchymatous degeneration with '1' score, congestion with '2' score and finally score '3' for the necrosis cell. Thus, the safety of the extract at this dose level should be considered because it is known that congestion and necrosis of the cells was a presence as a pathological condition.

The scores of renal damages in both male and female rats at the extract group with dose of 600 mg/kg BW were significantly different from the control group (Tables 3 and 4). The high score of renal damaged in the extract group at 600 mg/kg BW may be cause by the necrosis cell that had been found in the male rats, and also by the extent of the renal area that has undergone parenchymatous degeneration in female rats. In the EJC group at a dose of 300 mg/kg BW, both male and female rats had renal damage scores which did not significantly differ from the control group. Although the score of renal damage in EJC group at a dose of 300 mg/kg BW did not significantly differ from the control, renal microscopic observations in Figures 1 and 2 precisely demonstrated a presence of renal damage as a congestion in male rats as well as congestion and necrosis in female rats.

Table 3. The result of histopathological scoring analysis in the renal of male wistar rats after 30 days administration of ethanolic extract of *J. curcas* (EJC) leaves

Groups	Treatment	n (Number of rats)	Score
I	Control Na-CMC 1 %	3	0,66
II	EJC 150 mg/kg BW	3	1,67
III	EJC 300 mg/kg BW	3	1,11
IV	EJC 600 mg/kg BW	3	3,11*

* **:** Significantly differ from the control group (One-Way ANOVATest; Tuckey HSD; $p < 0.05$)

Table 4. The result of histopathological scoring analysis in the renal of female wistar rats after 30 days administration of ethanolic extract of *J. curcas* (EJC) leaves

Groups	Treatment	n (Number of rats)	Score
I	Na-CMC 1 %	3	1,11
II	EJC 150 mg/kg BW	3	0,89
III	EJC 300 mg/kg BW	3	1,89
IV	EJC 600 mg/kg BW	3	2,89*

* : Significantly differ from the control group (One-Way ANOVATest; Tuckey HSD; p <0.05)

2.4. Examination of Hematology Parameters

The results of hematology studies are shown in Tables 5 and 6. From the results, the laboratory data including WBC values and RBC levels after treatment in all groups were still within the normal range. There was no significant differences between WBC levels after treatment in male rats compared with the control group. Based on the results of levels in RBC, there were no significant changes after treatment with the EJC leaves with a dose of 150, 300 and 600 mg/kg BW in male rats. There were statistically significant differences of RBC levels after treatment with EJC with a dose of 600 mg/kg BW in female rats. There was decreasing in RBC levels after administration EJC with the dose of 600 mg/kg BW for 30 days.

Table 5. Hematological parameters in male rats after administration of ethanolic extract of *Jatropha curcas* (EJC) during 30 days

Group	Parameters	
	WBC (Mean ± SD)	RBC (Mean ± SD)
Na-CMC	11,44 ± 2,56	7,44 ± 0,43
EJC 150 mg/kg BW	12,50 ± 2,40	7,55 ± 0,59
EJC 300 mg/kg BW	12,82 ± 4,05	7,54 ± 0,32
EJC 600 mg/kg BW	14,48 ± 6,17	6,79 ± 0,68

* : Significantly different from controll group (one way anova, p<0.05)

Table 6. Hematological parameters in female rats after administration of ethanolic extract of *Jatropha curcas* (EJC) during 30 days.

Group	Parameters	
	WBC (Mean ± SD)	RBC (Mean ± SD)
Na-CMC	11,54 ± 2,20	7,32 ± 0,35
EJC 150 mg/kg BW	10,78 ± 2,06	6,83 ± 0,33
EJC 300 mg/kg BW	11,76 ± 2,97	6,92 ± 0,43
EJC 600 mg/kg BW	12,58 ± 3,77	6,52 ± 0,16*

* : Significantly different from controll group (One-Way ANOVATest, Tuckey HSD; p <0.05)

3. DISCUSSION

The main objective of this study was to evaluate the safety in rats treated with the ethanolic extract of *J. curcas* (EJC) leaves on hematology parameters and renal function. Considering that the renal are the body's primary excretion organ and receive 20–25% of cardiac output, there is a greater likelihood that they will be exposed to toxins [8]. Then, both serum creatinine level and histopathological appearance are the main parameters that can be used to evaluate the renal function itself. Nevertheless, within subchronic toxicity effect evaluation, overall clinical toxic symptoms of the animal test also should be reported well.

The presence of black stools in this study can be interpreted as hemorrhagic inflammation in the test animal's gastrointestinal system. A substance called phorbol esters is found in EJC leaves, and it can promote the production of histamine, which leads to vascular remodeling and inflammation [9, 10]. Inflammation that resulted in black stools in this study was significant to be considered because it related to the cell lesion.

The previous study showed that both the small and large intestines of mice administered phorbol esters had black digest in them, and the rectum had dry beads of feces [11]. Baldini *et al*. [12] assert that there was no phorbol ester present in *J. curcas* leaves. According to Rakshit *et al*. [13], there were significant changes in terms of food intake and gain in body weight of rats after treatment with phorbol ester. But in this study, EJC leaves with a dose of 150 mg/kg BW was confirmed as the safest dose level because it led briefest toxic symptom as a black stool compared with the dose level of 300 and 600 mg/kg BW. It can be concluded that the EJC leaves did not elicit muscle spasms in animal experiments because the observations also stated that neither male nor female rats experienced convulsions at all of the extract dose levels. The male and female rats at all dose groups also experienced toxic symptoms such as watery stool. According to the Bristol Stool Chart, the watery stools in this study were classified as belonging to stool types 5 and 6 based on their consistency (BSC). Whereas stool type 6 was described as fluffy bits with ragged edges and mushy stool, stool type 5 was described as soft blobs with clear-cut edges [14]. Stool type 5 and 6 according to the BSC were interpreted as a marker of diarrhea. The presence of watery stool (diarrhea) in this study may cause by the compound called curcan oleat acid which acts as a purgative substance in the *J. curcas* leaves. Laxane *et al.* [15] reported that curcan oleat acid had the same activity as ricin oleic acid, a compound that had been known as purgative. Purgative properties of curcan oleat acid are explained by the inhibition of water absorption in the small intestine after this substance was activated by lipase. Curcan oleat acid was also known to trigger ion secretion in the small intestine, and also act as an activator of the EP3 receptor which led contraction of the intestine's smooth muscle [16].

Assessment of serum creatinine level can be used to determine the normality of renal function, in which elevated levels of creatinine in the blood followed by a decrease in glomerular filtration rate would lead to an indication of renal impairment [17]. Renal impairment can be regarded as significant when there was an increment in serum creatinine level ≥0,5 mg/dl which reflects a change in renal glomerular filtration rate up to 40% [18]. Because it is generally known that creatinine is only excreted from the renal through the filtration process without tubular reabsorption, a serum creatinine level within normal range was a sign that the renal glomerular filtration function was still functioning well after the administration of EJC. Consequently, it can be inferred from the results of this study that subchronic administration of EJC leaves to male and female Wistar rats was safe and non-toxic to their renal systems. The serum creatinine level, a measure of renal function, which did not increase until the value indicated renal impairment, was used to demonstrate its safety. The severity of the damage in the renal structure that was seen in this investigation can be categorized. The lightest cell lesion is a parenchymatous degeneration, which is followed by necrosis and congestion. Additionally, further analysis using the semiquantitative scoring approach was carried out to identify the extract groups with renal injury that were most substantially different from the control group.

The extract is a strong candidate to be developed as a phytopharmaceutical for antiarthritics based on the pharmacological investigation [1]. The safety of *J. curcas* leaves has been investigated in some toxicity studies. The lethal dose of *J. curcas* leaves was reportedly greater than 5734 mg/kg BW, according to reports. No relative toxicity associated with *J. curcas* therapy at dosages of 5734 mg/kg BW has been documented [7]. Igbinosa et al. [19] investigated that the methanolic extract of *J. curcas* was safe and tolerated in hematology parameters after treatment extract with the dose of 500, 1000 and 2000 mg/kg BW. There was phorbol ester from *J. curcas* seed, that was reported toxic in renal [11]. The result revealed that red blood cells (RBC) and white blood cells (WBC) in male white rats did not alter as a result of the *J. curcas* leaves extract. After administering female white rats, a dose of 600 mg/kg BW of *J. curcas* leaves extract, there were noticeable alterations in RBC value, but they were still within the normal range. Poon *et al.*[20] reported that there was decreasing in leucocyte and lymphocyte levels after treatment *J. curcas* oil with dose of 50 and 500 mg/kg BW for 28 days in female rats. In this study, there were no changes in white blood cell in both female or male rats.

In this present study, it has been found the same type of organ damage in both groups present as a parenchymatous degeneration. Thus, it can be concluded that the safest dose level of the extracts to the renal function based on the histopathology examination in this study was the extract at 150 mg/kg BW. Overall results from the renal histopathology examination reported that the subacute administration of EJC leaves in all dose levels can cause such a cell injury in the renal. This cell injury can occur due to the phorbol ester substance which still present in the extract and then entered into the systemic system of the animal test. *J. curcas* leaves were known to contain phorbol ester compound between 1,83 to 2,75 mg/g of dry samples [21]. Phorbol ester was a toxic compound that belongs to diterpene secondary metabolite. Phorbol ester was amphiphilic and could bind with phospholipid receptor in the vascular endothelial membrane [11]. Phorbol esters could cause prominent lesions mainly found in the lung and renal, with diffused hemorrhages in the

lung, glomerular sclerosis and atrophy in the renal at dose ⩾32,40 mg/kg BW [11]. Impaired endothelial cell membrane caused by this substance led to inflammation and then caused damage to the blood capillaries or congestion. According to Pereira et al. [22], it needs an efficient method for the quality control of the detoxification process in leaves of *J. curcas*. Furthermore, the optimal detoxification of the phorbol ester compound in *J. curcas* leaves should be conducted first.

4. CONCLUSION

In our study, we found that oral administration of the ethanolic extract of *J. curcas* (EJC) leaves for 30 days at doses of 150, 300, and 600 mg/kg BW did not result in mortality or somatomotor system disorder, but it did cause gastrointestinal toxic symptoms in male and female Wistar rats, such as dark and watery stools. The extract with the dose of 150 mg/kg BW was safe to use because it only briefly produced toxic dark-stool symptoms as compared to the control group. In both male and female Wistar rats, oral treatment of the ethanolic extract of *J. curcas* (EJC) leaves for 30 days at doses of 150, 300, and 600 mg/kg BW did not result in an increase in the serum creatinine level until the value that indicates a renal impairment. Wistar rats' RBC and WBC levels were shown to be unaffected by the ethanolic extract of *J. curcas* leaves.

5. MATERIALS AND METHODS

5.1 Materials

Fresh leaves of *J. curcas* were collected from Sukoharjo village, Ngaglik Sleman-Yogyakarta. Ethanol 96% was used for the maceration process for as long as 4x24 h. Eighty rats (40 female rats and 40 male rats) of 1,5 to 2 months of age and 100-200 g weight from the Laboratory of pharmacology and Toxicology-Medical Faculty of Gadjah Mada University, Yogyakarta, were used as an animal test. A maceration tube, stirrer stick, dustcloth, Buchner funnel, rotary evaporator and water bath were used to maceration process. Wistar rats were housed in a rat cage (40cm x 30cm x 18 cm). Injection spuit with volume of 1 and 5 ml were used to adminstration of *J. curcas* leaves extract during this study. Binocular light microscope (Nikon Eclipse E 100-Microscope Digital Camera System) was used to histophatological examination.

5.2 Methods

This research was an experimental study using the post-test-only controlled group design. Randomization of the animal test in this research uses a completely randomized design (CRD) unidirectional pattern. The exposure design of this repeated oral dose toxicity study was based on the OECD Test Guideline 407 [8]. All procedures conducted in this study were approved with ethical clearance by Faculty of Medicine, Jenderal Soedirman University Ethical Committee.

5.2.1 Extraction of plant materials

Powdered *J. curcas* leaves (1000 g) were extracted with 1200 mL ethanol 96% in a maceration tube for 4x24 hours. The obtained methanolic extracts were filtered and evaporated by using a rotary evaporator to obtain the ethanolic extract of *J. curcas* (EJC) leaves. From the maceration process, 76,5 g crude extract (yield 7,65 % w/w) was obtained. The extract was stored in a desiccator for use in a subsequent 30-days subacute toxicity studies.

5.2.2. Animal experimental studies

Before the subacute-toxicity study was conducted, animal tests were provided with food and water ad libitum and allowed to acclimatize to the facility for 7 days. All Wistar rats were allowed to fast 24 hours before day-1 administration of the extract. Eighty rats consisting of 40 female rats and 40 male rats were randomly divided into 4 groups. Each group consists of 10 animal test (5 male and 5 female). Group I was control group with 1 ml of Na-CMC 1%, Group II, III, and IV were given EJC leaves with a dose of 150, 300 and 600 mg/kgBW, respectively, while the extract was given orally once a day for 30 days.

Rats were weighed daily. Water consumption and food intake are also monitored daily. Three kinds of observations were made in this study. Observation of clinical toxic symptoms was made within 3 minutes in each rat at 1 (one) hour after the administration of the extract. The data output from the clinical toxic symptom observation is the number of mortality and morbidity of the animal test. The morbidity observation was concerned with the disorder in the SSP-somatomotor and gastrointestinal systems of the animal test. The SSP-somatomotor system disorder was marked by a change of body posture and convulsion, meanwhile gastrointestinal disorder was marked by dark and watery stool. Clinical toxic

symptom observations were done by the Open-Field Observations method, which allowed the observer to observe each animal test in a special box/opened box sized 50x50x20 cm. Special for stool observation, assessment was done with specific tools called *Bristol Stool Chart* (BSC) *Bristol Stool Chart* (BSC) [14, 23].

After 30 days of exposure, blood was collected from animals for hematological (Red Blood Cells and White Blood Cells) and serum creatinine levels. Three male and female rats from each group were killed by cervical dislocation to renal histophatology examination on day-31. Furthermore, the renal organ collected from each rat was used for histology cleansing preparation by H&E staining technique.

5.2.3. Subacute-toxicity study design

A subacute toxicity study was conducted by giving the animal test with three dose levels of *J. curcas* leaves extract. Three dose levels were represented as toxic dose (highest dose), middle dose, and the lowest dose as the dose level EJC leaves that predicted not caused a toxic symptom. The toxic dose (highest dose) in this study was obtained from 10% of LD_{50} in the previous acute-toxicity study by Baroroh and Rahmani [7] which reported that LD_{50} EJC leaves using Balb/C male mice is 5734 mg/kg BW or rounded to 6000 mg/kg BW. Thus, the highest dose level was 600 mg/kg BW. The middle and lowest dose respectively were obtained from fix-divided by factor 2 (two) from the higher dose before. So, the middle dose was 300 mg/kg BW and the lowest dose was 150 mg/kg BW.

5.2.4. Statistical analysis

Clinical toxic symptoms were analyzed descriptively. Serum creatinine, RBC and WBC level were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. The result of the histopathology examination from necropsy on day-31 were analyzed descriptively followed by scoring system analysis. The results from statistical analysis were presented as mean with standard deviation. p-value <0.05 was considered significant.

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