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Research Article

Acute toxicity of the galactagogue phytomedicine containing *Sauropus androgynous*, *Trigonella foenum-graecum*, and *Moringa oleifera*

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ABSTRACT

This study aims to evaluate acute toxicity of the herbal preparation on rats as an early step to evaluate its safety. This study used 25 females *Rattus norvegicus* strain Sprague Dawley rats aged 8 weeks with a body weight of at least 120 g divided into 5 groups of doses of herbal preparation (0/aquadest, 50, 300, 2,000, 5,000) mg/kg BW. After administration of the herbal preparation, rats were observed using a camera continuously for 14 days and manual observation intensively for the first 24 hours and then once a day for up to 14 days. The toxic effects including death, behavioral changes, neural symptoms, and other abnormalities were recorded. The weight of the rats was monitored every three days. On the 15th day, the rats were sacrificed to collect vital organs for macroscopic and histopathological examinations. The LD_{50} was estimated based on OECD Guideline. No mortality and significant toxicity signs in any of the rats after receiving the herbal formula at highest dose of 5000 mg/kg was reported during the 14-day observation period. Bodyweight and organ weight did not show significant variation between controls and treatment groups. In addition, no abnormalities of liver, heart and lungs were also observed in macroscopic and histopathological examinations. In conclusion, the herbal preparation shows the LD_{50} of greater than 5,000 mg/kg can be classified as category 5 or unclassified. Further sub chronic toxicity study will be conducted to evaluate its safety after repeated exposure.

Keywords:
Galactagogue, Acute toxicity, *Moringa oleifera*, *Sauropus androgynous*, *Trigonella foenum-graecum*

1. INTRODUCTION

Breast milk is the best food for the healthy growth and development of newborns[1-2]. In addition to a balanced nutritional content, breast milk contains a complex and variable mixture of active constituents[3] each contributing either singly or in combination to maintain the newborns health[4]. Breastfeeding is essential for the development and growth of infants, particularly in the first six months of life. To achieve optimal growth, development and health of the baby, The World Health Organization (WHO) and UNICEF recommend breastfeeding mothers

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to give breast milk as soon as possible (1 hour after birth), exclusively breastfeed their babies for the first six months, give complementary foods and continue breastfeeding for 2 years, and give breast milk whenever the baby asks for it[5-6]. In 2020, the Ministry of Health of Republic of Indonesia reported that the exclusive breastfeeding rate increased to 66.1% from targeted 40%[7]. However, this figure had not fulfilled WHO recommendation of breastfeeding rate of 80%, due to several reasons[8]. Low supply of breast milk (hypogalactia) or absence of breast milk production (agalactia) is one of the most common reasons[9-10].

Non-pharmacological and pharmacological interventions have been performed to overcome hypogalactia or agalactia. Some drugs have been used to induce, maintain or increase breast milk production such as chlorpromazine, sulpiride, metoclopramide and domperidone[10-11]. However, their use is restricted due to remarkable side effects both in mothers and infants[11-13]. Medicinal plants have been used traditionally to boost breast milk product in almost all regions worldwide[9-10]. Medicinal plants are believed relatively safe after their use for long time in community and could be an alternative therapy to avoid side effect from conventional galactagogues. However, their use is still based on empirical experiences rather than scientific evidence. Therefore, the development of the medicinal plants as phytomedicine is needed.

People in various countries have traditionally used natural materials around them to improve breast milk production. Various plants have been empirically proven to be used as breast milk promoters such as fenugreek (*Trigonella foenum-graceum*), katuk *(Sauropus androgynous)*, kelor (*Moringa oleifera)*, and others[14]. *S. androgynus* leaves, *T. foenum-graceum* seeds, and *M. oleifera* leaves are three plants that have been proven to have complete nutritional content as well as contain a stimulator of breast milk formation. *S. androgynus* leaves contain balanced micronutrients and macronutrients[15]. *M. oleifera* leaves contain high complete nutrients, especially protein and carbohydrates[16], while *T. foenum-graceum* seeds contain high micronutrients of saponins and alkaloids as antioxidants[17], so it is necessary to try to combine them.

In addition, their molecular mechanism of action has been also reported[9,15-19]. In order to develop a phytomedicine for galactagogue, the herbal preparation containing combination of extracts of *S. androgynus* folium, *T. foenum graceum* seeds, and *M. oleifera* folium has been successfully developed. Moreover, this herbal preparation was proven to be able to stimulate the production of milk in lactating rats through upregulation of mRNA smooth muscle α-actin (ACTA2), cytokeratin 14 (CK14), α-lactalbumin and aquaporin expression[20-21]. However, toxicity evaluation of this herbal preparation has not been conducted, yet.

Toxicity assessment is paramount before a clinical study of an herbal preparation conducted on humans. It is not only to evaluate the safety, but also to characterize the possible toxic effects. In this study, oral acute toxicity of this herbal preparation on female rats was reported. It is an early step to evaluate the safety of this preparation before further sub chronic or chronic toxicity studies will be performed.

2. MATERIALS AND METHODS

2.1. Herbal preparation

The herbal preparation was developed and produced by PT Swayasa Prakarsa, a traditional medicinal industry located in Yogyakarta, Indonesia, following the good manufacturing practice for herbal medicine. The herbal preparation (per 100 mg) contains combination of extracts of 60 mg *S. androgynus* folium, 30 mg *T. foenum graceum* seeds, and 10 mg *M. oleifera* folium[20-21].

2.2. Experimental animals

Female healthy *Sprague Dawley* rats (*Rattus norvegicus*), 8 weeks old, weighing about 120 g were obtained from The Integrated Research and Testing Laboratory, Universitas Gadjah Mada, Yogyakarta where the study conducted. Rats were acclimatized in group of five per cage under standard environmental conditions i.e., in a controlled temperature of 22±3°C, 70±4% relative humidity and under schedule of 12 h/12 h light/dark cycle for 7 days before treatment. Standard diet and water were available *ad libitum*[22].

2.3. Dose and clinical observations

The acute oral toxicity study of the herbal preparation was conducted using conventional method according to the national guideline of non-clinical toxicity test *in vivo* issued by The National of Drug and Food Control, Indonesia (2014) adopted from Organization for Economic Cooperation and Development (OECD) Guideline Testing of Chemicals (such as 420 and 425)[22-23]. Twenty-five rats were randomized divided into five groups with five rats in each group. Rats were fasted for 14-18 h prior the start of the experiment but had access to water. All rats in the experimental groups were administered the herbal preparation graded single oral doses of 50, 300, 2,000 and 5,000 mg/kg, respectively at 08.00-10.00 am. As control group, rats were given aquadest. Clinical observations were performed strictly and individually for mortality, behavior, neurological, and any other abnormalities. The observations were performed for the first 30 min after dosing and periodically every 4 h during the first 24 h, with distinctive concern during the first 4 h and daily after that for 14 days. Every 3

days, the body weights were recorded. At the end of study, the rats were exterminated and subjected to necropsy. The vital organs were taken out and weighed for gross pathological observations and histopathological examinations. The lethal dose 50% (LD_{50}) was calculated and the herbal preparation was then categorized according to globally harmonized system (GHS) for the 26 classifications of chemicals[14].

2.4. Pathological examinations

All rats were sacrificed to necropsy on the 15th day or earlier in case of death by 0.1 mL/100 g BW ketamine intramuscular injection and continued by cervical dislocation. The vital organs i.e., heart, liver, lungs, kidney, stomach, intestines, kidneys and ovaries were taken out and washed with physiological saline (NaCl 0.9%). The vital organs were dried with filter paper, observed for gross pathological including color abnormalities and consistency, the presence of lesions and masses (abscesses or tumors)[24-26], and weighed. Relative organ weights are calculated and recorded in proportion to body weight[27]. The vital organs were then preserved in 10% neutral buffered formalin for histology slide preparation.

2.5. Histopathological examinations

The general technique for making organ histology preparations is tissue preparation, fixation, dehydration, clearing, immersion in paraffin wax, cutting, and staining[28]. Organ sections were taken at random, fixed in 10% neutral buffered formalin overnight at room temperature, and then embedded in paraffin wax in square metal plates forming tissue blocks. The tissue blocks were kept at room temperature until they were cut. The tissue blocks were thinly sliced into ribbons with a thickness of 2-3 μm. Histological preparations were made of three bands from different locations. The ribbons of the section were collected at every section and put onto the surface of a warm water bath. The floating ribbons over the surface of warm water were mounted onto pre-cleaned slides which was smeared with egg albumin. The slides containing paraffin wax were placed in an oven and allowed to cool at room temperature. Slides were stained regressively with routine stains of hematoxylin and eosin (H and E)[29-30]. Then, the slides were observed under a light microscope for examination of histopathological features by a pathologist. After the examination, photomicrographs of selected samples were taken from all groups.

2.6. Statistical analysis

The experimental data were analyzed using SPSS 21 statistical software. Data were presented as mean± the standard error of the mean (SEM). Based on normality and homogeneity result, the data were analyzed by one-way ANOVA or by Kruskal Wallis test. The difference among groups with respect to investigated variables would be performed if the result showed significant difference (post hoc test, either Tukey test for ANOVA or Mann-Whitney test with Bonferroni test for Kruskal Wallis test).

2.7. Ethical considerations

The protocol of the study was approved by The Medical and Health Research Ethics Committee, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta (Reference number KE/FK/0327/EC, March 27th, 2020).

Table 1. Clinical signs and behavioral patterns of rats after administration of the herbal preparation during the study.

Parameters	Control	Dose (mg/kg BW)			
		50	300	2000	5000
Skin	N	N	N	N	N
Fur	N	N	N	N	N
Eyes	N	N	N	N	N
Mucous membrane	N	N	N	N	N
Respiratory system	N	N	N	N	N
Peripheral nervous system	N	N	N	N	N
Central nervous system	N	N	N	N	N
Somatomotor activity	N	N	N	N	N
Behavior	N	N	N	N	N
Shaking	N	N	N	N	N
Salivation	N	N	N	N	N
Defecation	N	N	N	N	N
Weak	N	N	N	N	N
Sleep	N	N	N	N	N
Coma	NF	NF	NF	NF	NF
Mortality	NF	NF	NF	NF	NF

N: normal; NF: not found

Table 2. Rats body weights (mean ±SEM) after administration of the herbal preparation during experimental.

Groups	Day of measurement					
	0	3	6	9	12	15
Control	131.88±1.30	150.94±1.40	159.80±1.38	171.60±1.38	178.14±1.32	186.82±1.25
Dose 50	140.46±2.89	156.74±2.99	164.42±2.80	174.94±2.51	180.88±2.61	188.78±2.78
Dose 300	135.54±3.38	153.14±3.87	162.28±3.91	174.46±3.99	181.68±4.21	191.36±4.53
Dose 2000	128.16±1.66	145.82±1.36	154.86±1.54	166.92±1.79	173.32±1.95	181.88±2.19
Dose 5000	133.62±4.11	147.64±4.05	156.66±3.74	168.62±3.42	175.90±3.42	185.62±3.61

2.8. Study limitation

Some oral acute toxicity tests are complemented by blood tests. This study did not evaluate blood parameters so it will be equipped with a 90-day sub chronic toxicity test. The sub chronic toxicity test, in addition to evaluating clinical symptoms, general pathology and vital organs, will also evaluate routine blood parameters, and blood biochemistry before continuing clinical trials in humans.

3. RESULTS

3.1. Clinical and behavioral observations

Any toxic signs or symptoms experienced by rats were observed at different time interval of 0, 30 min, 1, 2, 3, 4, 8, 12, 16, 20, 24 h and then daily for a period of 14 days. The observations did not show any physical and symptoms as well as any possibilities of toxic signs (Table 1). Moreover, mortality was not also observed, even in the highest dose of the herbal preparation (5,000 mg/kg BW). Therefore, the LD_{50} of the herbal preparation could be considered >5,000 mg/kg BW. According to the global harmonization system criteria for acute toxicity, the herbal preparation can be categorized into category 5 or unclassified (2,000 mg/kg BW < LD_{50} < 5000 mg/kg BW).

The body weights of rats after administration of the herbal preparation increased progressively throughout the study period in all treated groups and control group (Table 2). The body weight gain was between 48.3 and 55.5 g at the end of the study in all treatment and control groups (Figure 1). However, there was no significant difference in the weight gain of all treated groups compared with control group (p>0.05).

3.2. Pathological examinations

Figure 2 shows the macroscopic of isolated vital organs included brain, heart, lungs, ren, liver, lien, gaster, intestines and ovaries of control group rats and rats after administration of the herbal preparation at highest dose (5,000 mg/kg BW). No significant difference found in the macroscopic changes in all of the treatment groups compared with control group.

The organs to body weight index of the control and all of the treatment groups were calculated and presented in Figure 3. The organs to body weight index tended to be lower at higher doses administration (300, 2,000 and 5,000 mg/kg BW) compared to control or dose of 50 mg/kg BW. However, there is no significant variation in the organs to body weight index among all groups (p>0.05).

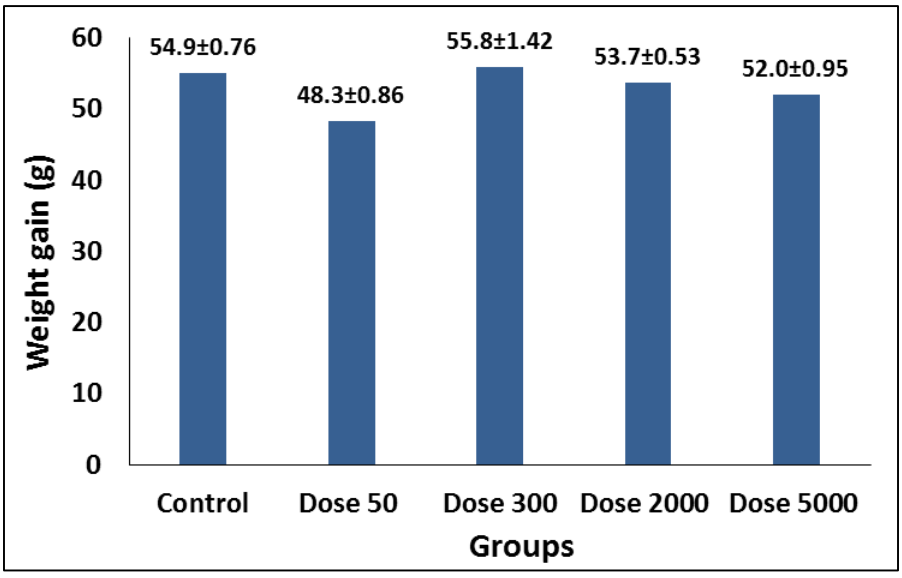

Figure 1. The weight gain of all treatment groups and control group (p>0.05).

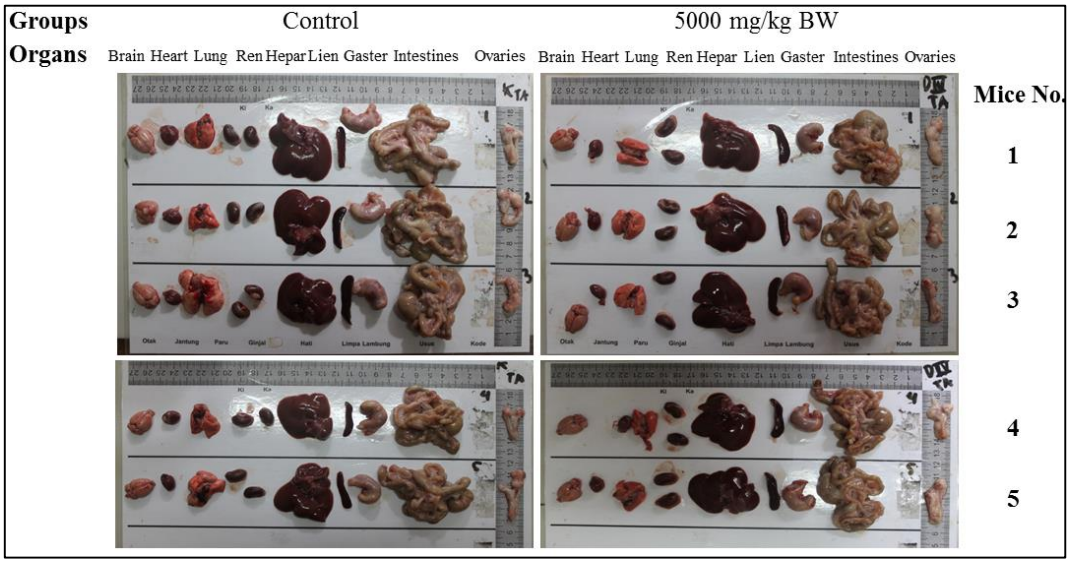

Figure 2. Macroscopic of isolated organs of control group rats and rats after administration of the herbal preparation at dose of 5000 mg/kg BW.

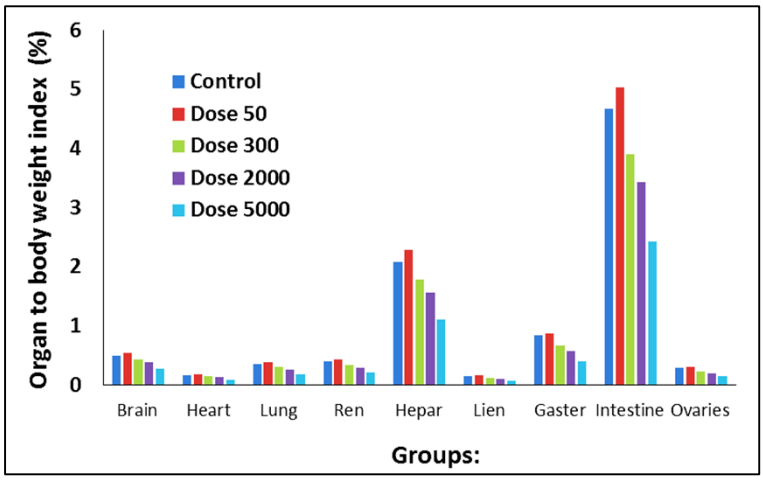

Figure 3. The organs to body weight index of the control group and the treatment groups.

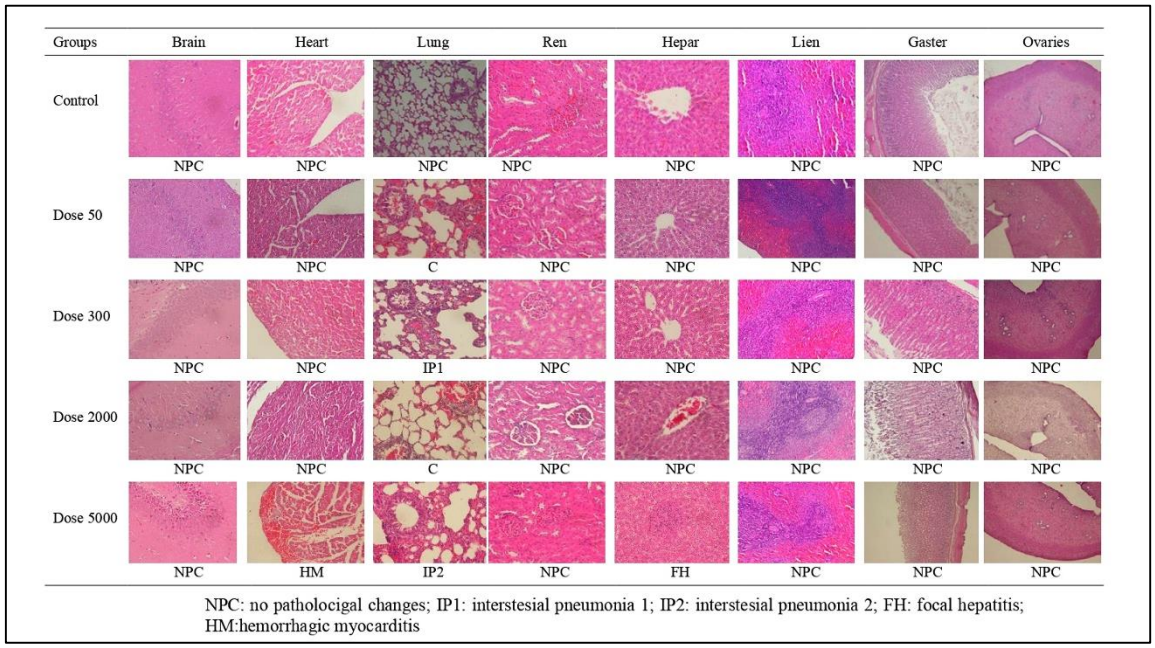

Figure 4. Histopathological features of organs in the control and the treatment groups.

Table 3. Histopathological examinations on selected organs of control and treatment groups.

Group	Liver		Heart		Lung			
	NPC	Lesion	NPC	Lesion	NPC	Lesion		
						IP1	IP2	Congestion
Control	5	0	5	0	2	3	0	0
Dose 50	5	0	5	0	0	4	0	1
Dose 300	5	0	5	0	1	4	0	0
Dose 2000	5	0	5	0	0	3	0	2
Dose 5000	4	1*	4	1**	0	3	2	0

NPC: no pathological changes; IP1: interstitial pneumonia level 1; IP2: interstitial pneumonia level 2; *: focal hepatitis; **: haemorrhagic myocarditis

3.3. Histopathological examinations

In general, no histopathological changes (n=5) of the isolated organs of the control group and the treatment groups were observed until administration of dose of 2,000 mg/kg, in liver and heart (Table 3 and Figure 4). While interstitial pneumonia 1 (IP1) was observed on the lung of both the control group and the treatment groups. Furthermore, congestion (C) was only observed in the dosage of 50 and 2,000 mg/kg and IP2 was only observed on dosage of 5,000 mg/kg. On the dosage of 5,000 mg/kg, focal hepatitis (FH) was observed (n=1) and hemorrhagic myocarditis (HM) was observed (n=1).

4. DISCUSSION

An herbal preparation containing combination of extracts of *S. androgynus* folium, *T. foenum graceum* seeds, and *M. oleifera* folium is being developed as a phytomedicine for galactagogue. This preparation has been produced by a herbal medicine industry (PT Swayasa Prakarsa, Yogyakarta, Indonesia) according to the good manufacturing practice for herbal medicine guideline. The galactagogue activity and its mechanism of actions of this herbal preparation were also proven in the preclinical studies[20-21]. In order to be phytomedicine approved National Agency of Drug and Food Control of Republic of Indonesia (NA-DFC RI), evaluation of its safety and efficacy of this herbal preparation is necessary.

In this study, temperature and humidity were set since those factors can affect the respiratory system. Several studies demonstrate different results regarding the relationship of humidity/temperature and pneumonia. A room with temperature 4°C and 50%±5 humidity can induce pneumonia[31]. High humidity also increases Sendai virus transmission to rats[32] and increases ammonia accumulation[33] which can lead to pneumonia in rats[34]. The accumulated ammonia can be prevented by cleaning the cage regularly (every day or every three day/week). It is also important to note that high humidity is beneficial to the lungs to maintain normal function against metacholin exposure[35], decrease the transmission of influenza virus[36-37], and lower the allergen (found in the rodent urine) concentration in the air[38].

Furthermore, previous studies demonstrated that the extract of *S. androgynus* folium, *T. foenum-graceum* seeds, and *M. oleifera* folium did not have lethal effect to rats. Methanol extract of *T. foenum-graceum* seeds with 2.5 g/kg/day can protect the liver[39-40]. Flour from *S. androgynus* folium prevents hepatic steatosis[41]. Extract of *M. oleifera* folium protects hepatorenal[42]. Administration of *M. oleifera* folium extract with serial dosages 16.1, 8.05, 4.02, 2.01 g/kg BW significantly increases total leucocyte, Cl^-, K^+, Ca^{2+}, alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), and bilirubin serum. In addition, histopathological analysis demonstrated a mild hepatitis symptoms, glomerulonephritis, dan myocarditis[43]. One of bacteria that can cause focal liver lesion is *Helicobacter hepaticus*[44].

Acute toxicity test is preliminary safety evaluation to determine the dose that causing deaths or serious toxicological effects after administration of single dose or multiple doses in short period time of a preparation. In this study, no death and abnormal clinical signs as well as behavioral patterns of rats after administration of the herbal preparation were found during 14 days observation in this study. The body weights of rats increased progressively in all treated groups and control group. However, there was no significant difference in weight gain of all treatment groups compared to the control group. Taking into account that at the highest dose (5,000 mg/kg BW) did not cause deaths or toxicological effects, the LD_{50} of the herbal preparation could be considered greater than 5,000 mg/kg BW. According to the Global Harmonization System Criteria for Acute Toxicity, this herbal preparation can be categorized into the fifth category or unclassified (LD_{50} > 2,000 mg/kg BW).

The toxicity of single extract of each plant contained in the herbal preparation have been reported in the previous studies. Based on the LD_{50} values of each plant, the three plants could be categorized into the fifth category. The oral LD_{50} of juice and soup *S. androgynus* leaves in Wistar rats were found to be greater than 5,000 mg/kg BW[45]. Furthermore, it was reported that the oral LD_{50} of *T. foenum graceum* seeds extract in Wistar rats is greater than 2,500 mg/kg BW. There were no abnormalities in feed, water intake, body weight and gross

pathology of *T. foenum graceum* seeds extract at a test dose of 2,500 mg/kg BW. Another study reported that oral LD_{50} of *T. foenum graceum* seeds extract in rats was more than 2,000 mg/kg BW[46]. Whereas the intraperitoneal LD_{50} of *M. oleifera* ethanolic leaves extract in rats was reported to be 6,616.67 mg/kg BW[47].

In general, no pathological changes were observed in the histological features of organs examined in rats treated with doses of 50, 300 and 2,000 mg/kg of the herbal formulation and control group, except the histological features of lungs. Interstitial pneumonia level 1 (IP1) was observed in the histological feature of lung of rats treated with the herbal formulation in all doses and also in the control, whereas the IP2 was only observed in dose of 5,000 mg/kg. This dosage was preferred since it has already consumed by many people[48] that was consisted of 3,000 mg *S. androgynus* folium, 1,500 mg *T. foenum graceum* seeds and 500 mg *M. oleifera* folium. Sub-chronic toxicity test of fresh *S. androgynus* folium with 10 g/kg BW dosage or dried *S. androgynus* folium with 8 g/kg BW showed a normal range of lymphocytes, Th1 gene expression, and blood biochemical parameters[49]. Acute and subacute toxicity tests of 1,000 mg/kg BW and 2,000 mg/kg BW dosage did not cause significant changes/side effects in mice[46,50]. Furthermore, an acute toxicity test of methanol extract of *M. oleifera* folium with 6.4 g/kg BW did not cause death to mice[51]. This present study presented that the acute toxicity test of 5,000 mg/kg BW did not cause death. It suggests that the IP1 of lunglungsthe rats is not caused by the adminnistration of the herbal formulation. Infections caused by viruses, bacteria, and fungus might be the cause of the interstitial pneumonia of the rats[52-53]. In addition, congestion was only observed in the lung of rats treated with doses of 50 and 2,000 mg/kg, whereas in doses of 300 and 5,000 mg/kg, there was not. It suggests that the congestion of the rats was not dose dependent or might not be caused by administration of the herbal formulation.

Focal hepatitis (FH) was observed in one rat after administration of this herbal preparation at the dose of 5,000 mg/kg, but it was not observed in other treatment groups and control. It is characterized by lymphocyte infiltration in hepatocytes. Liver plays an important role in the metabolism of plant active constituents of this herbal preparation. These active constituents and their metabolites might result in toxicity or cell damage on the liver. *S. androgynus*, *T. foenum graceum* and *M. oleifera* contain active constituents specifically alkaloids, flavonoids, steroids, terpenoids, tannins, saponins and glycosides which might be toxic in high dose[46-47,54-55]. Although the herbal preparation is practically nontoxic based on the LD_{50} value (> 5,000 mg/kg), further subacute toxicity study will be conducted to confirm the toxic effect of the herbal preparation on the liver and other organs.

5. CONCLUSION

In conclusion, administration of the herbal preparation containing combination of extracts of *S. androgynus* folium, *T. foenum graceum* seeds, and *M. oleifera* folium at the highest dose of 5,000 mg/kg BW does not cause mortality or serious toxicological effects. Therefore, the LD_{50} of the herbal preparation could be considered greater than 5,000 mg/kg BW and this herbal preparation can be categorized into the fifth category or practically nontoxic. Further subacute toxicity study will be conducted to investigate the toxic effect of the herbal preparation after multiple dose administration.

Author contribution

Zulkhah Noor: developed the idea and research proposal, performed experimental, evaluated clinical signs and behavioral patterns of animal, analyzed histopathological data and wrote manuscript under supervisor Mustofa and Dwi Liliek Kusindarta.

Dwi Liliek Kusindarta: supervised Zulkhah Noor performing experimental, evaluated clinical signs and behavioral patterns of animal, analysed histopathological data, performed the analytic calculations, and wrote manuscript.

Ahmad Hamim Sadewa: research consultant, helped analyzed results and involved in wrote manuscript.

Didik Setyo Heriyanto: histopathological consultant, and contributed in final discussion of manuscript preparation.

Diah Rumekti Hadiati: research consultant and contributed in the final discussion of manuscript preparation.

Mustofa: supervised Zulkhah Noor in developing the idea and research proposal, supervised during performing experimental, helped analysed result finding and wrote manuscript.

Conflict of interest

None to declare.

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Ethics approval

The protocol of the study was approved by The Medical and Health Research Ethics Committee, Faculty of Meddicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta (Reference number KE/FK/0327/EC, March 27th, 2020).

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