SAFETY OF GAHARU TEA (*Aquilaria malaccensis* Lamk) FROM INDUCTION TREE THROUGH 28 DAYS ORAL SUBCHRONIC TOXICITY TEST

SURJANTO[1], RIDWANTI BATUBARA[2,3♥], RIKSON T MANIK [2]

[1]Faculty of Pharmacy Universitas Sumatera Utara, Jln.Tri Dharma. No. 5, USU, Medan 20155

[2] Faculty of Forestry Universitas Sumatera Utara, Jln. Tri Dharma Ujung No. 1, USU, Medan 20155

Tel./Fax. +628-776-932-0308, ♥email: ridwantibb@yahoo.com

[3] Mangrove and Bio-Resources Group, Center of Excellence for Natural Resources Based Technology, Universitas Sumatera Utara. Medan 20155

Manuscript received: Revision accepted:

Abstract.

Subcronic toxicity test is a test to detect the effect that appear after the implementation of preparation test with repeated doses by orally in tested animal for 28 or 90 days. Gaharu (*Aquilaria malaccensis* Lamk) is used by the gaharu farmers in Langkat as a brewed beverage. The aim of this study is to know the availability of toxic symptoms appearing from induced gaharu tea products. This study used the male and female mice which is divided into 5 treatment groups, 130, 260, 390, and 520 mg/kgBW doses, and control group. The observation results of clinical toxic symptoms indicating the availability of weak symptoms, modification of fur and anxiety in male and female mice, macropathological observation of the organ of mice were still normal with brownish red color, slippery surface, and rubbery consistency. Histopathologic results indicated the availability of hemoghia and dilation of vein. The results showed that the administration brewed gaharu tea induced on mice begun from the doses of 130, 260 , 390 and 520 mg/kgBW doses there were not dead mice, but at 390 and 520 mg/kgBW doses steeping induced gaharu tea causing toxic symptoms. The further research was needed for safety of long-term consume and repetitive.

Keywords : gaharu tea, subcronic toxicity test, mice, doses

A. INTRODUCTION

Plants of gaharu producer (*A. malaccensis*) is one of important forest plants in Indonesia and also some countries like India, Singapora, Malaysia, Japan, Middle east Tengah, and United State. In world trade, gaharu known as agarwood, aloewood, eaglewood, because of the smelled fragrant, so that including luxury commodities for industrial use, perfume, cosmetics, incense, raw materials of medicine, and tea. Gaharu is an aromatic substance light brown color, dark brown and blackish brown to black formed on gaharu producer wooden trunks (*A. malaccensis*) as a self-defense response to pathogen attack. Nowadays, demand of gaharu in the world market is increasing, while producers approach obstacles in obtaining gaharu from farmers. According to CITES (Convention on International Trade of Endangered Species) at convention who was to IX in Florida in May 1995, that plants of gaharu producer (*A. malaccensis)* are included in appendix II which means that logging and export should be limited in quotas and obtain to all countries where a species of this plant is found.

Gaharu (*A. malaccensis*) as germplasm, in the last decade is the focus of various parties especially on the products produced, namely resin (oleoresin) which is in a wooden tissue that is black and smelled fragrant which is usually called agarwood, this product has a very high value especially gubal that has super quality. With a very high value causes the community and some parties to hunt gaharu natural tree. Gaharu wood that was only obtained from the direct nature, now can be cultivated more carefully as other plantation crops (tea, coffe, chocolate, rubber etc).

Tea is beverage that has been widely known in Indonesia and in the world. This brown is commonly served as guest drinks. Smelled fragrant and typical taste make this drink widely consumed. In the cultivation of gaharu leaves are also used as raw materials brewed drinks (gaharu leaf tea). Gaharu leaves can come from trees that have not been or have been induced.

Based on the above, it is necessary to do research about the safety of non-clinical gaharu tea consumption. The aim of this research is to know the safety of gaharu leaf tea through 28 days oral subcronic toxicity test.

B. MATERIAL AND METHODS

Time and Study Area

The study was conducted from July to November 2016. Sampling was conducted in Langkat, North Sumatra. Tea making, and oral acute testing were

conducted at the Pharmacology Laboratory, Research Laboratory of Faculty of Pharmacy, Universitas Sumatera Utara. Organ histopathology at Anatomy and Pathology Laboratory Faculty Of Medicine, Universitas Sumatera Utara.

Materials and Research Tools

The materials that used including fresh gaharu leaves (*A. malaccensis* Lamk.) non induction that has been dried for 1 month, mice *(M. musculus)*, histo of organ liver and kidney, aquades, and hot water. The material chemicals that used including 96% ethanol and distilled water (aquades), Na_2HPO_4, and Formaldehyd, infusion of NaCl.

The tools that used including a cage of mice, teats, vials bottles, oral sonde, syringes, watch glasses, stirrer bars, tissues, filter paper, parchment paper, desiccators, digital cameras and dryer cabinets, microscopes, scales, stationary, measuring cups, baker glass, and plastic polyethylene.

Research Procedure

The research method refers to the Guidance of Toxicity Non-Clinic Test in in Vivo, Ditjen POM RI, 2014. The research procedure includes sampling, tea making and testing of test animals and observation of test results.

Sampling plant

Sampling is conducted purposively without compare with the same plant from other area. Sampling is conducted on trees that have been induced. The sample used in this research is gaharu leaves from Langkat, North Sumatra.

Making tea

1. Cleaned gaharu leaves samples from dirt that stick with water flow
2. Withered gaharu leaves samples with distributed on parchment paper until the water is absorbed
3. Dried in the dryer cupboard at ± 40°C until dry (indicated when crumbly brewed)
4. Blended the dried leaves
5. Put into polyethylene plastic
6. Brewed gaharu leaf tea into tea beverage with 150 ml hot water

Oral Toxic AnimalsTest

Oral toxic animals test used male and female mice ddY strain is approximately two months old with weight 20-30 g each consisting of 50 tails from the Nonruminant Section Faculty of Pharmacy, Universitas Sumatera Utara.

Preparation

Mice were acclimatized for two weeks with the aim adapted to experimental enclosure environment. At this step carried out the observation of the general condition of the test animals. This study used 25 male mice and 25 female mice which is divided into 5 treatment groups. The grouping of the test animals was done completly randomized, each consisting of 5 tails.

Dose Determination

Dose of gaharu tea is 1 g. The convertion factor from human to mice, that is 0.0026, then the dose of the test preparation for the mice is = 2.6 g/20 g BW mice= 0.13g/kg BW mice. This dose be appointed as the lowest dose to be administrated. The largest dose of determination was conducted with preliminary test to know the largest dose that can be condensed to mice, obtained dose 0.52 g/kg BW mice. To get good results are used doses in sequence that should follow geometric development that is :

YN = Y1 x RN-1

With ; Y1 = First dose, YN = N-Dose, R = Geometric factor ≠ 0 or 1 multiple of dose. By entering the lowest dose (1st dose) and the highest dose (4th dose) into the equation, then obtained geometric factor 0.52 = 0.13 x R4-1, so obtained R = 2. Based on the calculationt, to get 4 doses used multiple of dose amount 2, so that the dosage calculation should be administration as follows :dose 1 = 0.13 g/kg BW, b. Dose 2 = 0.13 x 2 = 0.26 g/kg BW, c. Dose 3 = 0.13 x 3 = 0,39 g/kg BW, d. Dose 4 = 0,13 x 4 = 0.52 gram/ kg BW

Determination of LD_{50}.

For detemination of LD_{50} value, used multilevel doses consisting of four dosage variations. The administration of the extract was administered in one oral administration using sonde, the mice were observed for 4 hours to see if there were any toxic symptoms present or not. The observations in mice were done 24 hours after administrated of the test solution by counting the number of dead mice from each group. LD_{50} value was calculated using Weil's formula.

Administration of Test Preparation

Animals should fasted before administration the treatment (the mice are fasted for 14-18 hours, for water may be administration), after fasted, animals weighed and administration test preparations. Test preparations are administration in single doses using sonde. In situation where it was not possible to administrated doses with one-time administration, the test preparation may be administrated within period of administration of the substance not more than 24 hours, after treatment, feed can be administrated again after 3-4 hours for mouse and 1-2 hours for mice. If the test preparation was administrated several times, then the feed may be administrated after treatment depending on the length of the test preparation period.

Observation

Test preparation (gaharu tea) was administrated orally every day for 28 days. After the test preparation was administrated, 1 hour later observed

for 2 hours. Observations that occur in the form of toxic symptoms and clinical symptoms like physical behavior (tremor, salivation, diarrhea, limp, animal gestures like walking backwards and walking using the stomach) (Rasyid, et al, 2012). The method of observation is as follows:

a. Salivation
   The salivation expenditure of mice that have been administrated gaharu tea extract compared with controls, using filter paper.
b. Diarrhea
   The excretion of mice that have been administrated gaharu tea extract compared with controls, using filter paper.
c. Tremor
   Animals that have been administrated extracts of gaharu tea, observed tremor or animal body vibrate.
d. Weakness
   Animals that have been administrated extracts of gaharu tea observed its activity in general.
e. Animals gestures
   Animals that have been administrated extracts of gaharu tea observed his gesture like walking backwards and walking using the stomach.

Observation of Weight

Weight monitoring was conducted once a week. The test animal was weighed to determine the volume of the test dosage provided. At the end of the study, the animals are still alive conducted an autopsy (BPOM RI., 2011).

Observation of Animal Death

Mice deaths were observed from the first day to 90th day and mice who died during the time of test preparation were immediately autopsied (BPOM RI., 2011).

Observation of Liver and Kidney Organs Macroscopic

The mice who died immediately autopsied and observed of the liver and kidney. Observations include the color, surface and consistencyof the liver visually.

Observation of Organ Histopathology

Histopathologically examined organs are the liver and kidneys. Organ that has been separated immediately inserted in 10 % formalin buffer solution and made histopathologic preprations with hematoxylin and eosin then examined under a microscope.

C. RESULTS AND DISCUSSION

Observation Result of Toxic Symptoms

The observation result conducted daily for 28 days on the behavior of mice such as seizures, diarrhea, salivation, weakness, sleep and coma (OECD, 2011) can be seen in Tables 1.

Table 1. The Observation Result of Symptoms Toxic of Female and Male Mice After Administration Gaharu Leaf Tea For 28 Days

Groups	Seizures		Diarrhea		Salivation		Weakness		Sleep		Coma	
	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male
T1	-	-	-	-	-	-	-	-	-	-	-	-
T2	-	-	-	-	-	-	-	-	-	-	-	-
T3	-	-	-	-	-	-	-	-	-	-	-	-
T4	-	-	-	-	-	-	-	-	-	-	-	-
Control	-	-	-	-	-	-	-	-	-	-	-	-

Note : T = Treatment; 1,2,3,4 = doses 130, 260, 390, 520 mg/kgBW;
(-) = not shows any symptoms; (+) = shows any symptoms

Based data on the Table 1 shows that administration the gaharu tea steeping which was administrated orally in mice for 28 days did not cause any toxic symptoms both at the treatment dose 130, 260, 390, and 520 mg/kgBW. Toxic signs that appear on organs and systems among others: in the gastrointestinal system include weakness, diarrhea, salivation, on behavior include sedation, anxious, severe depression, aggression or defensive behavior, fear, confusion, strange activity, etc. (Lu, 1995).

The main concept of toxicology is dose dependent. A substance can caused the desired effect associated with the administration dose of side effects, adverse effects and toxic effects that can be observed with parameters of toxic symptoms.

Observation Results of Death

The results of observation of animal mortality during the time of test preparation can be seen in Tables 2. The number of deaths of mice is 0 (non)

Table 2. The Observation Result of Death of Female and Male Mice After Administration Gaharu Leaf Tea For 28 Days

Groups	Number of mice	Number of deaths	
		Female	Male
T1	5	0	0
T2	5	0	0
T3	5	0	0
T4	5	0	0
Control	5	0	0

Note : T = Treatment; 1,2,3,4 = doses 130, 260, 390, 520 mg/kgBW;

Based on Tables 2 were not found test animals who died during the time 28 days treatment. From data contained above can be known that the administration of test preparation gaharu tea with dosage 130, 260, 390, and 520 mg/kgBW it can be said to be relatively safe because did not cause death in test animals for 28 days. The behavior and toxic symptoms after treatment are observed to see the toxic effects that result from the administration of gaharu tea.

Observation Results of Weight Body

The weighing of weight was conducted every week from day 0 until day 28 to determine the volume of the test preparation administration. The test animals were observed daily to see the presence of toxic symptoms and weight was weighed on a regular basis. Drastic and significant weight loss can be one of the observation indicators of potential toxic symptoms in test animals checked. The average of weight can be seen in Table 3.

Table 3. The Average Results of Weight of Female and Male Mice After Administration Gaharu Leaf TeaFor 28 Days

Weighing to	Average of Weight (g) ± SD (Famale)				
Control	T1	T2	T3	T4	
1	29.80 ± 3.62	25.34 ± 3.02	26.14 ± 2.99	24.92 ± 2.73	27.76 ± 3.77
2	30.20 ± 3.90	25.64 ± 2.83	26.38 ± 2.45	25.20 ± 2.61	27.56 ± 3.59
3	30.72 ± 3.28	25.40 ± 3.14	26.06 ± 1.27	24.52 ± 3.64	29.76 ± 2.11
4	29.82 ± 2.98	26.48 ± 2.93	25.08 ± 1.21	22.18 ± 2.87	28.40 ± 3.75
5	29.98 ± 3.03	26.80 ± 2.87	25.34 ± 1.07	22.76 ± 2.70	28.66 ± 3.60
Weighing to	Average of Weght (g) ± SD (Male)				
Control	T1	T2	T3	T4	
1	29.94 ± 1.31	27.98 ± 2.44	31.02 ± 4.40	28.22 ± 4.83	28.66 ± 4.78
2	31.88 ± 1.30	27.64 ± 2.73	28.28 ± 3.11	25.50 ± 3.79	24.42 ± 4.12
3	33.24 ± 1.44	31.32 ± 3.55	34.24 ± 2.66	26.96 ± 5.73	27.14 ± 6.02
4	30.54 ± 1.84	27.90 ± 2.72	32.04 ± 4.65	26.52 ± 6.17	31.50 ± 4.01
5	31.04 ± 1.91	28.24 ± 2.65	32.14 ± 4.30	26.90 ± 5.86	31.76 ± 3.84

Note : T = Treatment; 1,2,3,4 = doses 130, 260, 390, 520 mg/kgBW; SD = standart deviasi

The weighing of the mice weight was done 5 times on Tables 3 shows that was not significant changes of weight between the control group and treated group. This can be showed by the level of significance $p \geq 0,05$ thus the administration of the test preparation in the form of gaharu has not significant effects to changes the mice weight. The weight loss rapidly and meaningful is usually a sign of poor health. The weight loss also can be caused by a lack of consumption of food and beverages, specific disease or toxic signs that can be used as parameters to observe the toxicity symptoms (Price dan Wilson, 2005).

Observation of Relative Organ Weight

After administration of the test preparation for 28 days has been done the mice were then autopsied to see the toxic symptoms in the organs represented by liver and kidney organs. Then mice organs are observed and weighed. The results of organ weight of mice can be seen in Table 4.

Table 4. Organ Weight Mice Relative After Administration Gaharu Leaf Tea For 28 Days.

Groups	Average relative of organ weight liver ± SD		Average relative of organ weight kidney ±SD			
			Male		Female	
	Male	Female	Left kidney	Right Kidney	Left kidney	Right Kidney
Control	1.78±0.22	1.34±0.26	0.26±0.20	0.22±0.05	0.14±0.03	0.16±0.06
Dose 130mg/kg	1.46±0.20	1.01±0.18	0.20±0.02	0.20±0.04	0.12±0.01	0.12±0.01
Dose 260mg/kg	1.55±0.09	1.29±0.18	0.21±0.25	0.23±0.04	0.13±0.02	0.14±0.03
Dose 360mg/kg	1.14±0.24	1.13±0.02	0.13±0.005	0.15±0.01	0.15±0.04	0.14±0.03
Dose 520mg/kg	1.53±0.40	1.20±0.06	0.20±0.05	0.21±0.06	0,14±0.03	0.13±0.01

Based on the results of the relative weight organ of the mice in Table 7 where has been statistically analyzed using *two way ANOVA* show was not significant different in the relative weights of the organs in each group although the control group's body weight was higher than in the other group. This shows that the administrated of test preparation in the form gaharu tea has not significant effects on the weight organs of liver and kidney mices.

Observation Results of Macropathological

The observation results of macropathological include observation of surface color and organ consistency. The observation results are presented in Table 5.

Table 5. The Observation Results of Liver Organ Color of Famale and Male Mice After Administration Gaharu Leaf Tea For 28 Days

Groups	Observation (Famale)		
	Color	Surface	Consistency
Control	Brownish red	Slippery	Chewy
Dose 130 mg/kg BW	Brownish red	Slippery	Chewy
Dose 260 mg/kg BW	Brownish red	Slippery	Chewy
Dose 390 mg/kg BW	Brownish red	Slippery	Chewy
Dose 520 mg/kg BW	Brownish red	Slippery	Chewy
Groups	Observation (Male)		
	Color	Surface	Consistency
Control	Brownish red	Slippery	Chewy
Dose 130 mg/kg BW	Brownish red	Slippery	Chewy
Dose 260 mg/kg BW	Brownish red	Slippery	Chewy
Dose 390 mg/kg BW	Blackish red	Slippery	Chewy
Dose 520 mg/kg BW	Brownish red	Slippery	Chewy

Based on the Table 5 show that the liver and kidney organs after administration the gaharu tea in the dosage group 130, 260, 290 and 520 mg/bbBW is still in normal condition that is brownish red, slippery surface and consistency chewy. The result from surface observations visible slippery and consistency chewy in all groups.

Result of SGPT and SGOT Level Examination

Liver disorders can be known with examination of enzyme levels, one of them is measuring the level of transaminase enzyme namely the Serum Glutamate Piruvat Transaminase (SGPT). Results of chemical analysis of mice blood for SGPT content testing conducted at the end of treatment that is 28th day can be seen in Table 6.

Table 6. Results of Measurement Mean of SGPT and SGOT Level of Mice After Administration Gaharu Leaf Tea For 28 Days

Groups	Average SGPT levels (IU/L)		Average SGOT levels (IU/L)	
	Male mice	Female mice	Male mice	Female mice
130 mg/kg BW	108	81	944	425
260 mg/kg BW	69	106	303	630
390 mg/kg BW	82	12	200	18
520 mg/kg BW	79	98	162	300
Control	109	309	224	4

Based on the Table 6 can be present that SGPT levels of male mice is different in each treatment groups but its different has not significant where SGPT levels in the dosage group 130 mg/kg BW (108 IU/L), 260 mg/kg BW (69 IU/L), 390 mg/kg BW (82 IU/L) dan 520 mg/kg BW (79 IU/L) and control group of male mice that is 109 IU/L. In female mice, SGPT levels the lowest there at doses 390 mg/kg BW that is 12 IU/L while the highest at control group that is 309 IU/L (Table 10). The normal SGPT levels is 17-77 IU/L. This abnormal condition almost occurin all treatments including control, that normal only in group of male mice 260 mg/kg BW treatments.This be alleged because before treatment the mice are not checked the SGPT levels.

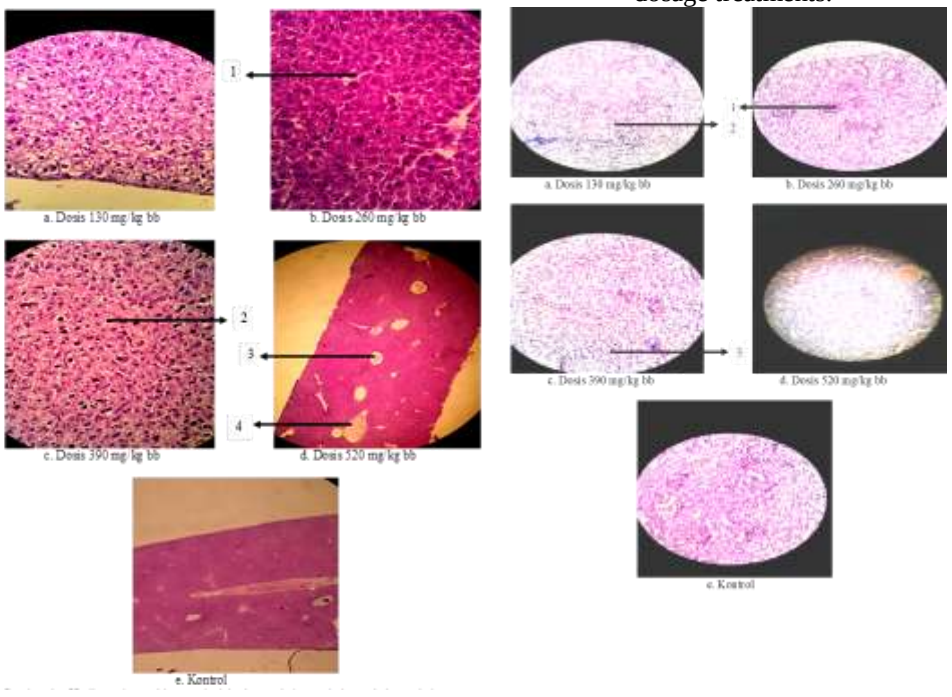
Based on measurement of SGOT levels of male mice on the Table 11 has not significant different between control group (224 IU/l) with dose 260 mg/kg BW (303 IU/L), dose 390 mg/kg BW (200 IU/L) and dose 520 mg/kg BW (162 IU/L), only the doses 130 mg/kg BW which is few different that is (944 IU/L), where the results measurement of SGOT levels in this group are above the normal limit. Different with SGOT levels in female mice, where only the control group and dose of 390 mg/kg BW are in the normal range while the dose of 130, dose of 260 and dose of 520 mg/kg BW above the normal limit. GPT and GOT are a strong and sensitive indicators against liver cell abnormalities. Enzyme

SGOT (*Serum Glutamic Oxaloacetic Transaminase*) or *Aspartateaminotransferase* (AST) is an enzyme that usually located in the liver cells. SGOT released in to the blood when liver or heart damaged. SGOT grade in the blood is significant with high of liver damage or heart damage (such as a heart attack). Some drugs may also increase levels of SGOT. This enzyme in small amounts is found in the heart muscle, kidney and sceletal muscle (Krysanti, 2014). The SGOT normal levels of mice is 54-298 IU/L (Research Animal Resources, 2009).

glutamate piruvat transaminase (GPT)/ALT is cytosolic enzymes that are mostly present in the lliver, heart and muscles. This enzyme as more spesific indicators for damage to liver cells was compared with GOT, because GOT is mitochondrial enzymes in large number in the heart, liver and sceletal muscle, if the heart liver level, ALT is high there is an indication of cell damage in the liver (Widjaja,2010).

Histopathology of the Liver and Kidney Organs

The observation results of liver and kidney organs (Magnification 400x) that have changes from the normal condition was presented in the figures (figure 1-2). Organ monitoring was conducted on all dosage treatments.



Based on the histopathology was contained in the figure can be seen that the administration of gaharu leaf tea with 4 doses did not showed the significant difference from the control group to the highest dose. The symptoms was found in microscopic observations of the liver are centralized

congestive veins and dilatation, dilated sinusoids, dilated portal veins and picnotic hepatocytes. While in the kidney organs the symptoms was found that the blood vessels have congestion but glomerulus and tubulus seen still in all normal condition.

The availibility of damage in the central vein can be caused by too much blood that is accommodated, this can causing concentration of toxic subtance that much larger thus clarifying the damage that occurs in the central vein (Price dan Wilson, 2005). Sinusoid is space between the liver cells which is an incomplete capillary and has a large diameter. In this study there is a sinusoid dilated blood vessels that is the treatments of 1 and 2 treatment. Sinusoid congestion occurs because of the rupture of capillary blood vessels that can cause blood goes into sinusoids. Hepatocytes are the main parenchyma in the liver that much acts in the path of metabolism, with a weight of about 80% of the liver mass, and single or double cell nucleus.

On the histology description of the kidneys there are symptoms of blood vessels that are congested. Congestion is a condition where there is excessive blood (an increase in the amount of blood) in the blood vessels at the certain area. Another word for congestion is hyperemia. Increased blood flow caused by the availibility of an arterior dilatation that acts as a valve regulating the flow into the local microcirculation. The boold vessels that are congeted occurs in all treatments from the lowest dose treatment (130 mg/kg BW) to the highest dose (520mg/kg BW).

Glomerular is part of the kidney which is a special capillary vein webbing whose walls are linked together with a bowman capsule wall. The renal glomerular serves to filter blood, glomerular filter products are a primary urine containing water, salts, amino acids, glucose, urea and other substances. On the histology figure of the kidneys, has not found glomerular problems in all treatments. According to Bevelander and Ramaley (1998) the changes that occurs in the glomerular and capsula should be effect disruption of filtrate function production and control of the composition of filtrate itself, while changes in the tubular result in disruption of the reabsorption process rather than the filtrate.

Same with glomerulus, the tubules in this study did not have any problems, the tubules is still look normal and function properly. The proximal tubular is the most common and susceptible to damage in nephrotoxic case. This can occur because of the accumulation of toxic materials and proximal tubule characters that have the weak epithelium and easly leak.The proximal tubular damage is a very important correlation between tubular segmental transport with accumulation, toxicity, and drug reactions in the proximal tubular target cells (Prasta, 2010).

CONCLUSION

Conclusion

On awarding gaharu tea (*A. malaccensis* Lamk), has not found toxic symptoms in behavior, weight, weight organ and macropathological organ observation of male and female mice. The awarding of steeping tea in liver organ of male mice which was administration a dose of 520 mg/kg BW dilated and hemorrhage in the veins of the porta veins and central veins and inflamed.

Recomendation

Based on the research that has been done, then it is recomendation to further research to test subcronic toxicity of liver, kidneys, heart, blood and other organs, and also test specific toxicities such as teratogenetic and carsinogenetic.

D. ACKNOWLEDGEMENT

This research was done by financed by: Directorate of Research and Community Dedication of Directorate General of Research Strengthening and Development of Ministry of Research, Technology and Higher Education 2016 on the Competitive Grant Scheme (Skim Hibah Bersaing).

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