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Hematology and Biochemistry Parameters on Sub Chronic Toxicity Rats by Using 70% Ethanol Extraction of *Citrus nobilis* Lour's Peel

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Abstract: *This research aims to assess hematology (hemoglobin, hematokrit, eritrosit total, and leukosit total) and blood biochemistry (SGPT, SGOT, BUN and creatinine) to both male and female rats in sub chronic toxicity condition. This research used 40 male 40 female white Galur Wistar rats and each divided into 4 groups (control group giving CMC Na 0,5%, dose group, dose group 40 mg/kg BB, dose group 200 mg/kg BB, and dose group 1000 mg/kb BB). Each group consists of 10 males and 10 females. Each cage contains 5 rats of the same age and gender, fed and drunk in ad libitum method. Furthermore, the rats are being weighing in. Next, 70% extraction of Citrus nobilis Lour peel is orally given to the rats, every day for 90 days accordance with the group. On the 90th days, the rat is not fed for 14 hours then drugged with ether to take their blood sample. The blood sample is then analyzed at labkesda (Local Lab of State), Surabaya Indonesia to get of hematology and biochemistry assessment result. Statistic results show that all variable, leucocytes, hemoglobin, and hematrocyte are considered as normal after the injection of different dose of ethanol extract 70% fruit peel Citrate nobilis Lour.*

Keywords: *Acute toxicity, Biochemistry, Citrus nobilis* Lour pee*, hematology, subchronic toxicity*.

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I. Introduction

Toxicity test is a test to detect a toxic effect of a substance in biological system and to obtain unique response dose data from the test supply. Toxicity test uses an animal as a model which is useful to observe the biochemistry, physiological and pathological reaction prediction of human toward the test preparation. The toxicity test result cannot used absolutely to prove the safety of substance or supply in humans. However, it can provide a guidance of relative toxicity and assist in identification of toxic effects in case of exposure to humans (BPOM, 2014).

Sub chronic toxicity level is used for several purposes as follow; to obtain the information on toxic effects of undetectable substances which is not predicted on acute toxicity test; to obtain the possibility of the information on toxic effects after repeated exposure of test preparation in a certain period, to obtain the dose information that has no toxic effect (No Observed Adverse Effect level / NOAEL) and to study the cumulative effect and reversibility effect of substance (BPOM, 2014).

Citrus nobilis Lour is one of the majority plants grown in Indonesia. The peel is known to contain variety of components that are Vitamin A, B, C, hesperidin, limonene, citral, and metal antranilat (Li, 2002). Oranges leaves and peels have secondary metabolites such as essential oil, flavonoids, saponins, steroid, and tannins (Prakash et al., 2013; Intekhab and Aslam, 2009). Moreover, citrus is one of the plants that produce atsiri essential oil. (Astarini et al., 2010).

Several previous studies determine the activity of *Citrus nobilis* Lour as anti-fertility. The main mechanism is through spermatozoa hyaluronidase enzyme resistance which is competitive and reversible. Hyaluronidase enzyme is an enzyme found at head (acrosom) spermatozoa (Gilbert, 1988). The ovum has 3 layers, namely oophorus cumulus, radiata corona, and pellucid zone. Fertilization occurs when spermatozoa can penetrate all third layers. Hyaluronidase enzyme has a function to penetrate of spermatozoa in cumulus oophorus, Corona Penetration Enzyme in radiate corona, acrosin in pellucida zone. These third enzymes work in a specific and in a series pattern. So that, spermatozoa penetration, along with fertilization process, will be failed if hialuronidase enzyme activity is inhibited. In previous study by Prajogo, hesperidins dose of 2,4 and 6 mg/20 g BB injected to lab rats would prevent spermatozoa penetration in fertilization process *in vitro* (Prajogo *et al.,* 1997).

Hesperidins is the substances of glycoside flavonoid and poly phenol which are part of inhibitor hialuronidase substance (Prajogo *et al.,* 1997). Hialuronidase is the first enzyme which is being secretion during enzyme binding penetration process to disperse cumulus oophurus. Thus, if hialurinidase enzyme is inhibited, the ability to disperse cumulus oophurus will be declined. Finally, there will be no penetration action at the end (Zaneveld, 1976). This penetration process becomes major reference in producing condoms for men (Fransworth and waller, 1982). From finding above, *Citrus nobilis* Lour peel could be an alternative materials for KB (Birth Control) for men. The using of this Birth Control medicine must be in continuity and long term patterns. That is why, toxicity experiment must be conducted.

II. Material And Methods

Plant and sample

Citrus nobilis Lour. sample is taken from Padang Rejo, Umbulsari, Jember, Eastern Java, Indonesia. The sample is also already registered at Conservation Institution of Botany Kebon Raya, Purwodadi, Pasuruan, Eastern Java, Indonesia.

Animals

The animals samples are male and female *Rattus norvegicus*galur wistar which are taken from Biochemistry Faculty of Doctor university of Airlangga. They are already fed by using *ad libitum* methods.

Preparation of extract

The process of creating 70% ethanol extraction of *Citrus nobilis* Lour's peel is by extracting 2180 gram of simplisia powder. By using maceration method, its process takes 3x24 hours of works, the samples are separated into 10 topples, each topples contains 218 gram of the sample. The samples then stirred out with shaker once every an hour for five minutes. The filtrate is patched and concentrated through evaporator within 45^0C until the strong liquid is obtained.

Acute toxicity study

The animals samples used in this research is both male and female BALB/c white rats within 7 weeks age weighing in 26-29 ram. Before the experiment day, the rats are being acclimated in the 22ºC degree of internal room with air conditioner attached on the building. The rats receive 12 hours of lights and another 12 hours without lights. The rats are fed with 50 gram for each cage, ad libitium is used as their beverage media. The animals are separated into 4 groups. The first group is control group which consist of 5 male rats. The second group is behavioural group which consist of 5 male rats. The third group is control group which consist of 5 female rats. The last group is behavioural group which consist of 5 female rats. The 0,5% of CMC-Na is injected to the control group rats it is also given 0,2mL/20 g BB of rats. For experimental group, 70% ethanol extraction of *Citrus nobilis* Lour's peel is given within 2000mg/Kg BB dose which is suspended into 0,5% of CMC-Na given 0,2mL/20 g BB of rats. In the first 30 minutes, the rats are dead after the samples were injected. After that, every 4 hours within 24 hours, it must continue once in a day for 14 days.

Sub-chronic toxicity

The animal samples used in this research are female and male within age around 6-7 weeks (*Rattus norvegicus)* galus *Wistar* which are taken from Biochemistry Laboratory of Doctor Faculty University of Airlangga. The male and female rats are being acclimated for a week in laboratory. All samples receive same treatment and same diet technique. Before the experiment day, all sample are being weighing in to measure the exact amount of dose regulation. The rats are placed at small cage which contains five rats of each cage. The air conditioner is set to 22°C with 12 hours full of light and another 12 without light condition. The rats are fed with 200 gram for each cage and ad libitium as their drinking liquid. The weight of samples is continuously reported before, during, and after the experiment. The monitoring of their weight is conducted twice a week. Meanwhile, toxicity symptom is reported every day.

Rats are grouped at random in such a way that the weight distribution is evenly distributed for all groups with weight variations are not exceeding 20% of the mean body weight. Each group consists of 10 rats divided into 40 cages, with the division of 20 male rats and 20 cages of female rats and each cage consisted of 5 males or 5 females. The group was divided into 4 groups: control group received 0.5% CMC-Na solution, dose group receive ethanol extract 70% fruit peel Citrate *nobilis* Lour.dose 40mg / Kg BB mouse suspended in 0.5% CMC Na, Group dose II get 70% of ethanol extraction *Citrus nobilis* Lour. peel dose of 200mg / kg of rat weight suspended in 0.5% CMC Na, Group dose III receive 70% of ethanol extraction *Citrus nobilis* Lour fruits dosage 1000mg / Kg BB rat suspended in 0.5% CMC Na. The administration was administered orally with a volume of 1mL / 100mg BB rat dose. Test solution was administered daily for 90 days. Monitoring weight gain is done twice a week. Monitoring of toxic and behavioral symptoms is done daily.

Blood hematology analysis is using the prestige analyzer tool xp 200i (labkesda, Surabaya Indonesia). The parameters consist of hemoglobin (Hb), leukocytes, erythrocytes, and hematocrit, all measured using the blood of male and female rats. As for the analysis of serum biochemistry using the tool prestige 24i automated analyzer (labkesda, Surabaya-Indonesia). The parameters consist of SGOT, SGPT, BUN, and Kreatinin, all measured using male and female serum rats.

Statistical analysis

The analysis is using oneway ANOVA method for parametric and the nalysis is using crustal wallis for non parametric data. In order to reveal the difference among samples, Least significancy difference (LSD) method must be implemented for parametric data. Meanwhile, Mann Whitney method is used for non parametric data.

III. Result

Acute toxicity study

The injection of 70% ethanol extraction of *Citrus nobilis* Lour peel within 2000mg/Kg of Rats mean weight dose does not cause any sign of death. The cause of toxicity characteristics such as stiffing fur, yellow eyes, and abnormal behavior (there is no movement or biting some body parts) could not be found on the samples. According to Organitation for Economic Co-operation Development (OECD) in 2001, the 70% ethanol extraction of *Citrus nobilis* Lour peel includes in the fifth category which is LD_{50} above 2000mg/Kg of Rats mean weight.

Sub-chronic toxicity

From sub-chronic toxicity test of 70% ethanol extraction of *Citrus nobilis* Lour peel, there is no sign of death found on the animal samples. The result of toxicity signal and abnormal behavior observation are negative, there is no signs like backward walking, biting other body parts, stiffing furs, or yellowing eyes.

Haematology

Table 1. Haematology data of male and female rats

	Male				Female			
	CMC Na 0,5%	40mg/Kg BB	200 mg/Kg BB	1000 mg/Kg BB	CMC Na 0,5%	40mg/Kg BB	200 mg/Kg BB	1000 mg/Kg BB
Haemoglobin (g/dL)	12,7±0,8	14,2±0,8	13,9±0,9	13,5±0,8	12,7±0,8	14,2±0,8	14,9±3,0*	13,4±0,5
Amount of Leucocytes ($10^3/\mu L$)	17,8±4,6	17,0±2,2	19,4±3,1	16,2±3,0	11,2±2,9	12,3±2,1	11,9±2,1	11,2±3,0
Erythrocytes ($10^6/\mu L$)	8,6±0,7	8,5±0,4	8,3±0,6	8,2±0,4	7,3±0,5	7,3±0,6	8,5±1,7	7,7±0,3
Hematocrit (%)	48±4	45±3	44±3	43±4	42±3	40±3	47±8	41±2

*Impact data

From male column and haemoglobin row, it is concluded that haemogoblin distribution experiment within *one sample kolmogorov-smirnov* method obtains 0,758 p-value, the p-value is bigger than 5% which means that the distribution is normal. According to result of ANOVA analysis, the p-value is 0, the p-value is bigger than 5% which means that the there is no significant effect of 70% ethanol extraction of *Citrus nobilis* Lour peel.

Meanwhile, from the data of female haemoglobin above it is known that the test result of haemoglobin data distribution with one sample *kolmogorov-smirnov* test obtained p-value of 0.01, p-value is less than 5% which means that the normal distribution data is normal. It is then tested with 2 independent sample known between the control group and group of dose 40 mg / Kg BB got p-value equal to 0,912 p-value more than 5% which means there is no effect of ethanol extract 70% peel of *Citrus nobilis* Lour fruit. Control group and dose group of 200 mg / Kg BB obtained p-value of 0, 01 p-value less than 5% which means there is influence of ethanol extract 70% *Citrus nobilis* Lour fruit peel. Control group and dose group 1000 mg / Kg BB obtained p-value equal to 0,055 p-value more than 5% which means there is no effect of ethanol extract 70% *Citrus nobilis* Lour fruit peel. Group dose 40 mg / Kg BB and group dose 200 mg / Kg BB got p-value equal to 0,015 p-value less than 5% which mean there is influence of ethanol extract 70% *Citrus nobilis* Lour fruit peel. Group dose 40 mg / Kg BW and group dose 1000 mg / Kg BB got p-value equal to 0,055 p-value more than 5% which means there is no effect of ethanol extract 70% *Citrus nobilis* Lour fruit peel. The dose group of 200 mg / Kg BW and dose group 1000 mg / Kg BW obtained p-value of 0, 003 p-value less than 5% which means there is influence of

ethanol extract 70% Citrus nobilis Lour fruit peel. From the description above can be seen that there is influence group dose 200 mg / Kg BB to female haemoglobin.

From male leucocytes table, it is concluded that leucocytes distribution experiment within *one sample kolmogorov-smirnov* method obtains 0,666 p-value, the p-value is bigger than 5% which means that the distribution is normal. According to result of ANOVA analysis, the p-value is 0,026, the p-value is less than 5% which means that the there is significant effect of 70% ethanol extraction of *Citrus nobilis* Lour peel.

From female leucocytes table, it is concluded that leucocytes distribution experiment within *one sample kolmogorov-smirnov* method obtains 0,835 p-value, the p-value is bigger than 5% which means that the distribution is normal. According to result of ANOVA analysis, the p-value is 0,705, the p-value is bigger than 5% which means that the there is no significant effect of 70% ethanol extraction of *Citrus nobilis* Lour peel.

From male erythrocytes table, it is concluded that erythrocytes distribution experiment within *one sample kolmogorov-smirnov* method obtains 0,682 p-value, the p-value is bigger than 5% which means that the distribution is normal. According to result of ANOVA analysis, the p-value is 0, the p-value is bigger than 5% which means that the there is no significant effect of 70% ethanol extraction of *Citrus nobilis* Lour peel.

From female erythrocytes table, it is concluded that erythrocytes distribution experiment within *one sample kolmogorov-smirnov* method obtains 0,070 p-value, the p-value is bigger than 5% which means that the distribution is normal. According to result of ANOVA analysis, the p-value is 0,018, the p-value is less than 5% which means that the there is significant effect of 70% ethanol extraction of *Citrus nobilis* Lour peel.

From male hematocrit table, it obtains 0,516 p-value, the p-value is bigger than 5% which means that the distribution is normal. According to result of ANOVA analysis, the p-value is 0, the p-value is bigger than 5% which means that the there is no significant effect of 70% ethanol extraction of *Citrus nobilis* Lour peel.

From female hematocrit table, it obtains 0,197 p-value, the p-value is bigger than 5% which means that the distribution is normal. According to result of ANOVA analysis, the p-value is 0,004, the p-value is less than 5% which means that the there is no significant effect of 70% ethanol extraction of *Citrus nobilis* Lour peel.

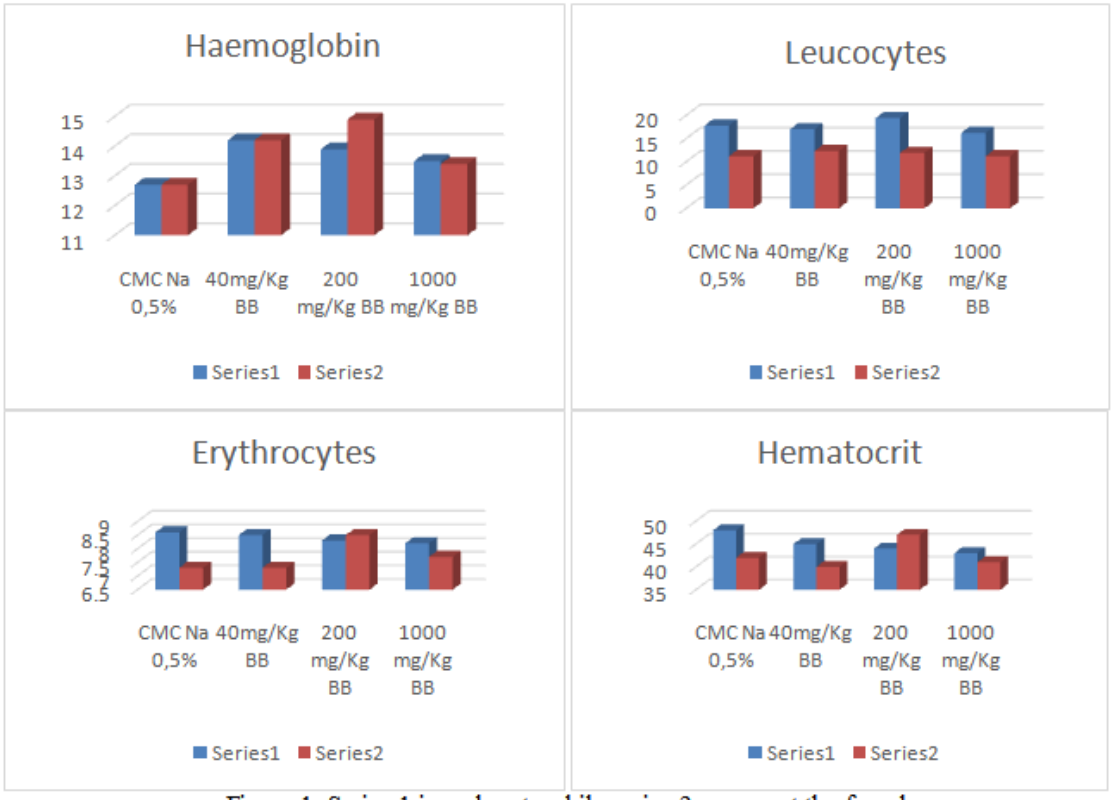

Figure 1. Series 1 is male rats while series 2 represent the female

From the charts above, we can conclude that rat female hemoglobin is increasing at 200 mg/Kg dose if it is compared with male rats. Meanwhile, at 1000 mg/Kg dose, the hemoglobin of female rats is decreasing if it is compared with male group. From the diagram, the result shows that the amount of leucocytes of female is lower if it is compared with the result of leucocytes male rats. Another result states that the amount of erythrocytes of female is lower if it is compared with the result of erythrocytes male rats. There is another

increasing erythrocytes of females when the dose is changed into 200 mg/Kg. the same case is occurred at hematocrit variable.

Biochemistry

Table 2. Biochemistry data of both male and female rats

	Male				Female			
	CMC Na 0,5%	40mg/Kg BB	200 mg/Kg BB	1000 mg/Kg BB	CMC Na 0,5%	40mg/Kg BB	200 mg/Kg BB	1000 mg/Kg BB
SGOT (U/L)	132±43	145±37	173±73	159±36	154±51	100±21	135±39	125±23
SGPT(U/L)	59±20	65±12	95±36	62±21	81±14	54±10	52±14	50±8
BUN (mg/dL)	20,8±3,9	26,1±2,5	24,3±2,9	29,6±2,7	23,6±1,5	21,8±3	30,9±3,4	28,0±6,4
Creatinine (mg/dL)	0,65±0,26	0,57±0,12	0,56±0,18	0,65±0,63	1,41±1,3	0,80±0,2	0,6±0,1	0,47±0,1

From male SGOT table, it is concluded that SGOT distribution experiment within *one sample kolmogorov-smirnov* method obtains 0,176 p-value, the p-value is bigger than 5% which means that the distribution is normal. According to result of ANOVA analysis, the p-value is 0,114, the p-value is bigger than 5% which means that the there is no significant effect of 70% ethanol extraction of *Citrus nobilis* Lour peel.

From female SGOT table, it is concluded that SGOT distribution experiment within *one sample kolmogorov-smirnov* method obtains 0,519 p-value, the p-value is bigger than 5% which means that the distribution is normal. According to result of ANOVA analysis, the p-value is 0,015, the p-value is less than 5% which means that the there is no significant effect of 70% ethanol extraction of *Citrus nobilis* Lour peel.

From male SGPT table, it is concluded that SGPT distribution experiment within *one sample kolmogorov-smirnov* method obtains 0,201 p-value, the p-value is bigger than 5% which means that the distribution is normal. According to result of ANOVA analysis, the p-value is 0,888, the p-value is bigger than 5% which means that the there is no significant effect of 70% ethanol extraction of *Citrus nobilis* Lour peel.

From female SGPT table, it is concluded that SGPT distribution experiment within *one sample kolmogorov-smirnov* method obtains 0,789 p-value, the p-value is bigger than 5% which means that the distribution is normal. According to result of ANOVA analysis, the p-value is 0, the p-value is bigger than 5% which means that the there is no significant effect of 70% ethanol extraction of *Citrus nobilis* Lour peel.

From male BUN table, it is concluded that BUN distribution experiment within *one sample kolmogorov-smirnov* method obtains 0,639 p-value, the p-value is bigger than 5% which means that the distribution is normal. According to result of ANOVA analysis, the p-value is 0, the p-value is bigger than 5% which means that the there is no significant effect of 70% ethanol extraction of *Citrus nobilis* Lour peel.

From female BUN table, it is concluded that BUN distribution experiment within *one sample kolmogorov-smirnov* method obtains 0,855 p-value, the p-value is bigger than 5% which means that the distribution is normal. According to result of ANOVA analysis, the p-value is 0, the p-value is bigger than 5% which means that the there is no significant effect of 70% ethanol extraction of *Citrus nobilis* Lour peel.

From male Creatinine table, it is concluded that Creatinine distribution experiment within *one sample kolmogorov-smirnov* method obtains 0,457 p-value, the p-value is bigger than 5% which means that the distribution is normal. According to result of ANOVA analysis, the p-value is 0, the p-value is bigger than 5% which means that the there is no significant effect of 70% ethanol extraction of *Citrus nobilis* Lour peel.

From female Creatinine table, it is concluded that Creatinine distribution experiment within *one sample kolmogorov-smirnov* method obtains 0,005 p-value, the p-value is bigger than 5% which means that the distribution is normal. The next step is to test 2 independent samples between control group and dose group of 40 mg/Kg. the result shows 0,164 p-value which is less than 5%. It means that there is no significant effect of 70% ethanol extraction of *Citrus nobilis* Lour peel. Meanwhile, both groups at 200 mg/Kg dose, the result shows 0.015 p-value which is less than 5%. It means that there is significant effect of 70% ethanol extraction of *Citrus nobilis* Lour peel. At 1000 mg/Kg dose, the p-value reach 0 which mean bigger than 5%. It means that there is no significant effect of 70% ethanol extraction of *Citrus nobilis* Lour peel. 40 mg/Kg dose group and 200 mg/Kg dose earn 0,055 p-value which exceed 5%. It means that there is no significant effect of 70% ethanol extraction of *Citrus nobilis* Lour peel. 40 mg/Kg dose group and 1000 mg/Kg dose group obtain 0,001 p-value which is less than 5%. It means that there is significant effect of 70% ethanol extraction of *Citrus nobilis* Lour peel. 200 mg/Kg dose group and 1000 mg/Kg dose group obtain 0,055 p-value which is bigger than 5%. It means that there is no significant effect of 70% ethanol extraction of *Citrus nobilis* Lour peel.

Figure 2. Series 1 is male rats while series 2 represents the female

From the diagram above, it shows that SGOT and SGPT of female are decreasing compared with male result. Another result state that the amount of BUN from female rat sample is higher than male group, the result could be concluded from the comparison of both control and 200 mg/Kg dose group. Meanwhile, at 40 mg/Kg dose group, BUN of female rats sample is decreasing compared with the result of BUN from male sample. Next, the result of creatinine states that behavioral rats possesses higher creatinine than control rats sample. From sex perspective, the creatinine of female is lower than male sample which is proven from control and 1000 mg/kg dose group.

IV. Discussion

From the data above can be known some values from hematology and biochemistry in blood male and female rats which given ethanol extract 70% *Citrus nobilis* Lour peel. One of the factors which influence the hematology and biochemistry value is a gender. Hematology and biochemistry of female rats is lower than male rats.

Hemoglobin is a component that serves as a transport of oxygen and carbon dioxide. Given the hemoglobin value of male and female control treatments is the same that is 12.7 ± 0.8. According to the mitruka, (1981) normal hemoglobin value of rats was 11.4-19.2 (16) g%. So it can be concluded that the value of hemoglobin rats is normal.

The main function of leukocytes is to fight infection, protect the body by phagocytes foreign organisms and produce, transport or distribute antibodies. Leukocytes are formed in the myelogenous, keep in lymphatic tissue (lymph, thymus, and tonsils) and transported by blood to organs and tissues. From the above results it is known that leukocyte value of male control treatment of male rats 17.8 ± 4.6 whereas leukocyte value of female rat control treatment is 11,2 ± 2,9. The existence of differences gender is what causes the differences in leukocyte values of male and female rats. According to mitruka (1981) the normal leukocytes of rats were 5-25,5 x 103. When viewed as a whole the control data, treatment 1, treatment 2, and treatment of 3 male and female rats were normal. It also occurs in the erythrocyte values obtained normal results. For more details, the results can be seen in the table above.

Hematocrit shows the presentation of red blood cells to total blood volume. A decrease in hematocrit values is an indicator of anemia due to various causes, hemolytic reactions, leukemia, cirrhosis, blood loss and hyperthyroidism. Hemoglobin and Hematocrit are used together to identify the presence of anemia (Hoffbrand, et al 2005). From the data above can be seen the hematocrit value of treatment 1, treatment 2, and treatment 3 decreased from the treatment value of male control 48 ± 4. Whereas for the data of female rats in treatment 2 occurred increasing to normal value. Based on the mitruka (1981) normal hematocrit of rats was 47.0 ± 3.1. The hematocrit value is down from the normal value can be said to be anemia symptoms but must be seen again with erythrocyte values and hemoglobin values. The hemoglobin and erythrocyte values are normal even though the hematocrit value drops does not mean an anemia indication.

Besides the data above also measured the values of SGOT, SGPT, BUN, and creatinine. The measurement is conducting to know the function of liver and kidney. The result is SGOT value is greater than SGPT value. SGOT value greater than SGPT occur due to necrosis. The occurrence of necrosis in liver cannot be seen by histopath, if SGOT is bigger than SGPT not surely yet occurring necrosis. In this case occur in BUN and creatinine. In the BUN measurement obtained the additional value than limits value that is more than 20, and creatinine value is down from the control treatment value. In this case can be indicated the presence of necrosis in kidney. The occurrence of necrosis in kidney cannot be seen by histopath, so that the BUN value more than 20 and creatinine value not surely yet occurring necrosis. Therefore, histopath reading of both organs (liver and kidney) are required.

V. Conclusion

Giving *Citrus nobilis* Lour peel extract orally for 90 days did not give subchronical toxic effect in hematology and biochemistry of male and female rats. One of the factors which influence hematology and biochemistry value is a gender. Hematology and biochemistry value of female rats is lower than male rats.

The hemoglobin value of male and female control treatments is the same that is 12.7 ± 0.8. So it can be concluded that the value of hemoglobin rats is normal.

Leukocyte value of male control treatment of male rats 17.8 ± 4.6 whereas leukocyte value of female rat control treatment is 11,2 ± 2,9. The existence of differences gender is what causes the differences in leukocyte values of male and female rats. From the control data above can be concluded that the treatment 1, treatment 2, and treatment of 3 male and female rats were normal. It also occurs in the erythrocyte values obtained normal results.

The hematocrit value of treatment 1, treatment 2, and treatment 3 decreased from the treatment value of male control 48 ± 4. Whereas for the data of female rats in treatment 2 occurred increasing to normal value.

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e thank every mentors for assistance as well as this research can be done in proper time, I'mgrateful for comments that greatly improved this manuscript.

However, the author concern that there are many less from this research. Hence, the evaluation and correction are welcoming for next paper.

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