Measuring the acute toxicity of earthworms (Pheretima javan-ica K.) through examining SGOT, SGPT, and liver histophatology corroborated by the observation on the physical characteristics of white rats (Rattus norvegicus L.)

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Abstract. *Pheretima javanica K.* is a type of earthworm potentially used as typhoid fever medicine because its contents, such as *Lumbricin I*,is an antimicrobial peptide which can nullify the permeability of bacterial membranes. In this study, fine earthworm powder was produced through drying and mixing process. This study aimed to test the acute toxicity of earthworm powder (*Pheretima javanicaK.*) by examining SGOT and SGPT levels, histopathological observations of white rats' liver (*Rattus norvegicus L.*), and theobservation of the rats's physical characteristics. Fifty white rats were divided into 5 treatment groups, including treatment 1 (dose 400 mg/Kg BW), treatment 2 (dose 800 mg/Kg BW,) treatment 3 (dose 1600 mg/Kg BW), and treatment 4 (dose 3200 Mg/Kg BW). The white rats were given earthworm powder dissolved in aquades for 14 days, and then the rats' liver were dissected, and histopathological preparations were done using Hematoxylin Eosin (HE) staining technique. LD50 calculation using Thomson and Weil method was operative to analyse the data. The analysis result indicated that the treatment involving 14-day interval of more than 5.000 mg/Kg BW was proven practically non-toxic. Anova test results focusing on SGOT and SGPT indicated no significant effect on liver function, as marked by significant value I, whereas the Histopathology test revealed no difference between negative control and treatments involving dosages ranging from 400 mg mg/KG BW to 3200 mg/KG BW. The observation of physical characteristics evinced that generally the rats were classified to be health, which was marked by soft dense furr, sharp red eye color, solid faeces, and increased weight gain on weekly basis

1. Introduction

Typhoid fever still remains a global health problem these days. Salmonella typhi is a type of bacteria triggers cause typhoid fever. WHO (World Health Organization) estimates that around 17 million people suffer from typhoid fever per year at world level, resulting in the death of 600,000 people. 70% of these deaths occur in developing countries of Asia [1]. The number of people with typhoid fever in Indonesia has been reported to be 81.7 per 100,000 people, and based on the distribution ofthe age group the illness is dominated by of those at the age of 2-15 years old [2].

The medication og typhoid fever is still dominated by using various various types of antibiotics such as chloramphenicol, amoxicillin, cotrimoxazole, ampicillin, and tiamfenikol. Based on research conducted by Haque et al. (2005), the use of antibiotics is considered less effective because of the bacterial resistance to typhoid. In 2001, the resistance increased and in 2007 about 6.8% of isolates

Salmonella typhi were found resistant to the three antibiotics, including ampicillin, chloramphenicol and cotrimoxazole [3].

A large number of antibiotics, in terms of both quantity and quality, are sold in the market, so people can easily get antibiotics without any prescription and use them inappropriately, which is why antibiotic resistance has been on the rise. Antibiotics such as chloramphenicol also cause side effects such as nausea in patients with ulcers, bone marrow suppression, and even aplastic anemia [4]. Therefore, it is imperative to come up with another alternative, that is earthworm (Pheretima javanica K.), which can be used as a natural cure for typhoid fever.

Earthworm (Pheretima javanica K.) has an antimicrobial peptide bioactive compound called Lumbricin I, which contains 15% proline of total dry weight and is composed of 26 kinds of amino acids with molecular weight of 7,231 kDa [5]. Lumbricin I compounds work by altering and destroying membrane permeability mechanism. Through electrostatic interaction with the cell walls, the bacteria forms Multimeric Membrane Protein Pore, thus causing the integrity of the membrane impaired and nullifying the cells [6].

With the potential of earthworms (Pheretima javanica K.), the present study deems necessary investigating the benefits of earthworm (Pheretima javanica K.) as an alternative typhoid fever drug before goes through massive commercial production. Before it is used for medicine and commercially produced, there are several test stages to carry out. These include preclinical testing and clinical trials. The preclinical test comprises of testing the conceived compound and the acute or subacute toxicity test of the pharmacodynamic data, which must be met before proceeding to a clinical trial later tested to humans [7]. In addition, one of the preclinical preconditioning requirements, the acute toxicity test, aims to determine the toxic effects of a compound on some specific organs exposed to the drug, such as liver and kidneys which is related to blood filtration process. Based on its function, liver is the most sensitive gland in detecting the presence of toxic compounds entering human's body. The determination of toxicity was done by measuring the levels of glutamate pyruvate transaminase (SGPT) and glutamate oxaloacetate transaminase (SGOT) as the benchmarks of acuto hepato cellular [8].

Drug excretion mainly occurs in the liver, so the risk of organ damage becomes higher compared to other organs. The liver is also an organ holds important plays a role in metabolism and excretion in the body. Almost all substances which enter the body and follow the systemic circulation will be metabolized in the liver [9].The liver is an organ of the body which has various functions, such as secretion organ, producing gall which is useful in the emulsification and absorption of fat, and the storage organ to some minerals, such as iron and copper, and fat-soluble vitamins, inter alia vitamins A, D, E, and K [10]. The liver also stores toxins and drugs which cannot be broken or excreted by the body. Upon detoxification, the liver can detoxify toxins and various drugs. This process is carried out through oxidation, methylation, and conjugation. Thus, the further research on organ damage through histopathologic images of the liver was done. Observations were done to determine the possible damage caused by earthworm powder (Pheretima javanica K.) during a certain period of time. Damaged liver cells are characterized by degeneration and necrosis [11].

2. Method

The samples in the research were fifty 3-4 months old white rats (Rattus norvegicus L.) weighing around 200-250 gram. The study consisted of 4 treatments and 1 negative control, with 10 replications. These animals were acclimated for 7 days. This aimed to homogenise and observe the early condition of white rats prior to the treatments.

The steps of making earthworm powder (*Pheretima javanica K.*) included washing, draining, drying the earthworm for 6-7 days, and baking it in an oven at the temperature of 40º C for 4 approximately hours. The earthworms which had been dried were blended to gain smooth powder, and the powder was filtered according to the desired powder size. After that, the dry powder was weighed with a predetermined doses of 400, 800, 1600 and 3200 mg/Kg BW. 2 ml blood of each rat, which had been acclimated and ascertained to be in good health, was taken through reorbitalis vein to be tested

for its SGOT and SGPT as preliminary data. After going through several stages of preparation, the rats were given treatment of earthworm powder with the various dosages of 400, 800, 1600 and 3200 mg/Kg BW for 14 days. During the treatment, the rats' condition were under observation for possible death in order to gain LD_{50}. On the 15^{th} day of the treatment, the second blood sampling was done to obtain SGOT and SGPT levels after treatment, and the results were compared with the initial levels to see whether there was an increase in SGOT and SGPT levels.

The histopathology preparation of liver was performed after investigating the SGOT and SGPT levels, followed by a surgery on the rats to examine the liver. At this juncture, the histopathology preparation employed Hematoxylin Eosin staining technique. The observation of liver damage was done using descriptive analysis and scoring method.

The observation of rats' physical characteristic was done during 4-week treatment, comprising of one week before adaptation, another one week after adaptation, and two weeks of treatment with parameters of furr condition, eye colour, faeces texture, and the weight gain.

The obtained data were calculated and the value of LD50 obtained was analyzed by using Thomson and Weil method. The data analysis of SGOT/SGPT applied ANOVA test, and the analysis of the liver damage was done in descriptive analysis with a scoring method. Furthermore, the analysis to investigate the effect of earthworm powder on SGOT/SGPT level at the was performed at the beginning and at the end of experiment. The weight gain was analyzed using Duncan test.

3. Results and Discussion

The examination on LD_{50} of earthworm powder (*Pheretimajavanica K.*) included various doses, *inter alia*, 400 mg/ Kg BW,800 mg/ Kg BW, 1600 mg/ Kg BW, 3200 mg/ Kg BW.

Table 1. Test results of LD50 earthworm powder (*Pheretima javanicaK.*) on white rats.

Dose (mg/Kg BW)	Number of Rats	Death Observation Period (Hours)	The Number of Deaths
0	10	336	0
400	10	336	0
800	10	336	0
1600	10	336	0
3200	10	336	0

The test of LD_{50} earthworm powder (*Pheretima javanica K.*), with a total of 10 animals tested for 336 hours, evince 0 death. Since there was no death in the test, the LD_{50} obtained more than 5000 mg/Kg BW, according to the Global Harmonization Classification System (GHS), was categorized in non-toxic practical category.

The results of SGOT and SGPT test before and after the exper-iment were determined by comparing the average results of the five treatment groups. The comparison results are presente in Table 2.

Table 2. Results of SGOT and SGPT levels before and after treatment

No	The Rat Code of Each Treatment	SGOT value (U/L)		SGOT value (U/L)	
		Before	After	Before	After
1	K-	188.9	194.2	89.7	88.2
2	P1	2015.5	187.7	80.9	76.4
3	P2	168	214.6	81.5	85.7
4	P3	195.3	193	70	72.7
5	P4	191	191.5	71.9	80.4

The result of ANOVA test to know the effect of earthworm powder (*Pheretima javanica K.*) on SGOT and SGPT levels before and after the treatment can be seen in the following tables.

The Anova data analysis results showed SGOT significance value of 0.461, it is greater than 0.05, thus indicating that there is no effect of earthworm powder (Pheretima javanica K.) on the rats' SGOT levels.

The result of ANOVA analysis on SGPT data indicated the significance value of 0.266, it is greater than 0.05, therefore indicating that there is no effect of earthworm powder (Pheretima javanica K.) on the rats' SGPT level.

The result of histopathologic observation of the rats' liver after treatment in five treatment groups can be seen in Table 3.

Table 3. Histopathological observation results of rat liver after treatment

Treatment	Repetition					Scoring
	1	2	3	4	5	
K- Male	-	-	-	1	-	1
K- Female	-	1	-	-	-	1
P1 Male	1	-	-	-	-	1
P1 Female	-	-	1	-	-	1
P2 Male	1	-	-	-	-	1
P2 Female	1	-	-	-	-	1
P3 Male	-	-	-	-	1	1
P3 Female	-	1	-	-	-	1
P4 Male	1	-	-	-	-	1
P4 Female	-	-	-	-	1	1

The results of histopathologic observation of white rats' liver (*Rattus norvegicus*) after 14 days of treatment showed a normal score of 1. The results of microscopic observation representing each treatment group can beseen in Figures 1 to 5, which applied with 400x magnification.

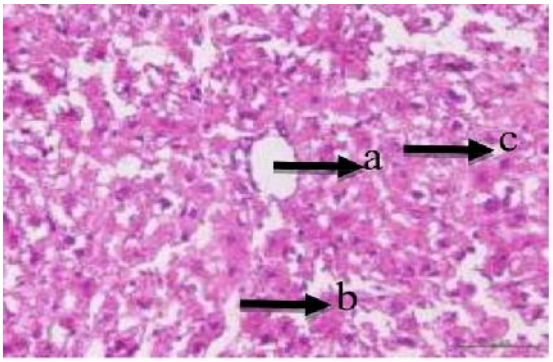

Figure 1. The Histopathology of Liver Cell in Control group (-) Observed under 400x magnification (a: central vein, b: sinusoid, c: hepatocyte).

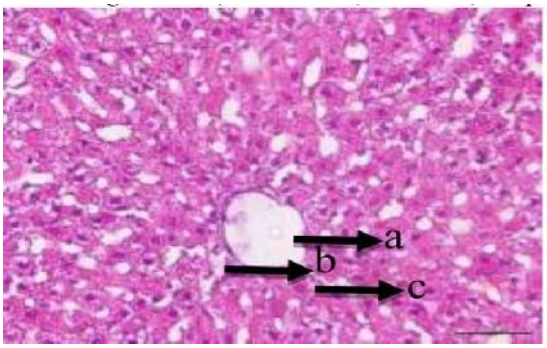

Figure 2. The Histopathology of Liver Cell in Treatment

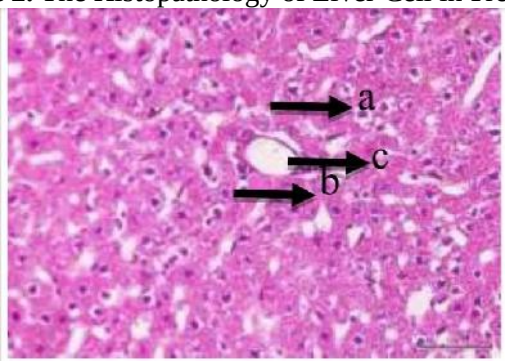

Figure 3. The Histopathology of Liver Cell in Treatment Group 2 Ob-served under 400x magnification (a: central vein, b: sinusoid, c: hepatocyte)

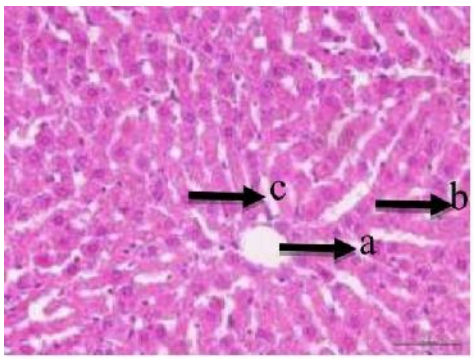

Figure 4. The Histopathology of Liver Cell in Treatment Group 3Observed under 400x magnification (a: central vein, b: sinusoid, c: hepatocyte)

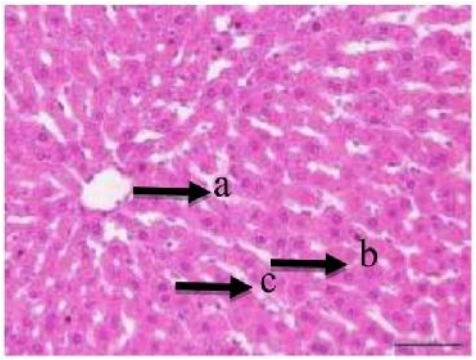

Figure 5. The Histopathology of Liver Cell in Treatment Group 4 Observed under 400x magnification (a: central vein, b: sinusoid, c: hepatocyte)

The observation of the physical characteristics of white rats showed that all tested animals passed the healthy criteria indicated by fine-grained furr condition, sharp red eye color, dense feces texture, and weight gain analyzed using Duncan test. The results of Duncan test are presented in Table 4.

Table 4. The result of Duncan test of white rats body weight

Week	N	Subset for alpha = 0.05	
		1	2
1	5	150.40	
2	5	160.80	
3	5		179.80
4	5		185.00
Sig.		.217	.530

The data clearly shows that the rats' body weight differ significantly in the second week to the third week in the treatment administering earthworm powder (*Pheretima javanicaK.*). This study was an experimental research aiming to generate an alternative drug, which is effective and safe to consume, in order to control the antibiotic resistance of typhoid fever. The use of antimicrobial peptide content of *Lumbricin I* in the earthworm powder (*Pheretima javanica K.*) was considered potential to generate such drug. It was necessary to perform preclinical testing, which was a compound activity test and an acute toxicity test. This study only dealt with acute toxicity tests to obtain an effective dose but still safe for consumption. Through the obtained value and range of LD_{50} (Lethal dose 50), the measurement of SGOT and SGPT levels relied on the transaminase enzyme as a benchmark of acute hepatocellular.

Based on the research results, the LD_{50} values obtained were higher than 5000 mg/Kg BW, which according to the chemical/material compound toxicity classification related to LD_{50} value on Globally Harmonized Classification System (GHS) as practically non-toxic because there was no death found.[12].

Thus, when the substances were administered to animals with high doses and no animals died, all dangerous acute toxicities were proven non-existent [13]. This study showed that earthworm powders did not contain any toxic. The ingredient at the highest dose was 3200 mg/Kg BW. Earthworm powder (*Pheretima javanica K.*) had no toxic effect since its content was dominated by

70% protein greater than meat and fish protein. High amount of protein in earthworms consisted of at least 9 essential amino acids and 4 kinds non-essential amino acids. The great number of amino acids indicated that earthworms (*Pheretima javanica K.*) were very useful for human health.

The abovementioned descriptions have concluded that the results of the LD50 test with an interval of 14 days on earthworm powder (Pheretima javanica K.) on rats, given at doses larger than 5000 m/Kg BW, are under the category of Globally Harmonized Classification System (GHS) toxic. The LD50 test was not the only test used to assess the toxicity of a drug or substance. Thus, this study was also supported by liver function test on the rats, in the form of SGOT and SGPT measurements which aimed to determine the effect of administering earthworm powder (Pheretima javanica K.) on liver function.

In some cases, enzymes can be used as a marker of damage to an organ due to certain diseases, including abnormalities that occur in liver cells. This especially happens on enzymes that catalyze various reactions in metabolism. Enzymes holding a role in metabolism are oxidoreductase, transferase, isomerase, liaise and ligase enzymes. The most common type of enzyme used to detect cellular damage, especially in the liver, is the transaminase enzyme. The enzymes normally used are the glutamate oksaloasetat transaminase (GOT) enzyme, also known as the aspartate Aminotransferase enzyme (AST), and the glutamate pyruvate transaminase (GPT), also called Alanine Aminotransferase (ALT). GOT enzymes are enzymes that catalyze the transfer of the alpha amino group from aspartate acid and a-ketoglutarate acid to produce oxaloacetic acid and glutamate acid. GOT itself can be found in the cytosol hepatic cell. By contrast, the GPT enzyme is a transaminase class enzyme which catalyzes the transfer of alpha amino group from alanine and a-ketoglutarate acid to

Detailed submission guidelines can be found on the journal web pages. All authors are responsible for understanding these guidelines before submitting their manuscript form pyruvate and glutamate acid. This enzyme is mostly found in mitochondria of hepatic cell. The damage of the liver's parenchymal cells and membrane permeability removed GOT and GPT enzymes out of the cell. Generally, the GPT and GOT enzymes work in intracellular fluid, so if any cells are dead or broken then the contents are poured out [14]. So, the enzyme runs into the blood vessels beyond the normal state and the level increases in the blood. Thus, the indicator of liver tissue damage is known by measuring the transaminase enzyme level on patient serum, which is Serum Glutamate Oxosacetate Transaminase (SGOT) and Serum Glutamate Pyruvate Trans-aminase (SGPT). Both of those enzymes will increase first, and their increase will be more drastic, compared to that of other enzym[15].

Any drug or toxic-containing substances can enter the body. The most sensitive organ to toxic is the liver. When the liver is damaged, the levels of SGOT and SGPT will increase signifi-cantly, i.e. 3-5 times higher than the normal value, so it later signify any disorders in the liver. To know the effect of earth-worm powder (Pheretima javanica K.) on the body, it is neces-sary to test the levels of SGOT and SGPT in animals.

The research samples involved in SGOT and SGPT measure-ment were 30 rats, divided into 5 groups consisting of 4 treat-ment groups with variant doses of 400, 800, 1600 and 3200 mg/Kg BW, and 1 control group. The entire group received treatment for 14 days. On the first day, before treatment, blood serum samples were taken and then measured to see the value of SGOT and SGPT before treatment. It can be seen from the average value of SGOT and SGPT before and after the treat-ment. There were groups that showed elevated level mean and some indicated a decline, but the evident increase was not too high and did noot reach 2x of the normal value. Likewise, those of the control group were also still within normale range. To know the significance increase taking place, Anova test was done to know whether there was influence of earthworm pow-der (Pheretima javanica K.) on SGOT and SGPT levels before and after treatment.

According to the result of Anova test, it was evident that the significance value between the early and the final SGOT value was $0.461 > 0.05$. Since the significance value was greater than 0.05, the study concluded that earthworm powder (Pheretimajavanica K.) had no significant effect on SGOT value, so it didnot affect the liver function or, in other words, it did not cause toxic effect on the liver. Meanwhile, according to the result of Anova test performed on the SGPT value, the significance val-

ue was 0.266> 0.05. Since the significance value was greater than 0.05, the earthworm powder (Pheretima javanica K.) was proven unable to increase the SGPT value, so it did not affect the liver function or, in other words, it did not cause toxic ef-fect on the liver.

Earthworm powder (Pheretima javanica K.) had no effect on the increase of SGOT and SGPT levels, since earthworm pow-ders contained several compounds such as Lumbricin I, arachi-donic acid, and lumbrokinase enzymes which were all benefi-cial for liver. Lumbricin I worked by forming Multimeric Membrane Protein Pore, which can affect the permeability of bacterial cell membranes causing cell death. [16] Another func-tion of Lumbricin I was to facilitate intracellular signaling pathways by inhibiting gamma-GT synthesis, which was a precursor of the compound triggering liver damage. In addition, Lumbricin I protein also contained lipid, lowering activity pre-venting fatty liver by repairing damaged liver cells.

Other ingredients, such as arachidonic acid and lumbrokinase enzymes, work continuously. Arachidonic acid can produce prostaglandin vasodilators, such as PGE 2 which initiates the inflammatory reactions and lumbrokinase enzymes to stabilize blood function by decreasing lipid peroxidase. The conditions, in which oxidative lipid degradation of the liver plasma mem-brane takes place, causes damage to liver cells. So, the liver function returns to normal (Lee et al., 2004: 535). These prem-ises have concluded that the earthworm does not affect the increase of SGOT and SGPT levels of white rats.

The increased levels of SGOT and SGPT occured due to other factors, some of which were different hormonal and metabolic effects in each tested animal. These factors affected the differ-ent levels of SGOT and SGPT.

Based on the aforementioned description, earthworm powder (Pheretima javanica K.) did not give any toxic effects on the liver. It was corroborated by the results of SGOT and SGPT examination, which showed insignificant increase. As a corol-lary, it was proven safe for liver function.

Based on the results of histopathologic examination on white rats' liver (Rattus norvegicus), the study found that there was no difference between control group C (-) and treatment group (T1, T2, T3, and T4) (Fig. 4.1- Figure 4.5) . In general, the histologic structure of the liver was affected by earthworm powder (Pheretima javanica K.) under normal circumstances. All the treatment groups showed that earthworm powder (Pheretima javanica K.), administered in several doses, was safe when given orally. Microscopic examination was done with 100x magnification, then continued by 400x magnification. Based on the histopathologic observation on white rats' (Rattus norvegicus) liver cell, there was no damage on normal cellular cells of regularly arranged hepatocyte cells, in all three zones of liver acetus. Also, the cytoplasm did not swell and it was not pale. The chromatin agglutination did not occur on nucleus, venous centralis (a), sinusoid (b), and hepatocytes (c). The indication of damage due to treatment was evident in zone III, which was closest to the central venous. When the damage happened to zone III, degeneration and nekrosis would occur. However, the observation results proved that there was no damage, and the damage mostly happened in zone I due to oxygen radicals since this was the most oxygen-laden area, resulting from its proximity to the hepatic artery. Based on those three zones, the histopathology of white rats' livers (Rat-tus norvegicus) were normal.

The results of observation scrutinizing the physical characteris-tics of white rats showed all tested animals were 100% healthy, and these criteria included smooth and dense furr, sharp red eye, and solid feces. During the treatment 2 up until treatment 4 on first week of treatment, the feces was black. This was probably caused by the dose given to the tested animal, especially on treatment 4, which administered 3200 mg/Kg BW powder and dissolved it in 4 ml Aquades. The solution, which was given to the tested animals, was very concentrated, so it affected the color changes of rats' feces. In addition, according to Palungkun (2010), besides high amount of protein, earthworm (Pheretima javanica K.) also contains fat, minerals, and water. The chemical components contained in earthworm include 0.4 to 1.6% iron (Fe). The excess iron level in the body was dis-posed through the feces; it made the feces turn out black.

Based on the results of weight analysis, an increase of weight in every week was found. A significant increase occurred in the third week. This was driven by the effect of of earthworm powder

(Pheretima javanica K.). This can occur because earthworm powder (Pheretima javanica K.) contained a lot of protein.

Consuming enough protein helps to nourish the body and repair damaged tissue, but consuming too much protein and doing less activity can cause weight gain. This happened to tested animal since the cage was small. One gram of protein contains about 4 calories, which means that the number of calories will increase as protein consumption rises. As a result, the admin-istration of earthworm powder (Pheretima javanica K.) at high dose increased the rats' weight.

4. Conclusion

The result of LD_{50} testing on earthworm powder (*Pheretimajavanica K.*) through Thomson and Weil method, administeringmore than 5000 mg/Kg BW powder, has been categorized in non-toxic practical category, according to the standard set by Globally Harmonized Classification System (GHS). Earthworm powder (*Pheretima javanica K.*) does not yield toxic effect on liver, which can be proven by the analysis result concerning the effect of earthworm powder on SGOT/SGPT level. The effect was proven insignificant, both in the beginning and in the end of experiment, with significance value reaching 0,461 on SGOT and 0,266 on SGPT. As a result, the powder has been categorized as safe for liver. The result of histopathological examination on the liver of all rats in both treatment groups and negative control group revealed no significant difference. In the same vein, the histopathologic picture of normal white rat's liver has proven no impact on the liver. The impact of earth-worm powder (*Pheretima javanica K.*), which is administered to the samples, has been evident of the physical characteristics of white rat (*Rattus norvegicus L.*). All samples gained 100% score in health criteria, marked by dense and smooth furr, sharp eye color, and solid feces, as well as the weight gain

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