In vivo sub chronic toxicity evaluation of the methanol extract of *Meretrix meretrix* Linnaeus in *Sprague dawley* rats

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Abstract. This study aims to determine the effect of the methanol extract of sea water clams (*Meretrix meretrix*) against blood chemistry profile in vivo in *Sprague Dawley* rats. After three months fed by two different methanol extract concentration i.e. 0.1 g. kg⁻¹ body weight (M/0.1) and 1 g. kg⁻¹ body weight (M/1), blood serum has been taken prior for the analysis using Biocon Diagnostic test kit quantitatively. Based on the observations, growth, feed intake, weight of liver and kidney were found in normal conditions. When comparing urea, creatinine and cholesterol extents among the control and treated mice were not significantly different (p>0.05) while the levels of bilirubin and albumin between control rats treated with M/0.1 and M/1 results in different significantly (p<0.05).

Keywords: blood chemistry, methanol extract, *Sprague Dawley* rats

1. Introduction

*Meretrix meretrix* of sea water is included in the class Bivalve. Bivalves are estimated to account for one third of the phylum mollusks that includes clams, and oysters’ gravestones. Biota have two shells flattened lateral and bilateral symmetry and the body is located inside the shell. *Meretrix meretrix* empirically believed by the public to cure various diseases including hepatitis, hypertension and may even improve stamina.

*Meretrix meretrix* was reported to have anti-cholesterol and antioxidant activity [1-4]. Other bioprospecting properties included the activity of immune modulator [5-9], antitumor and anticancer [9], as well as anti-hyperglycemic and anticholesterol [1, 7, 10].

*Meretrix meretrix* has a wide range of health benefits, but must be supported by data from toxicity testing. This information is intended for security in the development of products derived from these shells as both food and medicine. Based on the results of toxicity testing of water extract sub chronic *Meretrix meretrix* have LD₅₀>15 g/kg or virtually non-toxic identified that a substance if it has LD₅₀ (>15 g/Kg) belonged to practically nontoxic materials [11-12]. But the use of *Meretrix meretrix* repeatedly can cause toxic effects on organs. Therefore, it is necessary to conduct sub-chronic toxicity testing which aims to determine the effect of the methanol extract of *Meretrix meretrix* on rats.
2. Materials and Methods

2.1. Chemicals
All solutions have used pro analysis (p.a) quality such as pure aquadest, methanol (Merck USA), rat feed, Sprague Dawley Rats (Central Veterinary Research Bogor), blood rats serum, EDTA (Merck USA), Whatman 42 and paper aluminium.

2.2. Preparation and sample extraction
The extraction step is carried out through two phases, including sample preparation and extraction of samples of shellfish. Stages of sample preparation begin with a sample taken from Cirebon, West Java. Sample mussels are then separated between the shell and meat. Meat is then dried using freeze dryer, then mashed into powder form. The extraction process is done by using the maceration method refer to [13]. Samples of 50 g which have been mashed and then macerated with 200 mL of methanol (p.a) (Merck USA) for 24 hours and shaked with Shaker JOANLAB at speeds of 150 rpm. Results of maceration form until a clear solution is then filtered with Whatman 42 filter paper to obtain a filtrate and a residue. The filtrate extract is evaporated at a rotary vacuum evaporator Cole-Parmer EW-28615-01 at 50 °C to obtain methanol extract in paste form.

2.3. Sub chronic toxicity test in vivo [14]
Sub chronic toxicity testing is done by observing the physical and chemical parameters. Chemical parameter testing is carried out in several stages. The first step is the preparation of samples of test mice. Test mice obtained from the Central Veterinary Research with the size of ± 150 g sex male. Test mice were separated into three groups: control mice, the treatment of extract at a dose of 0.1 g/kg BW (M/0.1) and treatment of extract at a dose of 1 g/kg BW (M/1). Each treatment consisted of 10 rats. The second stage was making the blood serum. After three months, the mice have blood drawn for serum chemistry analysis. Making the blood serum is done through the following steps, the first which is that the rats are anaesthetized and then blood is drawn from the heart using a syringe. Blood was collected into centrifuge tubes, and then allowed to stand for 15 minutes and then centrifuged at 3000 rpm for 10 minutes. Serum is taken and stored in the refrigerator at a temperature of 12-15 °C. The second stage is to test blood serum chemistry parameters such as urea, bilirubin, creatinine, albumin and cholesterol. Each parameter uses a pipette of 20 ml sample of serum, and then added to the mixture of reagents 1 (buffer) and reagent 2 (starter) with the type of reagents varying for each parameter. Mixing between the sample and reagents as well as the reading is done automatically by the Auto-analyzer Cobas Mira Instrument Dirui BCC-30000. This analysis method Biocon Diagnostic test kit MCD-900 quantitatively.

2.4. Statistic test
The experiment will be conducted using a completely randomized design. Factors that are used there are two levels such as control rats and extracts of methanol with 10 repetitions. Data was processed using SPSS 15.

3. Results and Discussion

3.1. Growth and feed consumption
The results showed the effect of the crude extract inhibiting the growth of mice when comparing to the control and treated experimentation. We noted that the crude concentration resulted in slower growth. According to [12], reduced body weight gain is an index of toxic effects is simple but sensitive. Here it can be seen the growth data of the mice during the observation in figure 1.
Figure 1. The growth of test rats during the 12 week trial

M/0.1 (Methanol extract dose 0.1 g/kg BW), M/1 (Methanol extract dose 1 g/kg BW),
control, ▽ M/0.1, ▲ M/1.

Based on the results of measurements of growth during the 12 weeks, the rats had increased in body weight in the range of 2.20 - 15.98%. Differences in body weight were associated with each treatment condition and feed intake of rats. The growth of normal mice by an average of 1.5-3.0% per day from the initial weight, or about 7.05-21.0% per week, if it is fulfilled with good nutrition and mice are still under the age of 5 months [14]. Based on these results, it was concluded that given the growth of mice was normal methanol extract. This shows that the extract of Meretrix meretrix allegedly does not contain compounds that can cause interference with the absorption of nutrients, so the body can utilize nutrients efficiently and can increase the body weight of mice.

Figure 2. The amount of feed intake of rats for 12 weeks

M/0.1 (Methanol extract dose 0.1 g/kg BW), M/1 (Methanol extract dose 1 g/kg BW),
control, ▽ M/0.1, ▲ M/1.

Increased body weight of mice is affected by the amount of feed consumption. Feed is a role for energy and growth in mice. Here it can be seen the data of feed intake of laboratory mice for 12 weeks of observation in figure 2. Based on observations, feed intake of rats for 12 weeks experienced fluctuations. Materials containing toxic compounds always affect the digestive enzymes, so that the
enzyme can work uninterrupted and tastes of fall dramatically widened. This factor is an early stage disruption of metabolism, the amount of feed consumption is low and will cause other metabolic disorders, and the growth of rats being affected [15].

3.2. Weight of rats liver
The liver is the one organ that has the most important metabolic functions. Liver damage will cause metabolic disturbances in the body. The liver is responsible for biotransformation of harmful substances into harmless substances, which is then excreted through the kidneys [17]. The results of rat liver weight measurements by the methanol extract can be seen in table 1.

| Treatment | Weight (g) |
|-----------|------------|
| Control   | 2.78<sup>a</sup> |
| M/0.1     | 2.60<sup>a</sup> |
| M/1       | 2.49<sup>a</sup> |

M/0.1 (Methanol extract dose 0.1 g/Kg BW), M/1 (Methanol extract dose 1 g/kg BW).

Based on observations in rat liver weight after 12 weeks by the methanol extract *Mertetrix meretrix*, liver weight control and treatment groups were not significantly different (p<0.05). The percentage of liver weight of rats ranged between 2.92% - 3.07% of body weight. The relative weighting of rat liver is 2.3 - 3:10% of body weight. It can be considered that the liver weights of rats is in the normal range [18].

The livers are showing appearances of below normal liver or liver had cirrhosis stage further so that the liver becomes smaller than actual size. The hearts are heavier than normal weight, suggesting adipose tissue or fat accumulation in the liver, which is the early stage of cirrhosis. Cirrhosis of the advanced stage cell organelles, so that the condition of cells are getting worse and this condition will be irreversible. If the death of hepatocyte occurs continuously and in large numbers, then it will make cirrhosis and liver weights will shrink [16].

3.3. Weight of rats kidney
The relative weights of the kidneys can be used as an indication of kidney damage. Kidney weight measurement results can be seen in table 2.

| Treatment | Weight (g) |
|-----------|------------|
| Control   | 0.64<sup>a</sup> |
| M/0.1     | 0.59<sup>b,c</sup> |
| M/1       | 0.56<sup>c</sup> |

M/0.1 (Methanol extract dose 0.1 g/Kg BW), M/1 (Methanol extract dose 1 g/kg BW).

Based on observations of the rats kidney weights after 12 weeks by the methanol extract, rats kidneys decreased slightly. Weight kidney control rats on average are around the amount of 0.64 g, the rats were given a methanol extract with a dose of 0.1 and 1 g/kg respectively in the amount of 0.59 g (p>0.05) and 0.56 g (p<0.05) or lower of the average kidney weight control mice. The relative weights of the rat kidney is 0.4-0.9% of body weight of mice [17]. Kidney weights of mice ranged between 0.63 to 0.88%. Based on these data the weight of the kidneys of rats is still in the normal weight category.

The kidney functions to remove metabolic waste by filtering blood plasma, then processes into the urine, regulate water content, certain electrolytes (Na, K, Ca) as well as other important ingredients in blood glucose, dispose of materials in excess or unnecessary by the body after the first overhauled, such as hormones and drugs [18]. The relative weight of the kidneys can be used as an indication of kidney damage.
3.4. Blood chemistry profile

Blood chemistry analysis is very useful for the diagnosis or for the benefit of research purposes. Blood can describe the situation or the object medical experiments at the time the blood was drawn. The blood chemistry parameters were analyzed such as, urea, creatinine, cholesterol, bilirubin and albumin. Here can be seen the results of measurements of the blood chemistry profile of the mice given the methanol extract in Table 3.

| Treatment | Urea (mg/dL) | Creatinine (mg/dL) | Cholesterol (mg/dL) | Bilirubin (mg/dL) | Albumin (mg/dL) |
|-----------|--------------|--------------------|--------------------|------------------|-----------------|
| Control   | 28.65<sup>a</sup> | 1.03<sup>a</sup>   | 61.47<sup>a</sup>  | 0.52<sup>a</sup>  | 3.48<sup>a</sup> |
| M/0.1     | 28.83<sup>a</sup> | 1.02<sup>a</sup>   | 60.86<sup>a</sup>  | 0.25<sup>b</sup>  | 3.60<sup>b</sup> |
| M/1       | 31.28<sup>a</sup> | 1.04<sup>a</sup>   | 58.44<sup>a</sup>  | 0.04<sup>b</sup>  | 3.66<sup>b</sup> |

Based on the results of measurements of blood chemistry parameters in experimental rats given the methanol extract, results obtained urea content between control mice treated with methanol extract of 0.1 (M/0.1) and 1 (M/1) g/Kg did not give significantly different results (p>0.05). Normal levels of urea in the blood ranges between 12-42 mg/dL [15]. Levels of urea of the experimental results are still within the normal ranges.

Levels of urea in the blood reflect the balance between production and excretion. Urea is derived from the metabolism of proteins, especially those from food. People who consume lots of protein have urea levels usually above the normal range. Low levels are usually not considered normal, as it reflects low levels of protein eaten. If there are very low levels it can indicate severe liver disease. Decrease of urea levels was also observed in the long-term protein malnutrition. Replacement of long-term blood loss, dextran and glucose can lower levels of urea due to dilution [19].

Creatinine is a non-protein nitrogen compound produced during metabolism phosphocreatine. Creatinine is formed from amino acids kind of glycine, arginine and methionine with phosphate to form creatinine phosphate. Based on observations obtained creatinine levels of control rats treated with methanol extract of 0.1 (M/0.1) and 1 (M/1) g/kg did not change significantly (p>0.05). Normal levels of creatinine in the blood is between 0.40 to 1.37 mg/dL [20]. So, it can be concluded that the experimental mice blood creatinine levels are still within the normal ranges.

There is increased blood creatinine when kidney function declines [21]. Therefore, creatinine is considered more sensitive and is a specific indicator in renal disease. Slightly increased blood urea levels can indicate the occurrence lack of fluid volume, but the serum creatinine level of 2.5 mg/dL may be an indication of kidney damage.

Cholesterol is also an indicator of blood chemistry parameters. Based on observations, the methanol extract of 0.1 and 1 g/kg did not change significantly (p>0.05) on cholesterol levels in rats. Normal levels of blood cholesterol ranged between 52-104 mg/dL [20]. So it can be concluded that blood cholesterol levels are under normal conditions. Highly saturated fat diet increases blood cholesterol concentrations of 15-25%.

Bilirubin is a yellow pigment that is produced of biliverdin and is reduced by biliverdin reductase [21]. 0.1 methanol extract and 1 g/kg give a significantly different effect (p<0.05). Normal levels of bilirubin in the blood is <1.50 mg/dL [20]. Thus, it can be concluded that the level of bilirubin in the blood of experimental animals was in the normal category.

Decreased levels of bilirubin contained in the blood serum of rats that were given the methanol extract of *Meretrix meretrix*, is due to the content of bioactive alkaloid and terpenoids which have an antioxidant effect. Antioxidant activity generated by these bioactive is allegedly able to protect red
blood cells from oxidation and other damage so the lifespan of red blood cells remain normal, resulting in total serum bilirubin levels becoming low. Extracts allegedly had the effect to treat diseases such as hyper bilirubin. Hyper bilirubin indicated that liver cells are not able to conjugate the majority of bilirubin, so high levels of bilirubin remain in the blood.

Albumin is the main protein in human plasma amounts to 3.4-4.7 g/dL [22]. Methanol extract of 0.1 (M/0.1) and 1 (M/1) g/kg gives a significantly different effect (p<0.05). Levels of albumin contained in test animal are still in the normal category.

The liver produces albumin 9-12 g/day [23]. Albumin was originally synthesized as a pre-protein. Production is controlled by changes in albumin colloid osmotic pressure and the heart. Albumin is not stored in the body. Albumin is catabolized as much as 9-12 g/day by cells adjacent to vascular endothelium. Albumin has a half-life of 16-18 hours, leaving the circulation of blood through the intestinal lymph to system and returned to circulation via the thoracic duct. Increased levels of albumin is likely due to dehydration, excessive use of glukokartokoid, or heart failure. While the reduction in albumin levels found in liver is associated with dysfunction, malnutrition, diarrhea, burns, inflammatory diseases and disorders and congenital idiopathic.

4. Conclusion

Based on the observations, growth, feed intake, weight of liver and kidney were found in normal conditions. When comparing urea, creatinine and cholesterol extents among the control and treated minces were not significantly different (p>0.05) while the levels of bilirubin and albumin between control rats treated with doses of 0.1 g/kg BW (M/0.1) and doses of 1 g/kg BW (M/1) are significantly different (p<0.05). Methanol extract of Meretrix meretrix do not affect sub-chronic toxicity in test animals. It can be concluded that the methanol extract is safe and can be developed into various products both food and non-food.

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