



Mulberry Leaves (*Morus alba*) for Diabetes Mellitus: Acute Toxicity Test in Male Wistar Rats

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Abstract

Diabetes mellitus (DM) is a chronic disease which becoming health community problem in the world. DM patients can be treated by antidiabetic drugs throughout their lifetime. Therefore, to alleviate the concern for the side effect, it is important to have an alternative therapy which has same effect but less side effect. One of alternative therapies which has been known for a long time ago is Mulberry leaves (*Morus alba*). For something to be clinically used as an alternative therapy, effective and lethal dose should be known. Here we tried to discover the lethal dose (LD_{50}) and the liver morphological changes, using the effective dose that had been known from previous study. In our experiment, we used *Morus alba* leaves infusion and 25 male Wistar rats. These rats were divided into 1 control group and 4 groups treatment dosage, 0.3, 3, 30, and 300 grams/kg bodyweight. We observed the mortality in 7 days and examined the liver morphological changes. From this study, we conclude that 300 grams/kg bodyweight or 48 grams/kg bodyweight in human was pseudo-lethal dose in male Wistar rats. We did not found any significant liver morphological changes. Therefore, we can categorize *Morus alba* as a harmless alternative therapy.

Keywords: Alternative therapy, diabetes mellitus, liver morphological changes, lethal dose, *Morus alba*.

Daun Murbei (*Morus alba*) untuk Diabetes Mellitus: Uji Toksisitas Akut pada Tikus Wistar Jantan

Abstrak

Diabetes mellitus (DM) merupakan penyakit kronis yang menjadi masalah kesehatan masyarakat dunia. Pengobatan DM dilakukan dengan pemberian obat antidiabetes yang harus dikonsumsi seumur hidup. Karena efek samping pengobatan yang besar, diperlukan alternatif pengobatan yang berkhasiat sama dan lebih ekonomis. Salah satu alternatifnya adalah daun murbei (*Morus alba*). Daun murbei telah digunakan luas sebagai antidiabetik, namun belum tersedia literature mengenai dosis efektif dan dosis toksiknya. Penelitian ini ingin mengetahui dosis toksik (LD_{50}) dan perubahan histopatologis dari dosis efektif daun murbei. Penelitian menggunakan bahan uji infusa daun murbei dan 25 ekor hewan uji tikus jantan galur Wistar. Hewan uji dibagi menjadi 1 kelompok kontrol dan 4 kelompok perlakuan yang diberikan infusa daun murbei dengan dosis 0.3, 3, 30, dan 300 g/kg berat badan (BB). Observasi kematian hewan uji dan analisis perubahan morfologi sel hati dilakukan pada hari ke-7 setelah pemberian infusa daun murbei. Didapatkan dosis letal daun murbei pada hewan uji adalah 300 g/kg BB atau 48 g/kg BB manusia. Tidak ada perubahan morfologi sel hati yang bermakna pada seluruh tikus di setiap kelompok dosis. Hasil ini menunjukkan bahwa daun murbei (*Morus alba*) termasuk dalam kategori antidiabetik alternatif yang aman.

Kata Kunci: antidiabetik, diabetes mellitus, LD_{50} , morfologi sel hati, *Morus alba*.

1. Introduction

Diabetes mellitus (DM) is one of chronic diseases that becomes serious health community problem in the world. As one of four priority non communicable diseases targeted action by world leader. In 2014, about 422 million adults were living with DM¹. It is predicted increasing into 529 million people in 2035². In adult population, presentation of DM patients increase from 4.7% to 8.5%. DM caused 1.6 million deaths in 2015, and projected to be the seventh leading cause of death^{1,3}.

Based on Diabetes Country Profiles published by WHO 2016, 6% from 250 million people in Indonesia were diagnosed DM. Based on Ministry of Health, in 2013, there are 6.9% people from 15 years old Indonesian population or 12 million people have DM sign and symptom. 30.4% positively diagnosed DM and 69.6% have not been diagnosed before⁴.

There are two types of diabetes mellitus, type 1 is happened caused by deficient in insulin production. Meanwhile, type 2 DM happened caused by ineffective use of insulin. DM patients are treated by insulin and or antidiabetic drugs for throughout their lifetime. For type 2 DM, there are many types of antidiabetic drugs. Among several mechanisms of action are stimulating insulin secretion, decreasing blood glucose, and inhibit glucose absorption. In Indonesia, mostly used antidiabetic drugs is glibenclamide, second generation of sulfonylureas. This drug could have side effect such as nausea, vomiting, and cholestatic jaundice^{5,6}.

Since this antidiabetic drugs should be consumed in for life, patient would concern for the side effect of this therapy and prepare for this expense. Therefore, an alternative therapy which have same effect, less side effect, and less expense for this disease is required. One of herb that is used as therapy since long time ago especially in China and abundantly available in Indonesia is mulberry leaves (*Morus alba*)⁷.

It has been proven that *Morus alba* leaves can decrease the blood glucose level by several mechanisms⁷⁻¹⁰. Specifically these

leaves consist moranoline that inhibit alpha amylase, alpha glucosidase, and sodium-glucose transporter in intestinal which convert carbohydrate, maltose, sucrose to glucose^{11,12}. The other mechanism is by increasing the amount of beta cell in pancreas⁹. Besides that, this leaves consist of flavonoid that has a variety of therapeutically properties. Generally, this is a safe substance, but in a few studies, they reported toxicity effect of this substance such as liver failure, contact dermatitis, hemolytic anemia, and breast cancer¹³.

In order to be used safely as a therapy, as well as chemical drugs, effective and lethal dose of this substance should be known. Previous study has shown the effective dose of *Morus alba*^{8,9}. For discovering lethal dose of such substance, toxicology test is need to be conducted. Several substances may have toxicology effect in different doses.

The toxic dose is the dose which could make biological changes more than homeostasis adaptation ability. There are several factors which affect toxicity test, such as the animal test, the material, and the procedure. The animal test factor are the species, strain, age, sex, bodyweight, and health status of the animal. The material factor are physicochemistry characteristic, purity of the material, and the dose. The procedure factor are how the animal test cared, the people who conduct the study, and the method.

In this study, we choose rat as the animal test as it practicality and convenient for toxicity test. For substance administration, it was given orally according to the human consuming route. The volume given is based on normal volume of animal stomach, in this study, with 100 grams bodyweight, maximal volume is 5.0 mL¹⁴.

There are two types of toxicity test, short term and long term toxicity test. Short term or acute toxicity test is conducted to get the lethal dose of the substance, clinical manifestation, and the mechanism of the toxicity. This "acute" is the sudden effect or harm effect that emerge in 24 hours, after single dose or recurrent dose of the substance.

It also observed after 24 hours until 7 days, for delayed effect detection. Then, the result is considered as lethal dose 50% (LD_{50}), the dose which caused 50% mortality in animal test. It could be calculated by Millter and Tainter graph, aritmatically by Reed and Muench, Karber, or C.S. Weil method¹⁵.

In toxicity test, morphological changes are also observed, such as abnormality of the tissue (location, size, color, etc). The vital organ is liver, because it is the potential organ which could be first damaged, indicated by necrosis, fatty liver, cholestasis, cirrhosis, or cancer¹⁵.

Therefore, we tried to discover lethal dose (LD_{50}) for this substance and morphological changes of the liver in those dose.

2. Material and Methods

In this study, *Morus alba* leaves infusion was used as material test and male wistar rats, 2-3 month old, with 125-200 grams body weight as the animal tests^{8,9}. The infusion was proceed by boiling 10 mg mashed *Morus alba* leaves with 100 mL water until 90 degree Celcius, and then waiting for 15 minutes. Then, we filtered it and added until 100 mL⁸. This infusion would be given by stomach sonde, in accordance with usual human consuming route. Therefore, we can simply convert the result from animal test to the human¹⁵. The procedure is conformed to animal ethics research principle of 3R and 5F.

The lowest dose was determined by converting the usual doses that used in human. In human, common dose is 0.1-0.5 g/kg bodyweight. Using Laurence and Bochrach formula, converted dose for rats from that human dose is 3 gram/kg bodyweight¹⁶. From this doses, the treatment doses were calculated for each group in toxicity test. For toxicity test, we have to use logarithmic multiples from lowest dose that couldn't give any toxic effect. Therefore, we started the doses from 0.3 g/kg bodyweight, then 3 g/kg bodyweight, 30 g/kg bodyweight, and 300 g/kg bodyweight. For treatment, rats were grouped into 5 groups, as 1 control group and 4 groups with each different dose. Each

group contain 5 animal tests, in accordance with Weil who said at least 4 animal tests in each group doses¹. Before tested by *Morus alba* infusion, each rat was measured its bodyweight and didn't eat anything except water for 8-12 hours. At control group we gave aquades only. Each doses gave in 5 mL, based on maximum volume of gastric rats¹.

After giving *Morus alba* infusion in single dose, we observed mortality in 24 hours for investigating acute toxicity and 7 days for delayed toxicity. Rats were sacrificed on 8th day using ketamine hydrochloride 0.4 mL. After laparotomy, blood in rat 's liver was drained by physiological saline through the left ventricle. The liver was taken and put in formalin 4%, then cut into size of 1 cm x 0.5 cm and samples were then embedded in paraffin wax, sectioned (5 μ m) with a microtome, and stained by hematoxilin-eosin (HE). One section from each rats was randomLy chosen, then ten fields were observed. Degrees of liver damages were estimated by counting the number of necrotic cells in each field under Olympus BX-51, applied spectral imaging, light microscope at magnification 10x40. Images were captured automatically. The grades of liver damage in different groups were assigned in percentage of necrosis . We investigated it as previous study stated that flavonoid in *Morus alba* can be caused liver failure². Liver failure could be seen as centrolobular necrotic, fatty liver, cholestasis, cirrhosis, or cancer¹.

For analyzing the data, Reed Muench method was used for getting LD_{50} value. This method stated that if certain dose causes death in animal test, surely higher dose will causes death too. Otherwise, if the animal test live at certain dose, it will live at lower dose ¹. LD_{50} value for rats would be converted back to human dose.

For hepatocyte interpretation, we use microscope for counted necrotic hepatocytes in each group. The existance of necrotic hepatocytes was characterized by rupture of the cel membrane, vacuolization of the cytolasm, karyolysis, pyknosis and karyorrhexis¹⁷.

Data were expressed as means and

Tabel 1. Frequency of Necrosis Hepatocytes (%) (SD: standard deviation)

Group	Rat						mean	SD
	1	2	3	4	5			
1	1	0	0	0	0		0.2	0.4
2	0	0	2	1	1		0.8	0.8
3	0	0	0	0	0		0	0
4	0	0	0	0	0		0	0
5	0	0	0	1	0		0.2	0.4

standard deviation. Statistical analysis was performed using IBM SPSS for Windows version 22.0. A p-value of less than 0.05 was considered as statistically significant.

3. Results

We investigated lethal dose of *Morus alba* leaves which caused mortality in 50% animal test group. We found there was only one animal death, in 0.3 g/kg bodyweight. There wasn't any death in other groups. As the lowest dose was resulted the lethal effect and no mortality at the highest dose, we could

consider that this mortality because of another causes.

In histopathology investigation to the liver tissue (Figure 1), the hepatocytes of group dose 3 and 4 were intact and observed in fairly radial position in relation to central vein.

The control group, group dose 2 and 5 had some defect in hepatic tissue but not significantly different (Table 1). We could consider as at this doses there wasn't any acute toxicity effect for the liver.

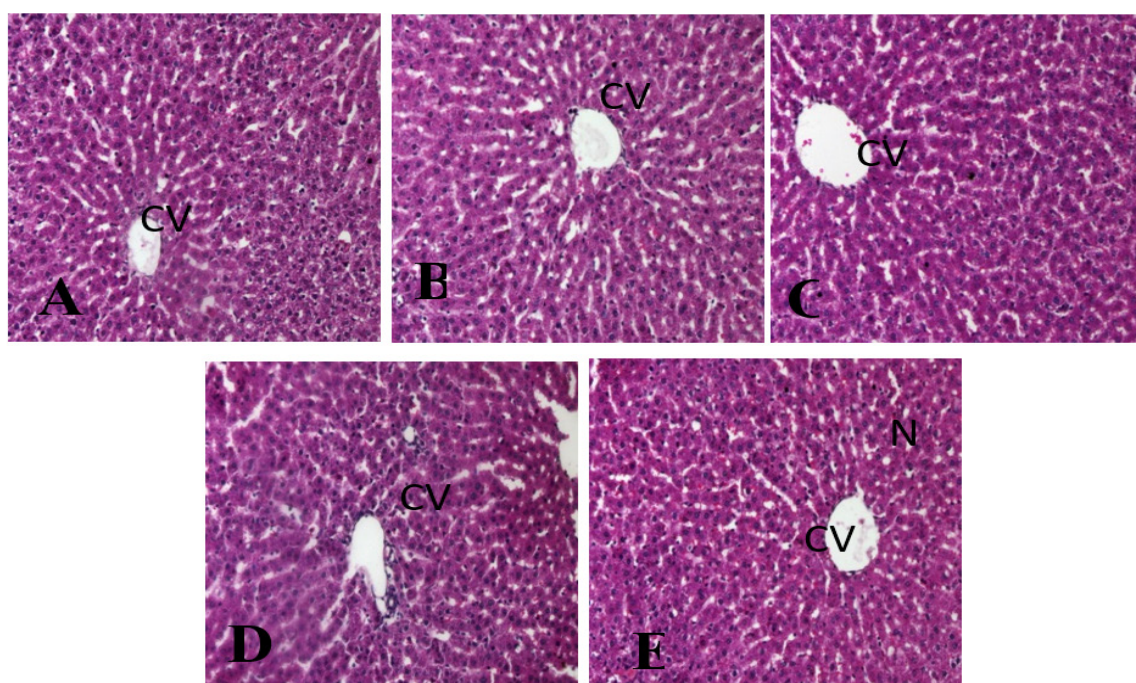


Figure 1. Histopathological appearance. A group1 (given aquades) B. Group 2 (given 0.3 g/rat's kgBW of *Morus alba* leaves infusion) C. Group 3 (given 3 g/rat'sBW of *Morus alba* leaves infusion) D. Group 4 (given 30 g/rat'sBW of *Morus alba* leaves infusion) E. Group 5 (given 300 g/rat'sBW of *Morus alba* leaves infusion) CV (central vein), N (necrosis)

4. Discussion

In this study, as there wasn't any dose that caused 100% mortality, we couldn't use Reed-Muench method. Based on reference, if there wasn't any mortality in animal test in each doses group, the highest doses group considered as pseudo-lethal dose¹. The highest doses, 300 g/kg bodyweight, was considered as 300 g/kg bodyweight is the pseudo-lethal dose (pseudo LD₅₀). This dose were highest dose that used in study of efficacy of mulberry leaves in animal model of diabetes^{9,18} and in earlier study in type 2 Diabetic patients⁷, after converted the dose.

The dose 300 g/kg bodyweight of rat was converted to human dose based on Laurence and Borcharch table¹⁶.

In this table, the dose for rats with 200g bodyweight could be converted as 56 times doses for 70 kg bodyweight of human. By this calculation, we considered 48 g/kg bodyweight as pseudo LD₅₀ for human. As the lethal dose more than 15 g/kg bodyweight, *Morus alba* leaves could be categorized as harmless alternative therapy¹.

5. Conclusion

From this study, there wasn't any dose that caused 100% mortality in animal test in each doses group, so we can conclude that the highest doses group considered as pseudo-lethal dose (pseudo LD₅₀). Pseudo-lethal dose of *Morus alba* leaves infusion in male wistar rat is 300 g/kg bodyweight or 48 g/kg bodyweight in human. Histopathologically, there wasn't any significant changes at each rats at each doses. No acute toxicity effect in the liver rat's. The infusion of *Morus alba* was harmless alternative therapy for DM.

We recommend further investigation for lethal dose and chronic toxicity test of *Morus alba* leaves. Furthermore, it would be better if conducting further observation in human, especially for effective dose of this alternative therapy.

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