CRUDE EXTRACT MULBERRY (MORUS ALBA L.) LEAVES CHLOROPHYLL IMPROVES URINE CREATININE LEVELS AND HISTOLOGY OF DIABETIC RAT KIDNEY

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Key words: Mulberry leaves chlorophyll crude extract (MLCE), Renal histology, Streptozotocin diabetic induced rats.

Abstract - Urine creatinine levels could be used as an indicator of the body's energy metabolism. The aim of this research is to determine the effect of mulberry leaves crude extract (MLCE) doses (90 mg/Kg 150 mg/Kg of body weigh, 200 mg/kg 150 mg/kg of body weigh, 150 mg/kg 150 mg/Kg of body weigh) improves urinary creatinine levels and renal histology in streptozotocin-diabetic rats induced (65 mg/Kg of body weight). Rats were randomly divided into five groups: control group 2 (positive and negative) and 3 groups; positive control (Glibenclamide 5 mg/Kg of body weight), negative control (streptozotocin-STZ), and groups of diabetic rats in 3 types MLCE doses given orally once daily for 14 days. Measurement of blood glucose levels on days 0,3,7,10, and 14 after induced by STZ. While the levels of urine creatinine and renal histology performed at the end of the experiment rats. The results of measurements of glucose levels in the blood (mg/dL) were tested for normality using the Kolmogorov-Smirnov test and Kruskal-Wallis. The results showed that MLCE did not lower blood glucose levels of diabetic rats. The results showed no significance with urine creatinine levels in all treatments. At levels of 150 mg/Kg of body weigh of MLCE (± 1.96 mg/dL) could be reduce the levels of urinary creatinine. Oral treatment of MLCE improve renal histology of diabetic rats.

INTRODUCTION

Diabetes has been widely reported association with the gene changes (Puspitaningrum et al., 2014a; Puspitaningrum et al., 2014b). This degenerative disease is undoubtedly caused by the weakening of the ability of the pancreas to secrete insulin or insulin sensitivity weakening against the insulin receptor (Takeda et al., 2011). This resulted in an increase in the concentration of glucose in the blood is known as hyperglycemia (Punithavathi et al., 2008). In normal circumstances, approximately 50% of glucose from the food you eat will have a perfect metabolism to carbon dioxide (CO₂) and water, glycogen and 10% to 20% to 40% is converted into fat. All metabolic processes are impaire in patients with diabetes due to insulin deficiency. Absorption of glucose into the cells decreased and impaires the metabolism. This causes most of the glucose to remains in the blood circulation resulting in hyperglycemia (Bellomo et al., 2008).

Physiological conditions such diabetes causes diabetic patients taking other energy generation pathways, namely by breaking the molecules of creatine phosphate in the muscle (Wallimann et al., 2007). Creatine is converted as an energy source of the body (Wallimann et al., 2007). The increase in end products resulting from the metabolism of creatine phosphate which is creatinine. Creatinine in large quantities, can influence the function of the kidney glomerular filtration (Pierre et al., 2011).

Creatinine is a waste product of creatine phosphate reshuffle that occurs in the muscles. Creatinine excretion will increase if an interruption occurs in the muscles (Megha et al., 2013). Increased creatinine levels were directly attributable to a decrease in glomerular filtration function, so that the creatinine test can be used to

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check the kidney filtration function (Rosenfeld and Dial, 2010). Kidney is an important organ for living beings.

The kidneys have a variety of functions such as removing solutes and water are selectively controlled by glomerular filtration, reabsorption, and tubular secretion. The main goal is the formation of glomerular filtration function of the primary filtrate in the proximal tubule (Sherwood, 2012). Changes in glomerular filtration function of the kidney may be an indicator of the presence of renal insufficiency (Marsik et al., 2008). Glomerular Bowman's capsule distance indicates an association between a decrease in glomerular filtration capabilities with the expansion of the lumen of the glomerulus, which is also occupied by the tubule lumen proteins. The smaller the distance between Bowman's capsule-glomerulus, the filtration capability will decrease as the blood results filtration can not get out through the Bowman's space, resulting in disturbances in renal fluid absorption (Jennette et al., 2005).

Diabetics prefer to consume natural ingredients to control physiological conditions. This is caused by other than the relatively expensive price that diabetes drugs command also long-term use of natural materials is considered sate. Alternative medicine is cheaper and natural ingredients safe for diabetics. Based on previous research, it is known that extracts of mulberry leaves (Morus alba L.) can be used as an alternative anti-diabetic drug (Kumar et al., 2012). Chlorophyll content mulberry leaves is very high, suspected to be high enough to act as an anti-oxidant for diabetics (Jamshid and Prakash, 2008). Chlorophyll consumption of molecule is expected to enter into the metabolic pathways of energy in the process of formation of ATP. Therefore, this study aimed to observe the effect of mulberry leaf crude extract-MLCE dose to urinary creatinine levels and renal histology in streptozotocin-diabetic rats induced.

**MATERIALS AND METHODS**

This research was conducted in the Laboratory Animal Center for Biomedical Try and Technology Faculty of Medicine and Health Association Gresik Laboratory, Central Jakarta from March to July, 2013. This study uses experimental design with completely randomized design (CRD) consisting of 4 treatment groups. Then the obtained data will be statistically analyzed. This study uses mulberry leaves (Morus alba L.) derived from Silk House, Bogor. This study used a sample of 20 male white rats (Rattus norvegicus) Sprague Dawley strain with body weight 200-250g.

a) **Preparation of mulberry leaves crude extract (MLCE)**

Fresh mulberry leaves were weighed using scales. Mulberry leaves + weighed 100 g (without bone leaves). Mulberry leaves that have been weighed and the crushed with a pestle and mortar. Extraction is done by heating the temperature of 60-65 °C for 15 minutes and an extract 80% Acetone was prepared (2L). Extract solution was concentrated on a water bath at a temperature of 60°C. Remaining solvent is removed by using a Rotary Evaporator so as to produce a crude extract thick blackish green. Furthermore, crude extract thick was aerated for 1 day in order to get a paste (without water). Paste stored at 50 °C in an oven for 1 day. Then stored at room temperature up to 37°C is used to test predefined parameters.

b) **Absorbance measurements**

Absorbance A measured at a wavelength of 645 nm for chlorophyll a and 663 nm for chlorophyll b (Hendry and Grime, 1993). Chlorophyll concentration are calculated with the following formula:
Chlorophyll a: 12.7 (A.663) - 2.69 (1.645) mg/L.
Chlorophyll b: 22.9 (A.645) - 4.68 (A.663) mg/L.
Chlorophyll total: 8.02 (A.663) + 20.2 (A.645)mg/L.

c) Chlorophyll levels

Crude extract chlorophyll levels of mulberry leaves in mice was given comparing with human dose which is 1 g/day. Comparison between the surface area is 0.018 mice with human (Paget and Barnes, 1964). So, 1gr x 0.018 x 50/50 (kg Indonesian people) to rats (200g) = 0.018g/200g rat. 5x 0.018 g/kg/day = 0.09g/kg day or 90mg/kg/day.

d) Animal preparation experiments

The next stage is the preparation of the rat as a research subject. Yag mice used in this study are male rats Sprague Dawley strain ± 2 months old. Each rat was confirmed given the normal blood glucose levels + standard feed 30g/head/day and drinking +200 mL/cage. Animal cage has a size of 34 x 40 cm. Rats in the early stages of acclimatization is + 2 weeks.

e) Treatment in animal experiments

Animal models for diabetes can occur spontaneously or induction of experimental results. How to make experimental diabetes in animal models such as the pancreatic β cell damage and the use of chemicals such diabetogenic Streptozotocin (STZ) (Szkudelski, 2010). Only mice with increased levels of blood glucose is >150mg/dL were used in this study as diabetic animals. To induce diabetic conditions required two weeks. Rats were divided into four groups randomly and housed per group. Each group consisted of four mice. Then from each group they were treated as follows: (1) positive control group (K+), DM conditions given Glibenclamid (Smg/kg of body weight); (2) negative control group, DM conditions; (3) crude extract mulberry leaf chlorophyll 90mg/kg of body weight (DM); (4) crude extract of mulberry leaf chlorophyll 120mg/ kg of body weight (DM); (5) crude extract of mulberry leaves chlorophyll 150 mg/kg of body weight (DM). Crude extract of mulberry leaf chlorophyll done every day for 14 days orally after rats experienced a state of diabetes mellitus with use of force-tool (sonde). Glucose measurements performed on days 0, 3, 7, 10, and 14 (after the rats had diabetes) with the aim that the crude extract of mulberry leaves can have a significant effect on the mouse body. Examination of blood creatinine levels is done with the Jaffe method (Syal et al., 2013)

f) Preparation of rat kidney histology

Making preparation rat pancreatic histology experiments were performed at the end of the experiment. Rats were anesthetized with ether, then surgery. Each rat isolated pancreas organs for histological preparations were then made with paraffin and staining method HE/(hematoxylin eosin).

RESULTS AND DISCUSSION

a) Mulberry Leaves Chlorophyll Crude Extract-MLCE

Mulberry leaves are used as much as ±400 g, ground into powder and then diluted with 80% acetone. After going through the process of evaporation and heating the obtained paste extract mulberry leaf chlorophyll 35.56 g. 80% acetone extract compounds are more selective in chlorophyll a (Prasad et al., 2012). The use of acetone solvent will cause denaturation of chlorophyll binding proteins that can be separated from the chlorophyll binding protein and participate in a solvent extracted. Furthermore, the extraction process is done with a heating temperature of 65 °C- 66 °C to obtain the maximum amount of chlorophyll. Heating at a temperature of 65°C-66°C is used to degrade chlorophyll klorofilase with the
help of enzymes. Enzyme catalyzes degradation activity of klorofilase chlorophyll between temperature of 62.2 °C to 80 °C (Fennema, 1996). Klorofilase enzyme is activated in a solution containing water, alcohol, or acetone, and causes the phytol group will be separated. When there is a process of heating or under acidic conditions, the Mg\(^{2+}\) contained in klorofillide will be separated to form pheophorbide derivative, the release of Mg\(^{2+}\) on pheophorbide cause a color change from green to brown.

Value of mulberry leaf chlorophyll absorbance was read at a wavelength of 645 nm and 663 nm. Absorbance value at a wavelength of 645 nm is 0.262, while the wavelength of 663 nm is 0.646. Furthermore, the calculation of mulberry leaf chlorophyll content. At a wavelength of 645 nm is obtained total chlorophyll a 7.5 x 10^7 g/mL, while the total chlorophyll b at a wavelength of 663 nm is 2.98 x 10^7 g/mL. Thus, the total chlorophyll extracted on mulberry leaves are 10.469 x 10^7 g/mL. The results using mulberry leaves have a higher value than research on previous research, that the results of the absorbance to the total chlorophyll a and b is 8.44 x 10^{-1} g/mL.

b) Blood Glucose Levels of Diabetes Rats and Orally Mulberry Leaf Chlorophyll Crude Extract-MLCE

Blood glucose levels to normal mice 2 weeks after acclimatization has a mean value of 76.34 mg/dL and blood glucose levels of mice on day 0 after 2 weeks of induction of STZ has a mean 210.19 mg/dL. Based on Figure 5 there are different levels of average blood glucose after streptozotocin induction is 210.19 mg/dL. Based on these data that the induction dose
streptozotocin 65mg/kg dose is low to make the mice became hyperglycemic. This is supported by previous research, that at a dose of 40-100mg streptozotocin/kg body weight to create a low-dose mice became hyperglycemic within 1 to 2 weeks (Arora et al., 2009).

STZ induced the same dose at 65 mg/kg of body weight. However, the results prove that the blood glucose levels after induction of STZ rats varied. Average blood glucose levels obtained at the highest rat group III with an average of 321.72 mg/dL. This is supported in the previous study, that one of the factors that cause variations in blood glucose levels of mice is durability (physiological conditions) different individual mice to diabetogenic substance causing the initial condition of diabetes is not uniform (Kim et al., 2006). STZ is a diabetogenic substance that can cause damage to the beta cells of the pancreas (Pierre et al., 2011). Streptozotocin can damage and oxidize B-cells of the pancreas produce insulin. The hormone insulin function speeding the transport of glucose into the cell. This is according to research by the disruption of the mechanism of action of the hormone insulin which is characterized by the increase of blood glucose in animal models of diabetes mellitus can lead to conditions (Figure 1).

c) Orally Mulberry Leaves Chlorophyll Crude Extract-MLCE

Crude extract of mulberry leaf chlorophyll in mice using three doses, the dose of 90mg/kg of body weight, 120mg/kg of body weight, and 150mg/kg of body weight. In addition to the three doses, these experiments add a positive and a negative control. Blood glucose levels of mice before administration of the crude extract of mulberry leaf chlorophyll (day 0) had a mean 210.19mg/dL.

Figure 2 shows that the blood glucose levels of diabetic rats after administration of crude extract of mulberry leaf chlorophyll with different doses increased and decreased for 14 days. Based on the results of the process of extraction of chlorophyll mulberry leaves obtained at 10.469 x 10^{-1} g/mL on mulberry leaves.

Based on the previous research revealed that chlorophyll is a green compound consisting of chlorophyll a and chlorophyll b (Limantara, 2009). Chlorophyll is a pigment that contains the basic ring tetrapirrol. Fourth ring binds to the Mg^{2+} ion and phytol chain (Sulanc et al., 2005). Although based on the Kruskal-Wallis test there was no effect of crude extract of mulberry leaf chlorophyll to decrease blood glucose levels of diabetic rats.

However, it can be explained some active components that have their own role in the Diabetes mellitus-DM condition. Tetrapirrol have a role in the body that play a role in oxidation reduction reactions and the transport of oxygen molecules include proteins, such as cytochromes, catalase, and peroxidase (Arora et al., 2009). Tetrapirrol porphyrin ring also has a role as an anti-oxidant (Prangdimurti et al., 2006). In conditions of DM rats had STZ-induced ROS. Chlorophyll is an antioxidant tetrapirrol especially the oxidation chain breaker works by donating electrons to free radicals (Palipoch et al., 2013).

McCarty, (2005) have derived the phytol fitanat acid. Fitanat acid (phytanic acid) has a role in regulating insulin sensitivity and balance blood glucose levels. This is supported by the theory that fitanat acid has a role in activating GLUT2 which facilitates the entry of glucose into the liver (Heim et al., 2002; Kim et al., 2000). Finatan acid can increase the receptor and translocation of GLUT 4 in heart and regulate glucose catabolism (Jay and Ren, 2007). Other components, magnesium acts as an enzyme cofactor. Magnesium has a role in facilitating the transport of glucose into the cells and is
also a co-factor of various enzymes (hexokinase or glucokinase) for the oxidation of glucose (Albertus, 2000). Magnesium acts as a cofactor in the glucose transport system and catalyzing is various enzymes related to glucose phosphorylation (Gums, 2004). In addition, magnesium plays a role in helping to maintain insulin secretion and pancreatic β cells (Elamin and Tuimeo, 1990).

However, due to the use of low doses there is no significant change in blood glucose levels during the 2 week administration of crude extract of mulberry leaf chlorophyll.

**d) Body Weight of Rats**

In the study, body weight of rats were weighed before treatment (initial weight), after induction of STZ 2 weeks, and during the 2 weeks of treatment. Weight of normal rats before induction of STZ has given the average weight is 295.95 g while the body weight of rats after induction of STZ has an average weight is 162.9 g (Figure 3). Changes in body weight occurred in all groups. Weight changes characterized by weight loss in rats after STZ induced. In the extract dose 90 mg/kg of body weight (II); 120 mg/kg of body weight (IV); and 150 mg/kg of body weight (V) there is a weight loss of each is 42 g; 12 g; and 45.25 g. Streptozotocin induced diabetes can cause decreasing and loss of muscle mass and protein in several tissues of the body weight of mice that decrease in rats. It is also supported by the other indicators of the effects of diabetes and its catabolite are the change of parameters in rats (body weight, muscle, and fat mass), that if the diabetic mice compared with control mice (normal) was there a significant difference (Motyl and Laura, 2009). Changes in body weight in rats after STZ induced diabetic rats caused by not being able to use glucose as a primary energy source due to a lack of the hormone insulin. Insulin deficiency in STZ induced diabetic rats due to damage pancreatic beta cells producing insulin as (Szkudelski, 2010). Lack of insulin causes the glucose can not enter the cells so that the body's energy needs to be obtained from the results of lipolysis. Fat in various tissues mobilized and degraded via the beta oxidation process to produce energy (Suarsana et al., 2010).

![Fig. 3 Comparison of body weight of normal rats, after induction of STZ and after orally administration of MLCE](image)

Fig. 3 Comparison of body weight of normal rats, after induction of STZ and after orally administration of MLCE

positive control group (I) and negative controls (II) there is a decrease in body weight that is 57.25 g and 58.75 g. In the group of mulberry leaf chlorophyll

After induction of STZ for 2 weeks, then the group III-IV rats were given orally crude extract of mulberry leaf chlorophyll. After 2 weeks the results obtained body weight of rats treated groups. The body
weight of rats in the treatment group decreased. At a dose of 90mg/kg of body weight (III); 120mg/kg of body weight (IV); and 150mg/kg of body weight (V) there is a decrease in body weight 17.75 g, respectively; 24 g; and 2 g. The results of the Kruskal-Wallis test p > 0.01. That is, there is no effect of crude extract of mulberry leaf chlorophyll on body weight of rats. Disturbed metabolism of diabetic rats due to insulin resistance and sensitivity to glucose transporter (GLUT) decreased in various tissues (Woerle et al., 2007).

Supplementation of Mg$^{2+}$ ions in the blood can affect insulin levels in the blood. Magnesium supplements may inhibit or prevent the secretion of insulin in patients with diabetes mellitus (Chaudhary et al., 2010). In addition to the inability of ion absorption by Mg$^{2+}$ induced diabetic rats for 2 weeks STZ treatment caused a significant decrease in body weight (Figure 3).

e) Mulberry leaf (Morus alba L.) chlorophyll crude extract improves urine creatinine levels and histology diabetic rat kidney

Measurement of urinary creatinine levels performed using reagents ST-creatinine Jaffe reaction method. Measurements with ST-creatinine reagents aimed to determine quantitative the levels of creatinine. The results of measurements of urinary creatinine levels can be seen in (Fig. 4).

Histological observation of kidney tissue performed using Olympus microscope at 400x magnification and image capture taken using Olympus microscope camera. A captured image can be seen as follows:

All mice treated histological changes in glomerular kidney glomerular histology when compared with normal mice. The arrows shown in the figure showed a shrinkage of the glomerulus to Bowman's capsule. Measurement of glomerular shrinking can be measured by using the ImageJ program (Fig. 5).

One of the causes of weight loss in mice DM is a change in the structure of muscle mass. Muscle is one of the energy storage (ATP) in the form of muscle glycogen.

![Graph showing creatinine levels](image-url)
Fig. 5 Histology of rat renal glomerulus stained with H & E magnification 400x
(n) = normal rat renal glomeruli
(a) = renal glomeruli of diabetic rats
(b) = diabetic rat renal glomeruli + dose of 90mg/Kg of body weight
(c) = diabetic rat renal glomeruli + dose of 120mg/Kg of body weight
(d) = diabetic rat renal glomeruli + dose of 150mg/Kg of body weight

Fig. 6 Graphs of difference blood glucose levels after treatment of crude extract of mulberry leaf chlorophyll (on day 14th)

Table 1. The average value of the distance measurements between the Bowman’s capsule Diabetic rat renal glomerulus

<table>
<thead>
<tr>
<th>Groups</th>
<th>The average value of the measurement of the between Bowman’s capsule with rat renal Diabetes (μm)</th>
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<tbody>
<tr>
<td>Control (diabetes)</td>
<td>3.48 ± 2.47</td>
</tr>
<tr>
<td>Dosis I (90 mg/kg of body weight)</td>
<td>3.61 ± 0.38</td>
</tr>
<tr>
<td>Dosis II (120 mg/kg of body weight)</td>
<td>6.39 ± 0.30</td>
</tr>
<tr>
<td>Dosis III (150 mg/kg of body weight)</td>
<td>6.71 ± 0.48</td>
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carbohydrate substance and creatine phosphate. In conditions of energy shortage, the two types of carbohydrates will be broken down to produce energy in the cells (Bellomo et al., 2008).

Creatinine is an endogenous metabolite that is very useful to assess glomerular function. This molecule is derived from the metabolism of muscle. Creatinine is excreted through the kidneys by glomerular filtration process. Under normal circumstances (kidney function, diet, muscle mass, and metabolism), creatinine will be produced in equal amounts and are excreted through the urine every day (John et al. 2009). Serum creatinine levels rise because the body needs energy in the form of ATP. The mechanism of the formation energy of creatine phosphate in the muscle is to break the phosphate group and the addition of a phosphate molecule to ADP (adenosine diphosphate), thus forming energy (Wijekoon et al., 2007). This reaction takes place reversible metabolism, meaning that creatine can still bind back with a phosphate group and reshaping of creatine phosphate in the muscle. Glomerular histologic changes can occur due to many factors that affect the performance of the kidneys, both in physiological function or renal function against the toxic effects. Creatinine is produced from muscle and carried in the blood stream which eventually excreted through the kidneys. As a result there will be a process of structural changes in the kidney itself, especially in the renal tubules because this is where the process of reabsorption and excretion of these toxic substances. Kidney function as the body's excretion of waste products of metabolism such as creatinine is always stable every day in normal conditions. Creatinine excretion of the excess above the normal range every day is excreted through the kidney glomerular filtration will result in a decrease in glomerular filtration function (Rosenfeld and Dial, 2010).

Deterioration of glomerular filtration function is suspected when it occurs in the long term will lead to changes in the structure of the glomerulus.

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