Effects of Gambir leaves extract (Uncaria gambir Roxb.) in preventing the aging process induced D-galactose on pancreas mice

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Abstract. Increasing level of reactive oxygen species (ROS) can damage the cell membrane and composition of DNA, leading to organ degeneration. Pancreas is among the organs that is sensitive to oxidative damage. Consumption of flavonoid-rich stuffs such as herbs, fruits and vegetables are believed to strengthen antioxidant capacity in the body in neutralizing free radicals. Gambir leaves have been used traditionally as remedies of various diseases. The most prominent active compounds in Gambir is flavonoid catechin which belongs to flavan-3-ols group. D-galactose is monosaccharide that in high concentration can increase ROS generation and induce senescence in animal models. In this study, we aimed to observe the effect of Gambir leaves extract (GLE) in preventing ROS production and pancreas damage in galactose-induced mice model. Twenty-four female mice were divided into 4 groups and injected with D-galactose 150 mg/kgBW intraperitoneally for 6 weeks. Three groups were supplemented with GLE 100; 200 and 400 mg/kgBW, respectively. Serum MDA, blood glucose and pancreas histopathology were analyzed. There is no significant difference among GLE-treated groups compared to control in the level of serum MDA (p=0.286), blood glucose fasting (p=0.051) and random (p=0.626), and pancreas histopathology: area of Langerhans islet (p=0.589) and perimeter of Langerhans islet (p=0.634). We did not find the histological damage in pancreas. Interestingly, we found a trend increase in area, perimeter and cell number in Langerhans islet. Our data suggested a potential effect of GLE in increasing the growth of pancreatic cells. Further study is necessary to elucidate the regenerative effect of GLE in damage pancreas such as streptozotocin-induced diabetes model.

1. Introduction
Aging is the process of losing the ability of the network to repair itself and maintain its normal structure and function, so that it cannot survive and repair the damage suffered. The aging process that is accompanied by a decrease in various functions of the body's organs can trigger the emergence of various diseases, which are referred to as degenerative diseases. Degenerative disease is a disease that arises as a result of the deterioration of the body's cell functions, from normal to worse.
In the aging process an increase in reactive oxygen species (ROS) due to oxidative glucose metabolism in cells. Oxidative reactions involving free radicals can damage the surrounding normal cell membrane and damage the composition of DNA so that it can cause a mutation. One effort to neutralize ROS in the body is through the consumption of foods that are rich in antioxidants. One of the natural antioxidants found in plants is flavonoids. According to Lucida this compound is not only able to directly neutralize ROS through its properties as an electron donor, it can also activate natural antioxidant enzymes in the body [1].

Gambir (Uncaria gambir Roxb.) is a typical North Sumatran plant, its location is mostly found in Siambaliang Village, Sidikalang District, Dairi Regency. Traditionally gambir (U. gambir Roxb.) is used by the community as a complement to betel betel and is believed to strengthen teeth. Besides that, gambir (U. gambir Roxb.) is also used by the community as a material for the treatment of inflammation of the gums (gum), headaches, diarrhea, dysentery, mouth sores, burns and medicine for skin pain (distributed). It was reported that research on gambier activity as an antioxidant by Chosdu and Sudrajat is a free radical test using ESR method on gambier leaves; and in Tarek's study which assessed the antioxidant and anti-inflammatory effects of catechins on cardiotoxicity induced by adriamycin in mice. The active ingredient of gambier leaves which has an antioxidant effect is a catechin. According to Anggraini, the highest amount of catechin content was found in leaves as much as 13.7%. Aside from being an antioxidant, catechins also have anti-inflammatory, antibacterial, antitumor, and antiviral effects [2,3,4,5].

D-galactose is widely used in several studies to model aging in experimental animals. D-galactose induction can cause an increase in MDA due to oxidative stress in various tissues through increased production of reactive oxygen species (ROS) and advanced glycation endproduct (AGE) which also occurs in normal aging [6,7]. One of the organs that is the target of damage due to increased ROS is the pancreas. Dysfunction of the pancreas will cause insufficient insulin production or its quality is not good so that the function of insulin to maintain homeostasis and metabolism of carbohydrates and fats is disrupted. Therefore, researchers are interested in analyzing the effects of gambir leaves extract (U. gambir Roxb.) In preventing pancreatic damage that occurs in the aging process which is induced D-galactose.

2. Materials and Methods

2.1. Animal Models
This was a laboratory experimental research with a randomized, post-test only control group design. In this study, 32 female swiss webster aged 8-12 weeks. The mice used were obtained from the Pharmacology Laboratory of the Faculty of Medicine, USU.

The mice were randomly divided into 4 groups. Negative control group (K-1) was peritoneal induced by D-galactose at the dose of 150 mg/kg daily for 6 weeks. The treatment groups (K-2, K-3 and K-4) were induced by D-galactose and orally administered with gambir leaves extract at the dose of 100, 200, and 400 mg/kgBW, respectively for 6 weeks.

2.2. D-Galactose and Gambir Leaves Extract
D-galactose (Gal; G0750) were from Sigma-Aldrich. Gambir leaves extract (U. gambir Roxb.) used was obtained from (Shen Mi Ye Sari Uncariae® Antioxidant (PT. Toyo Brothers) Jakarta, Indonesia with BPOM permission: TR 053 248 721.

2.3. Examination of Fasting and Random Blood Glucose
At the end of week 6 of induction treatment and examination, fasting and random blood glucose were examined. According to Permatasari (2012), blood sampling procedures in mice are taken through the tail vein (V. Lateral tail). This is because the tail vein is thicker than the dorsal metatarsal and medial saphenous veins, a larger quantity of venous blood can be obtained. The procedure is that the mice is held then the mice tail is extended then cut 0.2-2 cm from the base of the tail with a blade or sterile scissors. Blood samples are placed on Blood Glucose Test meter strip: Easy Touch GCU model CE-0197, which will measure blood glucose in mg/dL [6].
2.4. Examination of Malondialdehyde (MDA)

MDA levels were measured in serum obtained from cardiac puncture at the end of the study. Mice were first anesthetized with a mixture of Ketamine (87.5 mg/kg) and Xylazine (10 mg/kg). The blood is drawn and then put into a blood tube. The blood is frozen for 30 minutes at room temperature and then centrifuged at 7000 rpm for 10 minutes at 4°C. For work procedures can be seen in Table 1.

### Table 1. Standard operation table

|                      | Blank tube | Standard tube | Sample tube | Control tube |
|----------------------|------------|---------------|-------------|--------------|
| Absolute ethanol (mL)| 0.1        |               |             |              |
| Reagent 4 (mL)       |            | 0.1           |             |              |
| Samples (mL)         | 0.1        | 0.1           | 0.1         | 0.1          |
| Reagent 1 (mL)       | 0.1        | 0.1           | 0.1         | 0.1          |
| Reagent 2 solution (mL)| 3.0       | 3.0           | 3.0         | 3.0          |
| Reagent 2 solution (mL)| 1.0       | 1.0           | 1.0         |              |
| 50% glacial acetic acid (mL) | 1.0 |               |             |              |

Mix fully. Fasten the tube with the plastic film, then prick a small hole with a needle. Incubate in 95°C water bath for 40 min, then cool the tubes with running water after incubation. Centrifuge at 3100 g for 10 min, collect the supernatant. Set to zero with double-distilled water and measure the OD value at 532 nm with 1 cm diameter cuvette.

2.5. Examination of The Pancreas Histopathological of Mice

Pancreatic tissue is taken and then fixed with 10% formalin immediately until all parts of the tissue are submerged for 24 hours. Next step is dehydration using 70% series alcohol up to absolute alcohol, clearing in xylol, infiltration, embedding in paraffin 56-58°C. Each organ from each repetition was made 4 incisions at intervals of 10 incisions using a microtome, then placed on a glass object that had been provided with adhesive. Pancreatic preparations were stained with HE staining. Histological changes in the pancreas of mice were examined with the aid of a light microscope at 10x40 magnification.

Pancreatic histopathological readings were assessed by looking at the average area and perimeter in units of μm². Followed by an average assessment of cell nuclei carried out by counting the number of cell nucleus on the islet of Langerhans. Islet data is randomly selected from 5 views and the number of nucleated cells in each islet is under 10 times magnification. The area and perimeter of each islet and the number of cell nucleus in the islet are calculated using ImageJ software.

2.6. Statistic Analysis

Data obtained from all sample groups were processed with a statistical computer program. The sequence of tests starts with a normality test, a homogeneity test. Data that are normally distributed and homogeneous are tested by ANOVA. If there is a real difference then continue with the Post-Hoc Test (α=0.05).

2.7. Ethical Clearance

The use and handling of experimental animals in the laboratory in this study was approved by the animal research ethics committee of the Faculty of Mathematics and Natural Sciences USU Medan (No.00278/KEPH/FMIPA-2019).

3. Result

3.1. Effect of Galactose and Gambir Leaves Extract (U. gambir Roxb.) on Blood Glucose in Mice

Fasting, and random blood glucose after administration of D-galactose and gambir leaves extract (U. gambir Roxb.) are shown in Table 2. Fasting blood glucose was obtained from capillary blood after mice were fasted for 14 hours and random blood glucose was measured from serum obtained from
cardiac punksi at the end of the study. Fasting and random blood glucose in all treatment groups did not change after induced D-galactose in the ANOVA statistical test ($p=0.051$ and $p=0.626$).

| Group                  | Average blood glucose of fasting (mg/dL) | Average blood glucose of random (mg/dL) |
|------------------------|------------------------------------------|----------------------------------------|
| K1 (D-Gal i.p)         | 69.33 ± 14.4                             | 192.33 ± 80.2                           |
| K2 (D-Gal & GLE 100 p.o) | 57.83 ± 6.69                             | 198.83 ± 44.2                           |
| K3 (D-Gal & GLE 200 p.o) | 74.00 ± 6.65                             | 156.33 ± 55.3                           |
| K4 (D-Gal & GLE 400 p.o) | 76.50 ± 15.3                             | 185.16 ± 52.0                           |

**Table 2. Average blood glucose of fasting and random**

| Group                  | Average area of Langerhans islet ($\mu m^2$) | Average perimeter of Langerhans islet ($\mu m^2$) |
|------------------------|---------------------------------------------|-----------------------------------------------|
| K1 (D-Gal i.p)         | 34970.02±12165.7                            | 694.73 ± 133.5                               |
| K2 (D-Gal & E.G 100 p.o) | 41668.81±4030.6                            | 741.53 ± 39.5                               |
| K3 (D-Gal & E.G 200 p.o) | 48372.71±21902.7                           | 820.60 ± 238.1                              |
| K4 (D-Gal & E.G 400 p.o) | 43716.20±22161.4                           | 805.10 ± 252.7                              |

**Table 3. Average area and perimeter of Langerhans islet**

From Table 3 we can see an increase in the area of Langerhans islet in the group given gambir leaves extract ($U.$ gambir Roxb.) compared to the group that was only injected with D-galactose ($p=0.589$). Similarly there was an increase in the perimeter of Langerhans islet in the group given gambir leaves extract ($U.$ gambir Roxb.) compared to the group only injected with D-galactose ($p = 0.634$).

In line with this, in Table 4 it can also be seen that there is a trend of an increase in the number of cell nuclei in the islet of Langerhans in the group given gambir leaves extract ($U.$ gambir Roxb.) $p=0.336$.

Although statistically using the ANOVA test there was no change in the area and perimeter after D-galactose induction, it can be seen that there is an increasing trend in the size of the area and perimeter and the number of nuclei of the Langerhans islet. This shows that gambir leaves extract ($U.$ gambir Roxb.) has the potential to increase the number of cells on the Langerhans islet.

**Table 4. Average number cells nuclei of Langerhans islet**
Figure 1. shows the microscopic structure of the pancreas of mice treated with gambir leaves extract (*U. gambir* Roxb.) and D-galactose. It can be seen that the largest area and perimeter are in group 3, followed by group 4, group 2, and the smallest one is found in group 1.

![Figure 1](image1.png)

Figure 1. Microscopic structure of the pancreas of mice (Magnification: 40x10, HE)

3.3 Effect of Galactose and Gambir Leaves Extract (*U. gambir* Roxb.) on The Levels of Malondialdehyde (MDA) Blood of Mice

MDA levels were measured in serum obtained from cardiac punksi at the end of the study. The mean value of MDA levels in all treatment groups is shown in Table 5. After analyzing the variance test with ANOVA, there was no change in MDA levels in D-galactose-induced aging animal models between treatment groups (*p*=0.286).

| Group                              | Average amount of MDA (nmol/mL) | *p*   |
|------------------------------------|---------------------------------|-------|
| K1 (D-Galaktosa i.p)               | 12,02 ± 5,7                     |       |
| K2 (D-Galaktosa & EDG 100 p.o)     | 18,17 ± 3,9                     |       |
| K3 (D-Galaktosa & EDG 200 p.o)     | 13,34 ± 4,5                     |       |
| K4 (D-Galaktosa & EDG 400 p.o)     | 15,40 ± 2,8                     |       |

Note: the mean value is displayed in the form (± SD) with α = 0.05
4. Discussion

Blood glucose is an index to evaluate glucose metabolism in the blood and also reflects the function of the pancreas, especially the function of the islets of Langerhans [8]. Normal blood glucose in mice are 62-175 mg/dL and random <200 mg/dL [9].

Based on these references, there was no increase in blood glucose after D-galactose induction in both fasting and random blood glucose. This can be seen in the group given gambir leaves extract (U. gambir Roxb.) and D-galactose (groups 2, 3, and 4). Group 2 showed a decrease in blood glucose from group 1 which was only given D-galactose alone. Then in groups 3 and 4 blood glucose continued to increase with increasing doses. Furthermore, in random blood glucose, it can be seen in group 2 showing the increase in blood glucose from group 1 which was only given D-galactose alone. Then in group 3 the blood glucose decreased and returned to increase in group 4.

Based on this, it is suspected that the catechin content of gambir leaves extract (U. gambir Roxb.) does not result in changes in fasting and random blood glucose.

According to information in the leaflet gambir leaves extract (U. gambir Roxb.) used by Shen Mi Ye Sari Uncariae Antioxidant (PT. Toyo Brothers), a catechin content of 81,37 was obtained. Catechins in gambir leaves (U. gambir Roxb.) are functional compounds containing phenols and belong to the flavonoid class [4].

The pancreas is an important digestive gland for mammals, especially in the human body. The pancreas secretes secretions which contain various digestive enzymes. Pancreatic secretions play an important role in the digestion and absorption of food as well as in the regulation of constant blood glucose in the body through the secretion of insulin and glucagon and other hormones.

D-galactose is now recognized as an inducible aging reagent that can be recapitulated in animal models of aging [6, 11]. The aging model of mice made with D-galactose has similarities with natural old mice at the level of oxidative stress free radical injury, non-enzymatic glycation. This can damage a number of organs including the pancreas [8, 12].

Based on the research that has been done, it can be seen based on the average area and circumference of the island of Langerhans (Table 1.3) an increase in the area and circumference of the pancreas of rats in each treatment even though it is not significant. Furthermore, based on mice pancreatic microscopic images (figure 1.1) showed that there was an increase in area and perimeter in the group given D-galactose and gambir leaves extract (U. gambir Roxb.) group 2, 3, and 4 compared to group 1 which only given D-galactose only. The area and perimeter of Langerhans islet increased in group 3 but tended to decrease in groups 4 and 2.

The increase in area and perimeter of Langerhans islet found in group 2 can be attributed to the existence of a compensatory mechanism for β cells of the islet of Langerhans to an increase in blood glucose levels in physiological limits which causes cells to develop hypertrophy [13, 14]. However, cells will undergo apoptosis if blood glucose levels have passed a certain critical threshold so that there can be a decrease in the area and perimeter of the islets of Langerhans [15].

Oxidative damage is the main theory of cellular aging. Oxidative damage produces more reactive oxygen species (ROS) and disrupts cellular redox signals [16], which ultimately causes damage to cell DNA, proteins, and lipids [17, 18]. MDA is used in the evaluation of biomarkers of oxidative stress which is the final product of ROS induced peroxidation. MDA is a marker used to investigate lipid oxidative damage in the course of disease in humans.
Based on research that has been done, the statistical results show that there was no significant change in MDA levels in mice induced by D-galactose. This is thought to occur because the administration of doses that may not be optimal, given the antioxidant content of gambir leaves extract (U. gambir Roxb.) which is a high enough catechin that must be able to neutralize the accumulation of free radicals contained in the blood thereby reducing MDA levels in treated mice.

Measurement of MDA levels used in the study is the Thiobarbituric acid reactive substance (TBARs) method. The principle of this method is based on the ability to form a pink complex between MDA and Thiobarbituric Acid (TBA) [19] at a wavelength of 532 nm using spectrophotometry.

MDA is the result of the lipid peroxidation process due to the reaction of free radical accumulation. Lipid peroxidation can produce a number of compounds derived from aldehydes [20] which are toxic [21]. Lipid peroxidation occurs because of an imbalance between free radicals and antioxidants in the body. Phenolic compounds have an important role in detoxifying ROS (reactive oxidative species) and helping to maintain cell balance [22]. When MDA levels increase, one endogenous antioxidant (GSH) will decrease [23], so to improve the performance of endogenous antioxidant enzymes, exogenous antioxidants from outside the body are needed. Antioxidant activity in capturing free radicals through several stages, namely: increasing enzymatic antioxidants, inhibiting prooxidant enzymes and maintaining free radical attacks.

5. Conclusion
Gambir leaves extract does not cause a decrease in blood glucose and serum MDA. There are potential effects of GLE in promoting pancreatic growth and regeneration.

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