Hypoglicemic activity of gambier (*Uncaria gambir robx.*) drinks in alloxan-induced mice

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Abstract. Diabetes may cause complications in various parts of the body and increase the risk of early death. Gambier contains bioactive compounds and can be used as raw material in preparing gambier drink. The purpose of this study was to extract gambier, to determine the hypoglycemic activity of gambier drinks in alloxan-induced mice. In this study, gambier were extracted by 3 types of solvents with distilled water, ethyl acetate, and ethanol. The gambier drinks was tested on 5 groups of mice wich consisting of 5 male mice. Group 1 was given 0.5% CMC 1%/bw, group 2 was given metformin 65 mg/kg bw, groups 3, 4 and 5 were given gambier drinks with dosage of 100, 200, and 300 mg/kg bw. The results showed that extraction using distilled water produced the best gambier drinks. Gambier drinks with dosage 100, 200 and 300 mg/kg bb given for 15 days decreased the blood glucose level 27.69%, 38.75%, and 50.62% respectively, and increased the body weight 13.14%, 10, 91%, and 10.18% respectively. Treatment with gambier drinks was found to improve the condition of pancreatic langerhans island of alloxan-induced mice.

1. Introduction
Diabetes is a condition signalized by blood glucose level above normal called hyperglicemia due to dysfunction of blood glucose regulation\(^1\). There are two hormones which are responsible and play important roles to regulate glucose level in blood including insulin and glucagon hormones\(^2\). Metabolism abnormalities caused by β cell pancreas breakdown resulting in decreased insulin secretion and also insulin appeared to be resistant resulting in hyperglycemic as one of the diabetes symptoms causing complications in body parts and increasing the risk of early death\(^3\)\(^4\). To prevent diabetes epidemics, it needs strategy to consider and prevent the complexity of disease progression, and medications cost so increased access and knowledge on prevention strategy and medications on the disease is necessary by using components of traditional medicine for centuries\(^5\). Indonesia is known as main producer of gambier in international market\(^6\). Gambier is mainly used as tanner, dye substances and beauty product components, as a medicine mixture to treat burns, headache, diarrhea, dysentery, mouthwash, sprue and skin medicine\(^7,8\). Gambier which obtained from Indonesian market has very good antihyperglicemic activity\(^9\), and it has been consumed to treat diabetics as traditional medicines. Public interest in gambier consumption decreases because of
the astringent taste and less attractive appearance\cite{10}. The astringent taste can be reduced by decreasing tannin content and other impurities by extraction. Gambier can be processed into functional drinks with better flavour and easy to consume\cite{10}. Moreover, the processed food is expected to gain public interest in consuming gambier, as it also functions as anti diabetes. The purpose of this study was to extract gambier, and determine the hypoglycemic activity of gambier drinks in alloxan-induced mice.

2. Materials and Methods
2.1. Materials
Gambier plants obtained from Mado Laoli Village, Gunung Sitoli City, North Sumatra. Other material used in this experiment were cinnamon, gom arabic, and synthetic sugar (Tropicana Slim DIABTX\textregistered). The chemicals used in this study were distilled water, ethyl acetate and ethanol technical grade, catechin standart (Sigma), gallic-acid (Sigma), methanol, phenol, H\textsubscript{2}SO\textsubscript{4}, KMNO\textsubscript{4}, Na\textsubscript{2}CO\textsubscript{3} and Folin-Ciocalteu pro-analyzed (Merck), indigocarmin, DPPH, glucose, alloxan tetrahydrat (Sigma), metformin, formalin, alcohol, paraffin, gliserin, xylene, xylol, eoisin and hematoxyline mayers. The equipments used in this study were analytical balance (AND GR-200), micrometer pipettes (Eppendorf), Whatman filter papers No. 41, spectrophotometer (Shimadzu UV-1800), glucose kit (GlucoDr), automatic tissue processor (Lens TP 1020), mikroturm (Leica), light microscope (Primo Star Zeiss).

2.2. Gambier Production
Gambier leaves were boiled for 30 minutes on boiling water, crushed by using mortar, squeezed until all juice out from the leaves then saturated for 24 hours. After that, gambier’s gum was molded and dried in oven at 50°C for 60 hours.

2.3. Gambier’s Extractions
Gambier’s extractions were done by maceration method, using three different solvents: distilled water (E\textsubscript{1}), ethyl acetate (E\textsubscript{2}), and ethanol (E\textsubscript{3}). Gambier was mashed and sieved with 60 mesh filter. 100 g gambier powder were dissolved in 500 ml solvent, stirred using shaker for an hour and maceration for 24 hours at 30°C then filtered using filter paper Whatman No. 41. The obtained residue was extracted again twice with 500 ml solvent. The extract was evaporated by using rotary evaporator to obtain dry gambier extract.

2.4. Gambier drinks production
The mixture of gambier drinks consisted of 2.67% gambier extract, 0.67% cinnamon, 0.33% gom arabic, 2.33% synthesis glucose (Tropicana Slim DIATBX\textregistered) and 94% of water. The mixture was shaken to homogenous, boiled for three minutes, cooled, and packed in a bottle.

2.5. Catechin content determination
Catechin content was determined with procedure as described in Indonesian National Standard\cite{11}. 50 milligrams dry catechin standard was transferred into a 50 ml flask, dissolved and diluted with ethyl acetate to the volume of 50 ml (solution A), put on ultrasonic bath for 5 min. Transferred 2 ml of solution A into a 100 ml Erlenmeyer and add 50 ml of ethyl acetate (solution B) and placed on ultrasonic bath for 5 min, and the solution can be used for measurement. One (1) gram sample transferred into 50 ml flasks, dissolved and diluted with ethyl acetate until it reached the calibration mark (solution C), put on ultrasonic bath for 5 min, filtered. The first 15 ml filtrate was discarded and the next filtrate was collected. Put 2 ml of filtrate from solution C into 100 ml Erlenmeyer and added 50 ml of ethyl acetate solvent (solution D) and put on ultrasonic bath for 5 min before measurement. Then, the absorbance of the standard solution and the sample solution using UV spectrophotometer at 279 nm wavelength were measured. Catechin content was counted by the formula:
Catechin (%) = (Et 279 / Ec 279) x (Ws/ W) x 100%  \hspace{1cm} (1)

Note: Et 279 is absorbance of sample solution, Ec 279 is absorbance of catechin standard solution, Ws is catechin standard weight (g) and W is weight of sample (g).

2.6. Tannin content determination
Tannin content was determined by AOAC method\textsuperscript{[12]}. Fife (5) gram of sample was added 400 ml of distilled water and boiled for 30 min. After cooling, the solution was added distilled water to a volume of 500 ml, and filtered (filtrate 1). Then, 10 ml of filtrate 1 was mixed up with 25 ml of indigocarmine solution and 750 ml of distilled water then titrated with a 0.1 N KMnO\textsubscript{4} solution until the color turned to golden color (titration A). Filtrate 2 was resulted from the mixture of 100 ml of filtrate 1, 50 ml of gelatin solution, 100 ml of acid salt solution and 10 g caolin powder. The mixture was then shaken for 5 minutes and filtered. Twenty five (25) ml of filtrate 2 was mixed with 25 ml indigocarmine solution and 750 ml of distilled water and titrated with a 0.1 N KMnO\textsubscript{4} solution (titration B). Tannin content was calculated by the following equation:

\[ \text{Tannin (\%)} = \frac{(50A - 50B) \times N \times 0.0416}{g} \hspace{1cm} (2) \]

Note: A is titration volume of sample (ml) and B is titration volume of blank (ml), N is normality of standard KMnO\textsubscript{4} solution, g is weight of sample (g). 1 ml of KMnO\textsubscript{4} 0.1 N is equivalent to 0.00416 gram of tannin.

2.7. Total phenols content determination
Total phenols content was determined by using Folin Ciocalteu with gallic acid as standard\textsuperscript{[13]}. The standard solutions of gallic acid was prepared with concentration of 250, 500, dan 1000 µg/ml and measured the absorbance at 765 nm wavelength using a spectrophotometer (Shimadzu UV-1800). 0.5 ml of sample dissolved in distilled water (1:100 b/v) added 2.5 ml Folin-ciocalteu 50%, 2.5 ml distilled water, and 2 mL Na\textsubscript{2}CO\textsubscript{3} 15%, homogenized and kept in a dark place for an hour. Then measured the absorbance at 765 nm wavelength. The concentration was calculated based on equation obtained from the standard curve. Total phenolic content of the sample were calculated by the formula:

\[ \text{TPC} = \frac{(C \times V \times DF)}{g} \times 100\% \hspace{1cm} (3) \]

Note: TPC is total phenolic content (µg GAE/mg), V is volume of sample (ml), C is concentration (µgGAE/ml), DF is dilution factors, g is weight of sample (mg).

2.8. Antioxidant activity determination
Antioxidant activity was assessed using the radical capture method using DPPH\textsuperscript{[14]}. Briefly 1 ml of sample solution (diluted with methanol) with concentration of 10, 30, 50, 70 dan 90 µg/ml, added 2 ml solution of methanol and DPPH respectively. The solution was shaken until homogeneous and allowed to stand for 30 minutes, then absorbance was measured at a wavelength (λ) 517 nm using a spectrophotometer (Shimadzu UV-1800). The percentage of inhibition were calculated with the following formula:

\[ \%\text{inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100\% \hspace{1cm} (4) \]

By using the value of the inhibition percentage as the ordinate (y) and concentration of the extract as the absciss (x) using the linear regression method, a linear equation was obtained. IC\textsubscript{50} values was determined based on inhibition concentration at 50%.
2.9. Total sugar content determination
Total sugar content was determined by using phenol method\cite{15}. Standard glucose solution was prepared with concentrations of 40, 50, 60, 70, 80 μg/ml. 2 ml of each solution was mixed with 1 ml of 5% phenol solution and shaken. Then, 5 ml of concentrated sulfuric acid was added, left for 10 minutes into a shaken water bath. The absorbance was measured using a spectrophotometer at 490 nm wavelength. 2 ml of sample solution (1: 100 m/v) was mixed with 1 ml of 5% phenol solution and shaken. Added 5 ml of concentrated sulfuric acid, left for 10 minutes, shaken then placed in a water bath for 15 minutes. The absorbance was measured using a spectrophotometer at a wavelength of 490 nm. The concentration of sugar in the sample solution by the equation obtained from the standard curve. Then calculated percentage of total sugar sample with equation formula:

Total sugar (%) = \frac{(C.V.DF) x 100}{g}

Note : C is concentration of sugar (μg/ml), V is volume of sample (ml), DF is dilution factors and g is weight of sample (μg).

2.10. Organoleptic test of color, flavor and taste
Organoleptic tests of color, flavor and taste were determined using a hedonic value scale\cite{16} and randomly tested by 20 panelists. Testing was done on a sensory basis based on the hedonic scale.

2.11. Selection of the best treatment
The best treatment of gambier drinks was chosen based on weighting technique\cite{17} with variables including weight of organoleptic value of color 1, organoleptic value of taste 0.9, organoleptic value of flavor 0.8, total glucose 0.7, tannin content 0.6, catechin content 0.5, total phenolic content 0.4 and antioxidant activity 0.3.

2.12. Hypoglycemic Effect Assay
The animal used in this study were male Wistar mice weighing 28-32 g. Before the experiment, mice were acclimatized for a week in a good cage for environmental adaptation, i.e. the reception of light, 12 hours dark and 12 hours light and all the mice were subjected to normal mice chow and distilled water ad libitum.

Alloxaan solution was prepared by dissolving alloxan in distilled water. The mice were induced with alloxaan solution of 125 mg/kgBW administrated intraperitoneally. The blood glucose level (BGL) of mice were measured on the third day. The mice with glucose levels greater than 200 mg/dl were separated and used as experimental animals. The mice were divided randomly into 5 groups consisting of 5 mice of each group.

| Group | Treatment |
|-------|-----------|
| I     | Diabetic mice were given a 0.5% CMC suspension, dose of 1% of body weight (BW) |
| II    | Diabetic mice were given a Metformin suspension, dose of 65 mg/kgBW |
| III   | Diabetic mice were given gambier drink 100 mg/kgBW |
| IV    | Diabetic mice were given gambier drink 200 mg/kgBW |
| V     | Diabetic mice were given gambier drink 300 mg/kgBW |

The suspension for the tested material was administered orally for 15 consecutive days and the BGL and body weight were measured on the day 1, day 3, day 6, day 9, day 12, and day 15 after administration of the tested material.

2.13 Histolopathological of the Pancreas Mice
The mice from each group was killed by cervical dislocation after completion of treatment, and pancreas were dissected out quickly, washed in saline (0.09 NaCl), and fixed in formaldehyde solution (10%). Tissue were processed and embedded in paraffin wax. Sections were cut at 5 μ thickness and stained with Hematoxyline-Eosin (HE). After completion of staining sections were observed under microscope for histological change.
A study approval was obtained from Animal Research Ethics Committees (AREC) of Faculty of Math and Science, University of Sumatera Utara.

2.14 Statistical analysis

Data were analyzed using SPSS software (22.0). Differences in mean values were tested by analysis of variance, and significance levels were obtained with Duncan’s test. A significance level of P<0.05 was used.

3. Result and Discussion

3.1 The Quality of Gambier Drinks

Quality parameters based on the bioactive components present in gambier drinks by different solvent extractions shown in Table 1.

Table 1. The effect of gambier extraction method on gambier drinks’ quality parameters observed

| Quality parameters                  | Extraction using distilled water ($E_1$) | Extraction using ethyl acetate ($E_2$) | Extraction using ethanol ($E_3$) |
|-------------------------------------|------------------------------------------|---------------------------------------|----------------------------------|
| Catechin content (%)                | 1.85±0.05$^a$                           | 1.98±0.05$^a$                         | 1.93±0.54$^{ab}$                 |
| Tannin content (%)                  | 0.21±0.04$^b$                           | 0.29±0.04$^a$                         | 0.19±0.02$^b$                   |
| Total phenolic content (µGAE/mg)    | 639.75±5.70$^c$                         | 732.34±4.86$^a$                       | 697.34±4.84$^b$                 |
| Antioxidant activity (IC50) (µg/ml) | 37.41±1.37$^a$                          | 35.96±0.50$^a$                        | 36.23±0.87$^a$                  |
| Total sugar (%)                     | 1.92±0.10$^a$                           | 1.96±0.09$^a$                         | 1.95±0.09$^a$                   |
| Organoleptic of color value (numeric) | 4.02±0.10$^a$                     | 3.73±0.16$^b$                         | 3.72±0.10$^b$                   |
| Organoleptic of flavor value (numeric) | 3.83±0.10$^a$                  | 3.60±0.25$^b$                         | 3.55±0.13$^b$                   |
| Organoleptic of taste value (numeric) | 3.70±0.13$^a$                     | 3.42±0.10$^b$                         | 3.49±0.05$^b$                   |

Note: Data is the means of three replicates

Table 1 shows that gambier drinks $E_1$ has low tannin level and higher organoleptics color and taste value. Therefore, the $E_1$ is more preferable for consumers because it has low tannin level lead to lower astringent taste. Tannin is a compound which cause astringent taste in gambier$^{[10]}$. In addition, gambier drinks $E_2$ has the highest of catechin because polarities of ethyl acetate value similar to the polarities substance extracted so it is more effective$^{[18]}$. Based on weighting technique, the best gambier drinks is a drink which used gambier as its raw material extracted with distilled water ($E_1$) and it is used for in vivo test on research.

3.2 Hypoglycemic activity of gambier drinks

Hypoglycemic activity of tested material was observed in interval time of 3 days or 15 days and the results presented in Table 2 and Figure 1.

Table 2. The effect of gambier drinks on blood glucose levels of mice induced with alloxan

| Groups       | Glucose blood levels (mg/dl) with interval time (days) |
|--------------|--------------------------------------------------------|
|              | D0          | D1          | D3          | D6          | D9          | D12         | D15         |
| Group I      | 120.0±8.22$^a$ | 393.2±6.91$^d$ | 402.6±9.13$^c$ | 412.8±4.38$^b$ | 423.0±3.39$^a$ | 416.2±3.03$^{ab}$ | 410.0±3.61$^{bc}$ |
| Group II     | 135.2±6.76$^a$ | 395.6±9.76$^d$ | 352.2±4.21$^b$ | 302.0±8.69$^a$ | 240.0±4.30$^d$ | 207.0±4.74$^a$ | 181.0±7.28$^f$ |
| Group III    | 130.4±9.24$^a$ | 390.0±9.35$^c$ | 355.4±4.51$^b$ | 329.6±10.74$^c$ | 307.6±9.40$^d$ | 295.6±7.30$^a$ | 282.0±7.07$^f$ |
| Group IV     | 141.2±7.50$^a$ | 349.4±7.50$^c$ | 319.6±6.50$^b$ | 293.4±9.61$^c$ | 264.2±6.76$^d$ | 238.6±5.86$^a$ | 214.0±4.74$^d$ |
| Group V      | 148.6±9.45$^a$ | 356.4±9.76$^c$ | 309.8±6.80$^b$ | 271.0±6.96$^a$ | 234.0±4.74$^d$ | 201.6±8.41$^a$ | 176.0±7.65$^f$ |

Note: Data is the means of 5 replicates
Table 2 and Figure 1 show that the BGL in the control group (group I) increased with the time, but the levels were decreased after 15 days of storage. Metformin (group II) was able to reduce blood glucose levels from 395.6 mg/dl to 181 mg/dl (~54%). Gambier drinks with dose of 100 mg/kgBW (group III) reduced BGL from 390 mg/dl to 282 mg/dl (~28%), dose of 200 mg/kgBW (group IV) reduced BGL from 349.4 mg/dl to 214 mg/dl (~39%), and a dose of 300 mg/kg BW (group V) reduced BGL from 356.4 mg/dl to 176 mg/dl (~51%). The gambier drinks’ ability in reducing BGL was caused by phenolics compound in gambier with strong antioxidant activities. The antioxidant activity for gambier is 6.4 μg/ml and phenolics compounds are 1142.5 μg GAE/mg[19]. Gambier also had inhibitory activity on α-glucoside of 40.65-89.87 μg/ml[20]. The main bioactive compound which play role to inhibit α-glucoside is catechin[20]. BGL decrease is also caused by coumarin compound in cinnamon which can fix glicemics control by inhibiting mechanism on α-glucoside enzyme in intestine, organizing the balance of enzym Pyruvate Kinase (PK) and Pyruvate Carboxikinase (PEPCK) which play role to arrange glucose metabolism in the liver[21].

3.3. Body weight of mice
The effect of gambier drinks on body weight of mice can be seen in Table 3. Statistically, gambier beverages did not give significant different effect (P> 0.05) on body weight of mice.

Table 3. The effect of gambier drinks on body weight of mice induced with alloxan

| Groups   | Body weight (g) with interval time (days) |
|----------|----------------------------------------|
|          | D0     | D1     | D3     | D6     | D9     | D12    | D15    |
|----------|--------|--------|--------|--------|--------|--------|--------|
| Group I  | 29.8±2.05a | 29.8±2.05a | 30.4±2.51a | 31.4±3.51a | 32.6±4.28a | 34.2±5.31a | 35.2±5.31a |
| Group II | 29.2±0.45a | 29.2±0.45a | 29.4±1.14a | 30.0±1.87a | 30.6±2.88a | 31.2±3.27a | 31.8±3.77a |
| Group III| 30.4±1.67a | 30.4±1.67a | 31.0±2.12a | 31.4±2.88a | 33.2±3.77a | 34.2±4.76a | 35.0±5.10a |
| Group IV | 29.4±0.55a | 29.4±0.55a | 29.8±1.92a | 30.4±3.51a | 31.2±4.44a | 32.0±4.85a | 33.0±6.04a |
| Group V  | 30.0±1.41a | 30.0±1.41a | 30.2±1.48a | 31.0±2.92a | 31.8±4.66a | 33.0±5.70a | 33.4±6.07a |

Note: Data is the means of 5 replicates

3.4. Histology of langerhans islet of mice
The histology of langerhans islet of mice can be seen in Figure 2.
Figure 2. Islets of Langerhans of pancreas stained with HE. A is pancreas of group I mice, B is pancreas of group II mice at 40X. C is pancreas of group III mice, D is pancreas of group IV mice and E is pancreas of group V mice at 40X.

The group of control mice (K1) had langerhans islet impairment caused by alloxan which induced damage to the DNA of β cells located in langerhans islet and induced by high blood glucose levels. Metformin as an oral hypoglycemic agent showed improvement of β cells in which β cell density increases because metformin provides protection against pancreatic β-cell apoptosis by a mechanism interference with the production of ROS and inhibits the influx of free fatty acids and CD36 (cluster determinant 36) and prevent cell death due to oxidative stress in non-neuronal cells.

Gambier drinks with various doses seem to improve β cell structure of mice’s pancreas, the higher dose of gambier drinks repair β-cell density of langerhans islet better because it can restore pancreatic β cells to secrete insulin, resulted in the decrease of BGL. Gambier drinks contain high phytochemical compounds, which play a role in repairing the β cells, improve the condition of blood glucose by preventing oxidative stress, improve damage conditions and prevent the occurrence of lesions on the islet of langerhans.

4. Conclusions
The gambier extraction method using distilled water produces the best quality gambier. This study indicates that gambier drink has the hypoglicemic effect to decrease blood glucose levels. The dose of 300 mg/kgBW has hypoglycemic activity which is similar to antidiabetic agent metformin, and can repair the damage of β-cells of langerhans islet.

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