Fortification of skim milk with whey protein xanthone and its effect on antihyperglycemic activities in animal model

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Abstract. Diabetes Mellitus (DM) is a disease characterized by the increase of blood sugar levels, due to the damage of insulin-producing cells in the pancreas. DM management includes taking glucose-lowering bioactive compounds from natural products such as xanthone and its derivatives from mangosteen peel. Bioactive compounds are susceptible to degradation; therefore, it needs to be protected by whey protein. Whey protein is believed to be a delivery system or carrier in the food system by means of nanocapsules. In addition, fortification of whey protein and xanthone combined into liquid skim milk is a way to diversify dairy products for diabetics. The purpose of this study is to determine the effect of fortification of skim milk with whey protein xanthone on blood glucose levels of hyperglycaemic rats. The results of in vivo study prove that xanthone from mangosteen peel extract and its combination with whey protein nor liquid skim milk significantly affects (α=0.05) the decreased level of blood glucose male Wistar rats.

1. Introduction
Diabetes mellitus (DM) is a disease marked by the increasing level of blood glucose in the body, as a result of the damage of insulin-producing cells in the pancreas [1]. In 2015, around 415 million adults in the world suffered from diabetes and it is estimated that in 2040, this number will increase up to 642 million people with diabetes. In 2015, Indonesia ranked seventh in the world as the highest prevalence of diabetics with an estimated population of 10 million people [2].

DM is generally initiated by the exposure of free radicals towards the body. Free radicals can cause damage to insulin-producing cells in the pancreas and result in the body to lack of insulin [3]. One of the compounds that can suppress the side effects of DM is bioactive compounds, one of which is found in mangosteen peel, namely xanthone and its derivatives from mangosteen peel. Xanthone found in mangosteen peel can neutralize free radicals and prevent damage to pancreatic beta cells caused by free radicals [4]. It is interesting that adding mangosteen peel extract at a dose of 250 mg/kg BW to Wistar rats is capable to reduce blood glucose levels by 47.63% for 28 days [5].

Bioactive compounds such as phenol compounds (xanthone) and flavonoids are susceptible to degradation. Therefore, it is necessary to shield bioactive compounds to increase their stability, solubility, and bioavailability in the body [6]. One way to protect these bioactive compounds is by using whey protein. Whey protein is believed to be a delivery system or carrier in the food system because it
can form capsules [7]. Based on the results of molecular docking, whey protein is capable to bind with polyphenol compounds from mangosteen peel extract, namely xanthone. The interaction between whey protein (α-lactalbumin and beta-lactoglobulin) and xanthone is stabilized by hydrogen bonds, Van der Waals, and hydrophobic interactions [8].

Fortification of whey protein xanthone into liquid skim milk is one of the ways to produce a diversity of dairy products for diabetics. The addition of polyphenol components into milk will affect the bonds contained in the milk; therefore, in the process of milk fortification, it is necessary to pay attention to the concentration of the added solution of whey protein-xanthone. Based on the background that reveals the ability of xanthone from mangosteen peel extract to reduce blood glucose levels, it is necessary to perform in vivo testing for each dosage form. This in vivo test aims to determine the effect and affectivity of the addition of whey protein xanthone to the skim milk towards decreasing blood glucose levels from alloxan-induced rats.

2. Materials and method

The mangosteen peel powder was obtained from Materia Medica Batu, whey protein isolate (WPI), aquadest and liquid skim milk. Chemicals used for extraction and for analysis include 96% ethanol, Folin-ciocaltéau reagent, Na₂CO₃, gallic acid, quercetin, aquadest, NaNO₂, AlCl₃, and NaOH. These chemicals were obtained from the Laboratory of Food Chemistry and Biochemistry, Laboratory of Biotechnology, Department of Agricultural Product Technology, Faculty of Agricultural Technology, University of Brawijaya, CV. Makmur Sejati, and CV. Kridatama.

Male Wistar rats were obtained from Malang Murine Farm which weighs between 180-200 grams were examined in the in vivo testing. Materials that induce diabetes in rats is alloxan. Sigma Aldrich’s alloxan was obtained from the Food Nutrition Laboratory of the Faculty of Agricultural Technology, University of Brawijaya, while the sterile aquadest was obtained from the Medical Faculty, University of Brawijaya. The rats feed used were a modified AIN-93M standard feed.

2.1. Total phenol analysis

Total phenol analysis was determined using Folin-Ciocalteau Method [9]. Each dosage of the sample which was dissolved at a certain concentration was taken as much as 0.5 ml and then added with 2.5 ml of Folin-Ciocalteau reagent 10% (v/v). The mixtures were homogenized using vortex and incubated in a dark room for 5 minutes, then added with 2 ml Na₂CO₃ 7.5% (w/v). The mixture is homogenized using vortex and incubated in a dark room for 30 minutes. The sample absorbance was measured using a spectrophotometer at a wavelength of 756 nm.

2.2. Flavonoids analysis

Each dosage (sample) which was dissolved at a certain concentration was taken as much as 0.5 ml, then added with 0.5 ml of NaNO₂ 5% (w/v). The mixture was homogenized using vortex and incubated in a dark room for 5 minutes, then added with 0.5 ml of AlCl₃ 10% (w/v). Further, the mixture was homogenized using vortex and incubated in a dark room for 6 minutes, and then 4 ml of 1 M NaOH was added. The mixture is homogenized using a vortex. The absorbance of the sample was measured using a spectrophotometer at a wavelength of 447 nm [10].

2.3. Animal model’s treatments

The animals’ models used in this research are male Wistar rats and were divided into five treatment groups (Table 1) and consisting of six rats per each group. All groups of the animal model were acclimatized for two weeks to provide the adaptation conditions before treatments. The experiment was carried out for 21 days.
Table 1. Animal model’s treatments employed in this research.

| Group                          | Treatments                                                                 |
|-------------------------------|---------------------------------------------------------------------------|
| Negative Control (P1)         | without alloxan injection                                                 |
| Positive Control (P2)         | alloxan injection                                                         |
| Xanthone (P3)                 | alloxan injection, given xanthone with the dose of 250 mg/kg BW           |
| Whey Protein – Xanthone (P4)  | alloxan injection, given a solution of whey protein-xanthone with the dose of 250 mg/kg BW |
| Whey Protein – Xanthone in Skim Milk (P5) | alloxan injection, given liquid skim milk fortified with whey protein solution – xanthone with the dose of 250 mg/kg BW |

The sample volumes are based on the calculation of the sample dose of 250 mg/kg, total phenol in the sample, and the weight of the rats. During the treatment, each group of rats was given modified AIN-93M as standard feed and drinking water in ad libitum. There were four groups of rats induced by alloxan to achieve hyperglycemic conditions. The alloxan dose given is 100 mg/kg BW intraperitoneally. The research protocols were applied by the Medical Ethics Committee University of Brawijaya as National Ethics Committee.

2.4. Testing blood glucose level
Blood collecting and the measuring of blood glucose level under a fasting condition are carried out every seven days. Taking blood of rats was carried out from their tail (intravenously). The glucose test tool is used to measure the blood glucose level. Glucose levels will be appearing on the glucose test screen after 10 seconds and expressed in mg/dl.

3. Results and discussion
Phenolic or polyphenols are natural bioactive components that possess antioxidant activity [11]. Polyphenols that are abundant in the mangosteen peel are xanthone and their derivatives, anthocyanins, and tannins. Total phenol was quantified to determine the content of phenolic compounds contained in a substance.

Flavonoids are a group of polyphenolic compounds found in plants. The composition of flavonoids that are measured to determine the concentration of flavonoids consists of flavones and flavanols. This is due to the ability of the two groups to form stable complexes with AlCl₃ compounds [12]. Results on total phenol and flavonoids of various preparation containing extracts of xanthone from mangosteen peel powder extract are shown in table 2.

Table 2. Average data of phenol and flavonoids for various preparation containing xanthone.

| Types of preparation | Total phenol (mg GAE/g) | Flavonoid (mg QE/g) |
|----------------------|-------------------------|---------------------|
| Xanthone             | 61.72±6.61              | 32.87±3.11          |
| Whey protein solution-xanthone | 24.22±2.60             | 3.60±0.40           |
| Liquid skim milk fortified with a solution of whey protein-xanthone | 5.20±0.71             | 0.93±0.03           |

Further, according to table 2, it presents the total phenol and flavonoid from the highest to lowest as listed, xanthone, whey protein-xanthone, and liquid skim milk fortified with whey protein-xanthone. The differences that exist in total phenol and flavonoid levels occur because of the amount of xanthone added are few in amount. To make the whey protein solution-xanthone, the amount of xanthone added was 0.05% (w/v). In the making of liquid skim milk fortified with a solution of whey protein-xanthone,
the solution of whey protein-xanthone added was 20% (v/v). Differences in flavonoid levels also develop due to genotype factors, phase of fruit maturity, cultivation techniques, and climatic conditions for planting, and post-harvest handling [13].

Moreover, the blood glucose level of male Wistar rats as an animal's model was measured every week during 21 days of treatment. Results on table 3 showed that the positive control group (P2) experienced an increase in blood glucose levels. The making of DM rats is done by the induction of alloxan. Alloxan reacts with two SH groups that bind to the side of protein or amino acid, forming a disulfide bond that activates proteins which in result impairs the function of the protein. Alloxan has specific cytotoxic features in Langerhans beta cells and will generate radical groups that cause damage to Langerhans beta cells. The damage of beta cell will be followed by a decrease in the secretion of the insulin hormone which causes glycogenesis reactions and reduces glucose transport into cells [14].

Table 3. Average decrease blood glucose levels of animal’s model.

| Group treatment | Average blood glucose level | Average decrease in blood glucose levels |
|-----------------|-----------------------------|-----------------------------------------|
|                 | Start (mg/dL) | Final (mg/dL) | (mg/dL) * | (%) * |
|-----------------|---------------|---------------|-----------|-------|
| P1              | 76.20         | 77.20         | 1.00±19.24 | 4.60±22.44 |
| P2              | 292.80        | 522.20        | 229.40±130.51 | 97.04±65.34a |
| P3              | 504.20        | 184.00        | -320.20±45.51a | -65.02±13.74b |
| P4              | 442.00        | 209.20        | -238.80±31.57a | -53.05±8.46bc |
| P5              | 337.40        | 276.80        | -60.60±32.42b | -19.57±13.51bc |

The results of ANOVA analysis show that the distribution of xanthone, whey protein-xanthone, and liquid skim milk fortified with whey protein-xanthone has a significant effect (α = 0.05) on Wistar rat blood glucose levels. The highest average on the decrease in blood glucose levels (Table 3) was found in the P3 group which was -320.20 ± 45.51 mg/dL. Previous studies show the decrease in blood glucose levels in Wistar rats that were treated with extracts of xanthone dose of 250 mg/kg BW was 105.92 mg/dL [5]. This difference in the level of decline in blood glucose levels can occur due to the range of conditions of the animal tested, varieties of mangosteen peel used to make extracts and mangosteen peel extraction methods, thus affecting the number of bioactive compounds such as phenol compounds which play a role in decreasing blood glucose levels.

Viewed by the notation provided in table 3, the P3 group had an average decrease in blood glucose levels which was not significantly different, compared to the P4 group. The group that was given xanthone and who were given whey protein-xanthone shows a significant amount of decrease in their blood glucose levels, compared to the group given skim milk fortified whey protein-xanthone, both positive control (P1), and negative control (P2). This is due to the existence of xanthone, included in the phenol compound in the xanthone. The presence of a hydroxyl (-OH) groups on xanthone allow xanthone compounds to work as antioxidants. Phenol/polyphenol compounds are known to be able to reduce oxidative stress by preventing the occurrence of chain reactions which converts superoxide to superoxide hydrogen by donating hydrogen atoms from polyphenol aromatic hydroxyl (-OH) groups to bind free radicals [15]. In cells that have insulin receptors (muscle cells, adipose cells, and liver cells), binding of free radicals will increase insulin signaling in intracellular GLUT-4 translocation to the cell membrane enabling it to take glucose from the blood [16].

Phenol compounds contained in each sample hold antioxidant activity, allowing them to provide protection and refining effect on pancreatic beta cells damaged by induction of alloxan. The improvement of pancreatic beta cells condition causes an increase in insulin secretion [17]. The compounds contained in mangosteen peel extract, aside from playing a role as an antioxidant by donating electrons to free radicals, can also function as an inducer which will trigger the expression of antioxidant encoding genes through the activation of Nrf2 [18].

Phenol compounds also have the potential as an anti insulysin. Insulysin is an insulin degrading enzyme [19]. With the presence of phenol compounds in mangosteen peel, which acts as anti insulysin,
it is expected to be capable of increasing the level of availability of insulin in the body of DM rats. Increased availability of insulin can reduce high blood glucose levels [20].

Mangosteen peel also contains flavonoids. The composition of flavonoids in each sample given to the rat groups can be seen in table 2. Flavonoids can act as antioxidants. Flavonoids can bring positive effects on diabetes through a mechanism of insulin secretion increase, reducing apoptosis, and supporting pancreatic beta cell proliferation, improving hyperglycemia through regulation of glucose metabolism, and reducing insulin resistance [17]. Flavonoids can prevent complications or progression of DM by clearing excess free radicals, breaking the chains of free radical reactions, binding metal ions, and blocking the polyol pathway by inhibiting the aldolase reductase enzyme [21]. Flavonoids also have an inhibitory effect on alpha glucosidase enzyme through hydroxylation bonds and beta ring substitution. Flavonoids are reported to have an antidiabetic activity that is capable of regenerating cells on the islets of Langerhans [22]. This explains the effects of flavonoids in reducing blood glucose levels.

In the sample solution of whey protein-xanthone and liquid skim milk fortified with a solution of whey protein-xanthone, also contains total phenol levels (seen from table 2). Although the total rate of phenol in the solution of whey protein-xanthone was lower than the xanthone, the decreasing effect in blood glucose levels was not significantly different. In other words, the ability to decrease blood glucose levels by xanthone with a solution of whey protein-xanthone is similar, seen from the BNT advanced test notation with a confidence interval of 5%.

The use of whey protein-xanthone is based on a study of molecular docking on whey which was able to bind with polyphenol compounds from extracts of mangosteen peel powder, namely xanthone. The interaction between whey and xanthone was stabilized by hydrogen bonds, van der Waals, and hydrophobic interactions [8]. Whey as a delivery system or carrier in the food system since it is able to form nanocapsules. α-lactalbumin and beta-lactoglobulin are the main components in whey protein. The main function of beta-lactoglobulin is as an agent to retinol molecules and other hydrophobic components. Beta-lactoglobulin has the potential as a delivery vehicle for bioactive compounds. Common antioxidants, polyphenols, have limitations to be absorbed by the body and easily degraded. This happens due to the conditions of the gastric pH and digestive system. Based on the factors above, the bioactive component is protected by other components, one of which is whey. Bioactive compounds protected by whey protein are expected to increase their stability, solubility, and bioavailability in the body [6].

The use of whey in the solution of mangosteen peel extract is due to the ability of bioactive peptides from whey to increase hormone secretion of the digestive tract and inhibit the activity of enzymes that play a role in glucose homeostasis. Most whey amino acids (leucine, isoleucine, valine, lysine, and threonine) can control blood insulin and glycaemic responses [23]. In addition, the use of whey, specifically whey protein isolate (WPI) is because WPI has a very high protein composition (more than 90%) with very low lactose and almost fat-free [24]. This positively correlates in reducing blood glucose levels because of the low contribution of lactose or fat from WPI which increases blood glucose levels.

The group of rats fed with skim milk fortified with whey-xanthone shows the lowest decrease in blood glucose level, compared to the group of rats given extract and whey-extract solution. This happens since skim milk contains lactose. Lactose is one of the causes of the increase in blood glucose levels. The composition of non-fat solids from skim milk is 52.15% lactose, 38.71% protein (31.18% casein and 7.53% whey), 1.08% fat, and 8.06% ash [25]. The lowest reduction in blood glucose levels was due to the composition of the liquid skim milk used in the study, namely "Diamond" liquid skim milk, which contains 12 g of carbohydrate per 250 ml.

According to this research, xanthone, whey protein-xanthone, and skim milk fortified whey protein-xanthone is proven to be capable of reducing blood glucose levels. This is due to the composition of phenol and flavonoids which both have antioxidant properties. Although the distribution of polyphenols and flavonoids from each sample were not able to reduce blood glucose levels to its normal levels, it has the effect of protecting pancreatic beta cells, holds back the continuing growth of DM disease and being able to reduce blood glucose levels [26].
4. Conclusion
The highest total phenol and flavonoid levels were found in xanthone, which was 61.72 mg GAE/g and 32.87 mg QE/g. Based on the results of in vivo testing, the distribution of P3, P4, P5 significantly affected ($\alpha = 0.05$) in decreasing blood glucose levels on the rat as an animal model. Based on further BNT testing with a confidence interval of 5%, the decreasing level of blood glucose in rat given P3 did not significantly affect the group of rats that were given P4, so it can be concluded that the ability to reduce blood glucose levels on group of rats given whey protein-xanthone shared the same result as the group of rats given xanthone.

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