

In Vitro α -Glucosidase Inhibition and Antioxidant Activity of Mulberry (*Morus Alba* L.) Leaf Ethanolic Extract

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ABSTRACT

This present work aimed to investigate the in vitro antioxidant activity and α -glucosidase inhibitory effect of mulberry leaf ethanolic extract. Antioxidant analysis was performed using Thiobarbituric Acid (TBA) assay at concentrations of 125, 200, 500, and 1,000 ppm. The results showed that the optimum incubation time was four days and the extracts could reduce the formation of MDA, i.e. 41.21%, 45.33%, 44.19%, and 36.00%, respectively. This suggests that concentration of 200 ppm was found as the best treatment. In addition, the result showed that ethanolic extract of mulberry leaf also showed inhibition against α -glucosidase with the IC₅₀ of 309.82 μ g/ml.

Keywords: α -glucosidase, antioxidant, mulberry, thiobarbituric acid

INTRODUCTION

Free radical is an atom or group of atoms that possess one or more unpaired electrons, which make it highly chemically reactive towards electrons in other molecules of human cells (Amrun *et al.* 2007). It causes disruption of important macromolecules such as protein, lipid, carbohydrate, and DNA, which in turn causing physiological disorders such as diabetes mellitus (DM). Nowadays, the prevalence of DM has been growing around the globe, especially type 2 diabetes mellitus (T2DM). Factors contributing to the disease are aging, social-economic problems, lifestyle, lack of physical activities, and obesity (WHO 2015). In addition, glycemia was proposed as another contributor to T2DM (Barclay *et al.* 2008).

A noticeable increase in glucose level after carbohydrate loading (postprandial glycemia) that exceeds the normal level (hyperglycemia postprandial) is deemed as one of the significant factors causing T2DM (Barclay *et al.* 2008). One way to control postprandial glycemia is via inhibition of glucose absorption in the intestine. In this case, inhibiting the action of intestinal enzymes, such as α -glucosidase, that converts

carbohydrate into glucose can be a promising attempt (Castellano *et al.* 2013). This inhibitory action against α -glucosidase is meaningful in management of DM.

Mulberry (*Morus alba* L.) has been reported as a source of glucose inhibitors (Efendi *et al.* 2010) and widely used for various diseases such as diabetes, hypercholesterolemia, and kidney diseases (Huang *et al.* 2013). The beneficial properties of the plant are attributed to its active compounds such as alkaloid, flavonoid, polyphenol, Calcium, Phosphor, Iron, Manganese, vitamins (A, B, C). Numerous reports have been published regarding the physiological effects of mulberry leaf extracts in controlling glucose level after carbohydrate consumption (Chung *et al.* 2013; Jeszka-Skowron *et al.* 2014), while the extract was known as capable of alleviating the absorption of sucrose and maltose in intestine. Intake of mulberry leaf ethanolic and acetone extracts in streptozotocin (STZ)-induced diabetic rats remarkably reduced glucose level, while also increased insulin concentration and antioxidant activity (Jezka-Skowron *et al.* 2014).

A study on antioxidant activity of mulberry twig and root bark extract estimated using DPPH assay showed protective activities on phospho-

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lipids against free radical attacks; the extract was also effective in avoiding biomolecules from oxidative disruption (Chang *et al.* 2011). The anti oxidant activity of mulberry leaves extracted using different solvents (acetone, methanol, aqueous) were also investigated. The research found that the methanol extract exhibited the highest content of phenolic and antioxidant activity (Arabshahi-Delouee & Urooj 2007).

Further, Jeszka-Skowron *et al.* (2014) demonstrated that mulberry leaf extract isolated with ethanol 65% showed the strongest action on alleviating glucose level of experimental animals. In addition, the experiment showed that such glucose level-lowering effect contributed to increase secretion of insulin and antioxidant activity. However, there is no any scientific report on in vitro experiment uncovering the effects of mulberry leaf ethanolic extract on inhibition of α -glucosidase. This present work aimed to investigate the antioxidant activity using TBA (Thio-barbituric Acid) assay, and evaluate the inhibitory properties of mulberry leaf ethanolic extract against α -glucosidase.

METHODS

Design, location, and time

Antioxidant activity was evaluated according to Completely Randomized Design (CRD) consisting of three groups: negative control (distilled water), positive control (α -tocopherol) at concentration of 200 ppm, and mulberry leaf ethanolic extract at various levels (125, 200, 500 and 1,000 ppm). Each experimental unit was carried out at triplicates.

Materials and tools

Mulberry leaves were collected from UKBB, Indonesia. Chemicals used were ethanol 65%. Ethanol 30%, chloroform, ammonia, H₂SO₄, Dragendorf reagent, Meyer reagent, Wagner reagent, FeCl₃, ether, NaOH 10% were used for phytochemical analysis. In terms of MDA analysis and incubation time determination, some chemicals were used, including ethanol 70%, absolute ethanol, linoleic acid 50 mM, buffer phosphate 0.1 M (pH 7), α -tocopherol (vitamin E), TMP (1,1,3,3-tetramethoxypropane) 6 M, TCA (trichloroacetic acid), TBA (thiobarbituric acid), and glacial acetic acid. Chemicals used for α -glucosidase inhibition analysis included

DMSO (Dimethyl Sulfoxide), α -glucosidase, p-nitrophenyl- α -D glucopyranoside (p-NPG), natrium bicarbonate (Na₂CO₃), buffer phosphate, bovine serum albumin (BSA), starch, alloxan, and acarbose (Glucobay). All chemicals used are analytical grade.

Experimental instruments for antioxidant activity analysis were oven, electrical blender, shaker, filter paper, spectrophotometer, centrifuge. While, the instruments used for sample preparation and inhibition analysis were oven, rotary evaporator, microplate, microplate reader Bio Rad, digital balance, vortex.

Procedure

Preparation of mulberry leaf powder (Jeszka-Skowron *et al.* 2009). Mulberry leaves were sorted (young leaf, bright green-dark green in color, no blackspots). The sorted leaves were washed, shredded, and sun-dried for 1 hour. Next, the leaves were re-dried in oven for 6 hours at 60°C, powdered and filtered.

Moisture analysis (AOAC 2012). Moisture content was analyzed using standard method of AOAC (2012). Briefly, mulberry leaf powder (2 g) was dehydrated in oven for 6 hours at 105°C, desiccated for 15 minutes, and weighed.

Extraction (Jeszka-Skowron *et al.* 2014, modified). Mulberry leaves were macerated for 24 hours using ethanol 65% at a ratio of 1:10 (leaf powder: solvent). At the first 6 hours, the mixture was shaken in a shaker at 125 rpm, and left. Macerated substance was collected after filtration using filter paper. This maceration condition was replicated 3 times. The solvent was then removed using rotary evaporator, yielding an extract paste. The % extract yield was then determined as follows:

$$\% \text{ yield} = \frac{\text{extract weight}}{\text{water} - \text{free simplicia weight}} \times 100\%$$

Phytochemical Analysis (Harborne 1987). In this section, determination of alkaloid, tannin, flavonoid, saponin, steroid, and triterpenoid was carried out.

Determination of incubation time using conjugated dienes (Esterbauer 1989). A mixture consisting of 2 ml buffer phosphate (0.1 M, pH 7), 2 ml linoleic acid (50 mM; in ethanol 99.80%), and 1 ml deionized water was prepared and then placed in screw pan bottle. The mixture

was incubated at 400°C, and its absorbance was measured till obtaining the maximum one, with a descending value. Absorption intensity was measured by adding 50 μ L of linoleic acid previously incubated with 6 ml of ethanol 75%. The absorption was read at a wavelength of 234 nm, while ethanol 75% was used as blank solution.

Determination of Antioxidant Activity using TBA assay (Kikuzaki & Nakatami 1993). Ethanolic extract of mulberry leaf was made at different concentrations: 125, 250, 500 and 1,000 ppm. The α -tocopherol (200 ppm) was used as positive control, while distilled water was used as negative control. All of these samples were taken 1 ml, added with 2 ml of buffer phosphate (0.1 M, pH 7) and 2 ml of linoleic acid 50 mM in ethanol 99.8%.

Solution was transferred into a dark bottle with a screw cap, and incubated at 400°C for a particular period of time as determined previously. MDA level was measured by TBA method exactly 2 days after incubation. Briefly, one ml of each solution was added with 2 ml of TCA 2% and 2 ml of TBA 1% in a glacial acetic acid 50%. Distilled water was used as blank solution, with a similar procedure. The mixture was heated at 100°C for 10 minutes, cooled, and centrifuged at 3,000 rpm for 15 minutes. The absorbance was read at 532 nm wavelength using spectrophotometer.

Furthermore, standard curve was made. TMP solution was prepared at the following levels: 1.5; 3; 6; 9; 12; 15; and 18 μ M. One ml of each solution was reacted with 2 ml of TCA 20% and 2 ml of TBA 1% in a glacial acetic acid 50%. The mixture was heated at 100°C for 10 minutes, centrifuged at 3,000 rpm for 15 minutes. The absorbance of each level of TMP was spectrophotometrically measured at 532 nm. Distilled water was applied as blank solution.

Determination of α -glucosidase inhibitory activity (Sancheti et al. 2009). Inhibition against α -glucosidase (Sigma-Aldrich, Singapore) was tested, using p-nitrophenyl- α -D glucopyranoside (p-NPG) as substrate. In this experiment, substrate p-NPG was hydrolyzed by α -glucosidase to form glucose and p-nitrophenol that produces yellow color. Microplate reader was used to check color changes spectrophotometrically at 410 nm. The measurement results were then used to calculate IC₅₀, representing concentration of the extract able to inhibit 50% of enzyme activity.

Enzyme solution was made by dissolving 1 mg of α -glucosidase in 100 ml of buffer phosphate (pH 7) containing 200 mg of bovine serum albumin (BSA). Prior to use, one ml of enzyme solution was diluted 25 times using buffer phosphate (pH 7) 100 mM containing 200 mg of BSA. The reagent mixture was presented in Table 1, consisting of 25 of μ L p-NPG 20 mM as substrate, 50 μ L of buffer phosphate (pH 7) 100 mM, and 10 μ L of sample in DMSO 1% (b/v) (concentration: 10, 100, 250, 500, 1,000, 2,500, 5,000, and 10,000 μ g/ml) and 25 μ L of enzyme. The reagent was incubated at 37°C for 30 minutes, and the enzymatic reaction was stopped by adding 100 μ L of Na₂CO₃ 200 mM. Spectrophotometric measurement was used to check absorbance at wavelength of 410 nm using microplate reader.

Negative control represented the reaction between substrate and enzyme in the absence of inhibitor, while blank solution represented reaction system in absence of both enzyme and inhibitor. All experimental units were made at triplicates. The % inhibition was calculated as follows:

$$\%Inhibition = \frac{control\ absorbance - sample\ absorbance}{control\ absorbance} \times 100\%$$

Acarbose was applied as positive control, acting as inhibitor of α -glucosidase. Acarbose was dissolved in buffer solution and HCL 2 N (1:1), at various concentrations: 0.10; 0.50; 1.00; 5.00; and 10.00 ppm (each 10 μ L). The solution was reacted with reagent mixture, with similar procedure as tested samples. The absorbance was read using microplate reader at 410 nm.

Table 1. Reaction system of α -glucosidase inhibition

Solution	Blank (μ L)	Control (μ L)	S ₀ (μ L)	S ₁ (μ L)
Extract	-	-	10	10
DMSO	10	10	-	-
Buffer	75	50	75	50
Substrate	25	25	25	25
Enzyme	-	25	-	25
Incubation at 37°C 30 minutes				
Na ₂ CO ₃	100	100	100	100

Note: S₀ = control; S₁ = tested sample

Data analysis

Data were expressed as mean \pm standard deviation, and evaluated statistically using One-Way Analysis of Variance (ANOVA) in IBM SPSS Statistics 22. The significant difference among means was compared using Duncan test at $\alpha=0.05$. IC_{50} was calculated using linear regression, plotting % inhibition vs ln concentration.

RESULTS AND DISCUSSION

A previous report showed that moisture content of mulberry leaf reached $7.26\pm 0.76\%$, with a yield of 24.52%. The desirable results of experiment could be achieved due to low moisture level of the simplicia, i.e. <10% (BPOM 2014).

In this research, ethanol 65% was used as solvent since some studies suggested that ethanolic extracts of the mulberry plant is the best for significantly reducing glucose level. Ethanol 65% exhibited a higher efficiency in extraction of active compounds in mulberry leaf, approximately twice higher than aqueous solvent (Jeszka-Skowron *et al.* 2009). Further findings are also reported that ethanol and methanol at concentration of 40%–80% can isolate more polyphenol, flavonol, glycoside, and flavonoid over other solvents such as water, absolute ethanol and methanol.

Phytochemical Profile of Mulberry Leaf Extract

As presented in Table 2, the extract was confirmed to contain flavonoid, tannin and steroid, evidenced by positive result, while other compounds such as alkaloid, saponin, and alkaloid were confirmed negative.

Table 2. Results of phytochemical analysis on mulberry leaf extract

Compounds tested	Results
Alkaloid	-
Flavonoid	+
Tannin	+
Saponin	-
Steroid	+
Triterpenoid	-

Positive sign (+) indicates that the compound is present in the extract; Negative sign (-) indicates absence of the compound in the studied extract

Agustina *et al.* (2014) found that ethanolic extract of mulberry leaf was confirmed to contain quercetin and anthocyanin. In addition, the 45-days aged leaf was also known to have β -carotene at concentration of 2,04 $\mu\text{g/g}$. Another report showed that mulberry fruit possessed antioxidant properties reaching up to 86.79% based on DPPH assay (Natic *et al.* 2015). In other parts of the plant, ethanolic extract of mulberry's twigs and root barks was also confirmed to exert antioxidant and anti-tyrosine activity, in which the twig extract showed a stronger activity (Chang *et al.* 2011).

Incubation Time

Determination of optimum incubation time was based on conjugated diene method. As depicted in Figure 1, absorbance tends to contently increase. This is due to the generation of carbon radicals during propagation phase as well as continuous reactions (Allouche *et al.* 2010). The increment of absorbance from day 0 to day 4 could indicate formation of conjugated diene hydroperoxide reaching maximum level. After this period, the absorbance showed a fluctuative condition, suggesting that conjugated diene hydroperoxides began to decompose, which in turn forms malonaldehyde (MDA) as product of lipid peroxidation.

We also found that the absorbance was recorded to rise in day 6, which could be ascribed to temperature level and less stable oxygen during period of incubation. Compared to absorbance in day 4, the value in day 6 was lower; thus, incubation time was considered to reach maximum level for 4 days. Based on this finding, analysis of potential antioxidant could be carried out about 2 days after optimum production of conjugated diene hydroperoxide. This is understandable that, in day 6, most hydroperoxide compounds were decomposed to form MDA.

Formation of conjugated dienes is influenced by several factors, such as heat, light, pH, oxygen, metal ions, and lipid radicals.

Oxidation of Linoleic Acid

Figure 2 showed that the highest concentration of MDA was attributed to negative control. This is clear that MDA radicals are extensively produced due to absence of antioxidant compounds. In this case, high quantity of MDA is a result of oxidation process towards linoleic acid.

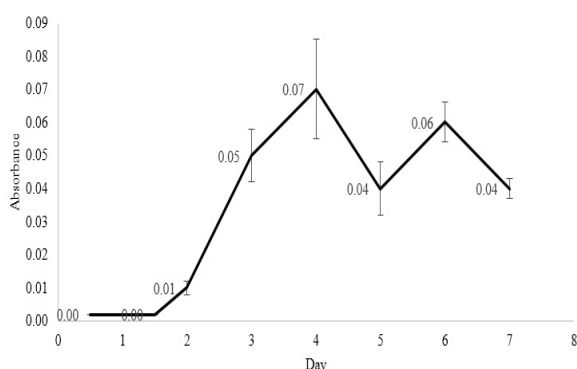


Figure 1. Changes in linoleic acid absorbance during 7 days of incubation time

The opposite result was found in group of positive control (α -tocopherol), in which the formation of MDA was significantly lower compared to other groups ($\alpha=0.05$), reaching up to 3.08 with inhibitory activity of 62.06% (exhibited in Figure 3). This discrepancy may result from some factors, such as purity and quality of α -tocopherol, as well as incubation time. However, the MDA concentration was remarkably altered by mulberry leaf ethanolic extracts. Administration of the extract at dose of 125, 200, 500, and 1,000 ppm caused a meaningful effect on level of MDA, resulting in inhibition of 41.21%; 45.33%; 44.20%; and 36.00%, respectively. Meanwhile, Alfarabi *et al.* (2010) investigated the linoleic acid inhibition generated by extract of Piper crocatum leaves at 25, 50, 75, 100, 200 ppm dose, yielding inhibi-

tion of 44.31%, 56.30%, 63.00%, 72.68% and 80.4% respectively. In this present study the % of inhibition up to 200 ppm dose of showed the highest alteration. Based on this finding, the recommended concentration of the extract was 200 ppm. However, we need to emphasize that statistical evaluation demonstrated a non significant difference in the level of MDA as well as in % inhibition among mulberry extract treatments. Additionally, our data confirmed that α -tocopherol, as positive control, was noted as the strongest inhibitor over all other groups of treatments in this experiment.

Research focusing on antioxidant properties of mulberry leaf has been discussed by other researchers. Katsube *et al.* (2009) reported the antioxidant activity of mulberry leaf ethanolic extract against oxidation of LDL. The leaf extract was isolated by ethanol 60% as solvent, in which quercetin and rutin were found as the predominant flavonol glycosides. Jezka-Skowron *et al.* (2014) revealed that mulberry leaf extracted by ethanol 65% contained an appreciable amount of total phenolic, being 20% higher than sample extracted by acetone, while also producing lower TBA value. All of these reports could enrich the scientific evidence of mulberry leaf as a source of antioxidative compounds.

Inhibitory activity against α -glucosidase

Our experiment successfully confirmed the inhibitory activity of mulberry leaf extracts

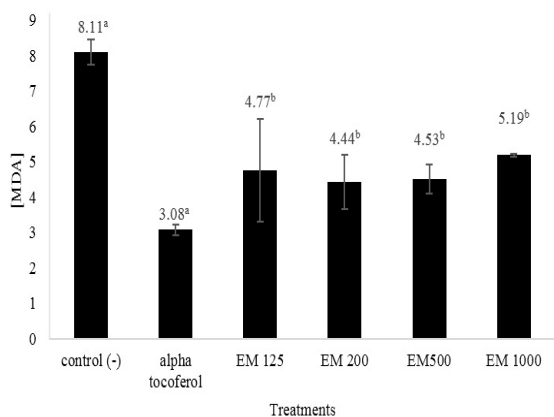


Figure 2. Effects of various treatments on MDA concentration. EM: extract ethanol mulberry. Those with different letters differ significantly at $p<0.05$

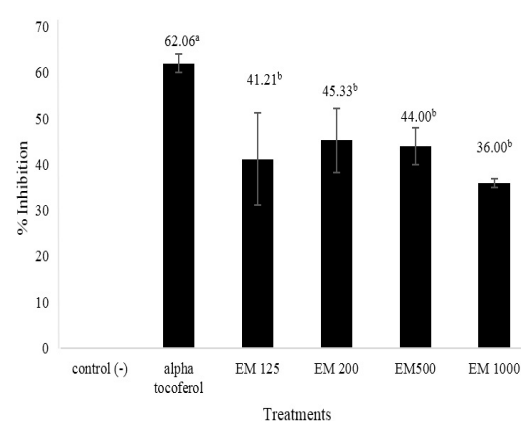


Figure 3. Effects of various treatments on % inhibition. EM: extract ethanol mulberry. Those with different letters differ significantly at $p<0.05$

towards α -glucosidase, as exhibited in Figure 4. In this case, inhibitory activity of the extract was expressed as IC_{50} , reaching up to 309.82 ± 5.72 $\mu\text{g/ml}$ (Table 3). The value of IC_{50} from mulberry leaf extracts was higher than that from acarbose (0.25 ± 0.02 $\mu\text{g/ml}$), meaning that acarbose was much stronger as inhibitor over the studied extracts.

Inhibitory properties of mulberry leaf extracts against α -glucosidase are linked to presence of bioactive compounds such as alkaloid which is present in form of 1-deoxynojirimycin (DNJ). As reported by Kwon *et al.* (2011), DNJ acts as competitive inhibitor against α -glucosidase, thereby causing the reduction of glucose level after carbohydrate intake (postprandial hyperglycemia). Another study also found that chemical structure of DNJ is identical to D-glucose; therefore, it could alleviate absorption of D-glucose in the walls of small intestine (Voss *et al.* 2007).

Regarding to phytochemicals, it is noteworthy that alkaloid is not found according to qualitative experiment, which caused the low content of DNJ. Absence of alkaloid in the studied extract could be the major reason of the weak inhibition against α -glucosidase. Besides alkaloid, flavonoid is also reported as a significant compound responsible for inhibition of α -glucosidase (Kazeem *et al.* 2013). Flavonoid compound may exist in many forms, such as rutin, quercetin, and chlorogenic acid, in which they are scientifically confirmed able to inhibit α -glucosidase (Hunyadi *et al.* 2012). Previously, *in vitro* experiments reported that flavonoid and polyphenol are bioactive compounds that enable to reduce activity of α -glucosidase in intestine and α -amylase pancreatic (Koh *et al.* 2010, Pereira *et al.* 2011).

Furthermore, inhibition of such enzymatic actions could be performed by other sources. Yilmazer-Musa *et al.* (2012) investigated the inhibition of α -glucosidase generated by extracts

of grape seed, green tea, teavigo, and white tea, yielding IC_{50} value of 1.20; 0.50; 0.30; and 2.50 $\mu\text{g/ml}$, respectively. IC_{50} of rich grape pomace extract was 1,630 $\mu\text{g/ml}$, reported by Hogan *et al.* (2010); meanwhile, Rubilar *et al.* (2011) reported IC_{50} of various extracts, such as murta (*Ugni molinae* Turcz.) leaf and fruit (215.70 and 61.30 $\mu\text{g/ml}$, respectively), and maqui (*Aristotelia chilensis*) leaf and stem (2.40 and 189.40 $\mu\text{g/ml}$, respectively). Gomathi *et al.* (2012) investigated IC_{50} of ethanolic extract of *Evolvulus alsinoides*, reaching value of 86 $\mu\text{g/ml}$. In this present work, the IC_{50} of mulberry leaf ethanolic extract was higher than that of other extracts reported. As conclusive remark, we need higher concentration of the extract enabling to generate a stronger inhibition against α -glucosidase.

CONCLUSION

Mulberry (*Morus alba* L.) leaf extract isolated by ethanol 65% could alleviate production of MDA. Some bioactive compounds (i.e. flavonoid, tannin, steroid) present in the extract are responsible for bioactivity of the extract. Moreover, the extract was also confirmed capable of producing inhibitory action against α -glucosidase, although the inhibition was lower compared to acarbose.

ACKNOWLEDGMENT

The authors would like to thank Department of Biochemistry, Faculty of Mathematics and Natural Sciences and Laboratory of Department of Food Science and Technology, Faculty of Agricultural Engineering and Technology, IPB University for research facilities. The authors have no conflict of interest.

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Table 3. Comparison of IC_{50} value between ethanolic extract and acarbose

Inhibitors	IC_{50} ($\mu\text{g/ml}$)*
Mulberry leaf extract (by ethanol 65%)	309.82 ± 5.72
Acarbose	0.25 ± 0.02

The mark * means that data are expressed as mean \pm standard deviation (n=3)

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