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Antidiabetic Potentials of Button Mushroom (*Agaricus bisporus*) on Alloxan-Induced Diabetic Rats

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Abstract

Button mushrooms (*Agaricus bisporus*) is an edible mushroom that is most widely cultivated in the world. It contains bioactive compounds that might provide beneficial effects on diabetes mellitus patient. The study aimed to determine the effects of *A. bisporus* administration on the blood glucose, and malondyaldehyd (MDA) levels as well as superoxide dismutase (SOD) activity of alloxan-induced diabetic rats. This study was also conducted to determine the secondary metabolites produced by *A. bisporus*. The method used was experimental methods with Completely Randomized Design. *A. bisporus* extract at the doses of 250, 500 and 750 mg/kg body weight (BW) per day were orally applied to alloxan-induced diabetic rats for a period of 14 days after the rats became diabetes. The results showed that the extract of *A. bisporus* could decrease blood glucose, and MDA levels as well as increase SOD activity ($p < 0.05$). *A. bisporus* extract 500 mg/kg BW is the most effective dose to be used. Based on Thin Layer Chromatography (TLC) test, it was known that secondary metabolites produced by *A. bisporus* are flavonoids, alkaloids, terpenoids and saponins. *A. bisporus* has potential as an antidiabetic through the ability to decrease blood glucose, and MDA levels, as well as increase SOD activity in diabetic rats. This research is able to provide information about the antidiabetic potential of *A. bisporus* extract so that it can be used as an alternative natural antidiabetic agent and can be applied in the community with ease and in a more controlled industrial scale.

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INTRODUCTION

Diabetes Mellitus (DM) is a chronic metabolic disorder characterized by high blood glucose (hyperglycemia) resulted from abnormality in insulin secretion produced by pancreatic β cells, action of insulin, or both of those conditions. Hyperglycemia in DM leads to free radical formation in form of Reactive Oxygen Species (ROS) which will cause a chain reaction of lipid peroxidation and produce malondialdehyde (MDA) (Robertson, 2003).

Pancreatic β cells contain endogenous antioxidants, one of them is superoxide dismutase (SOD) with fewer amounts compared to the other organs so it is very susceptible to ROS exposure and oxidative damage (Grankvist *et al.*,1981). The damage of pancreatic β cells makes the cells unable to produce insulin, so that the blood glucose levels will increase (Forbes *et al.*, 2008).

Oxidative stress can be known through the measurement of MDA which represents the number of free radicals exposed to the body and SOD that represents the endogenous antioxidant activity. The measurement of blood glucose and MDA levels along with SOD activities for diabetics need to be done to monitor the therapy as an effort to prevent the complications.

The use of long term oral antidiabetic drugs can cause side effects in the form of drug resistance and toxicity to the body, while, the administration of insulin, not only has an expensive price but also has a major side effect that is hypoglycemia. This is one of the considerations for the community to switch to the use of medicines from natural ingredients, one of them is edible mushroom. Many of the common edible mushroom species have potential properties of bioactive compounds for medicinal purpose. Several studies on edible mushroom have focused more on the potential of edible mushroom as immunomodullator and anticancer agents (Wasser, 2011; Ekowati *et al.*, 2017). However, study on the potential of edible mushroom as an antidiabetic agent has not been carried out comprehensively.

Button mushroom (*Agaricus bisporus*) is one of the most widely cultivated mushroom species in the world, which is about 38% of the total world mushroom production (Sharma *et al.*, 2015). This mushroom is very popular among Western nations and is routinely consumed by adding the mushrooms to their foods. *A. bisporus* are rich in proteins, amino acids, polyphenols, polysaccharides, ergothionins and vitamins. According to Jeong *et al.* (2010), the consumption of *A. bisporus* as a part of the daily diet is expected to reduce or prevent the risk of degenerative diseases such as cardiovascular disease and diabetes.

The purpose of this study was to determine: 1) The effect of *A. bisporus* extract administration on blood glucose and MDA levels as well as SOD activity in diabetic rats, 2) The most effective dose of extracts on decreasing the blood glucose and MDA levels as well as increasing SOD activity of diabetic rats and 3) The secondary metabolites produced by *A. bisporus*.

This research was expected to provide information about the antidiabetic potential of *A. bisporus* extract so that it can be used as an alternative antidiabetic treatment with natural ingredients and can be further developed by the pharmaceutical industry in Indonesia.

METHODS

Experimental Design

The study used experimental methods with 6 experimental groups as follow: K_S (healthy control), K_D (diabetic control), K_G (glibenclamide control), P_1 (diabetic rats with administration of 250 mg/kg BW of *A. bisporus* extract), P_2 (diabetic rats with administration of 500 mg/kg BW of *A. bisporus* extract) and P_3 (diabetic rats with administration of 750 mg/kg BW of *A. bisporus* extract). The treatment was carried out after the rats had the diabetes. The extract of 5 ml is given once a day for 14 days.

Male *Rattus novergicus* Wistar strains rats (180-230 g) were used as experimental animals. There were 5 rats in each group so that the total number of experimental animals were 30.

Extraction

Ethanol 96% was used for *A. bisporus* mushroom extraction with maceration method (1:10). The remaceration was done up to 3x24 hours. Vacuum rotary evaporator was used to evaporate the macerate.

Induction of Diabetes in Rats

Diabetes was induced in rats by intraperitonial injection of a single dose of 150 mg/kg BW (Tiwari *et al.*, 2014). After 2x24 hours, the measurement of blood glucose levels of rats was conducted, the rats were considered diabetic if their blood glucose levels were ≥ 200 mg/dL.

Blood Glucose Levels Measurement

Rats blood glucose levels were carried out by biosensor method of glucose oxidase using blood glucose test meter *GlucoDr*™. The measurements were carried out after 7 days and 14 days

administration of *A. bisporus* extract.

Biochemical Analysis

In this study, MDA and SOD measurement were made in serum. Blood samples from rats were collected in polystyrene tubes without anticoagulant.

1. Malondialdehyde (MDA) measurement

Tiobarbiturate acid (TBA) method was used in MDA levels measurement. The absorbance was read using a spectrophotometer 532 nm. The standard solution using 1.1.3.3. tetraethoxypropane which was dissolved with a concentration of 0.65 µM, 1.25 µM, 2.5 µM and 5 µM.

2.Superoxide dismutase (SOD) measurement

The measurement of SOD activity of rats was carried out using RanSOD® kit. The absorbance was read using a spectrophotometer 520 nm.

Statistical Analysis

The obtained data were initially tested by using Shapiro-Wilk test for a normality test and Levene Test for homogeneity test. Homogeneous and normally distributed data were then tested by ANOVA test followed by Post Hoc Duncan test. Inhomogeneous and not normally distributed data were tested using the Kruskal-Wallis test followed by the Mann-Whitney test.

Thin Layer Chromatography (TLC) Test

Thin Layer Chromatography (TLC) test was conducted to determine the active compound of secondary metabolites of *A. bisporus*. Silica gel G_{60} F_{254} were used as the stationary phase and chloroform: methanol (9:1) as the mobile phase.

RESULTS AND DISCUSSIONS

Simplicia of *A. bisporus* obtained from 6 kg of fresh mushrooms was 450 g. Based on extraction results, 32.49 grams of *A. bisporus* extract were obtained. The percentage of yield obtained from *A. bisporus* extraction using ethanol solvent was 7.22%. The fruit body of white button mushroom *Agaricus bisporus* (Figure 1).

The measurement of blood glucose levels in rats was carried out on 2x24 hours after alloxan induction. The result of measurement showed that groups with alloxan induction (K_D, K_G, P_1, P_2, P_3) had blood glucose levels with significantly different values compared with healthy controls (Table 1). The blood glucose levels after alloxan induction showed a value of >200 mg/dL, this indicates that those rats had diabetes. The increase of blood glucose levels accompanied by physical changes in rats in the form of weight loss and increase in urine volume (polyuria).

Figure 1. The fruit body of white button mushroom (*Agaricus bisporus*)

The alloxan induction produced diabetic rats with varying blood glucose levels, which were between 299-454 mg/dL. The variations of blood glucose levels of rats after the induction of alloxan occured due to different physiological responses. The presence of variations in pancreatic β cell damage due to alloxan induction was influenced by the resistance of each individual, so that the initial condition of blood glucose level was not uniform (Kim *et al.*, 2006; Frode and Medeiros, 2007).

Alloxan selectively damages pancreatic β cells which have a function to secrete the insulin hormone. Alloxan causes damage of ß cells through ROS production, inactivation of glucokinase and causes intracellular calcium homeostatic disturbances (Szkudelski, 2001; Walde *et al.*, 2002). The hypoglycemic effect of *A. bisporus* extract on diabetic rats was already seen on the day-8, after 7 days of treatment. *A. bisporus* extract can reduce the blood glucose levels with an effect that was comparable to glibenclamide. The better effect of *A. bisporus* extract on a decrease of blood glucose levels,can be seen after the treatment for 14 days.

The results showed that *A. bisporus* extract of 500 and 750 mg/kg BW was effective in reducing the blood glucose levels in diabetic rats with comparable effect to the one with glibenclamide. The group with *A. bisporus* doses of 250 mg/kg BW had a final blood glucose level that was not significantly different compared with the diabetes control group. It was inferred that the dose of 250 mg/kg BW extract was not enough to repair the damage of the pancreatic β cells of rats due to alloxan induction.

Control of diabetes group had increase in

Table 1.Blood glucose levels on day-0, day-8, day-15 and the percentage of reduction in blood glucose levels in the six treatment groups

Treatment	Blood Glucose Levels (mg/dL)			The percentage of reduction in blood glucose levels (%)
	Day-0 (pre)	Day-8	Day-15 (post)	
K_S	107.75± 4.91 ^{a}	112.25 ± 11.53 ^{a}	107.25 ± 4.15 ^{a}	0.34 ± 1.39 ^{a}
K_D	443.75 ± 70.19 ^{bc}	477.50 ± 112.66 ^{b}	510.25 ± 52.79 ^{c}	-21.20 ± 18.42 ^{a}
K_G	454.25 ± 42.67 ^{c}	270.00 ± 137.63 ^{a}	255.00 ± 69.87 ^{b}	43.59 ± 15.83^{b}
P_1	298.50 ± 27.79 ^{b}	257.75 ± 145.61 ^{a}	267.25 ± 75.02 ^{bc}	8.49 ± 28.28 ^{ab}
P_2	449.25 ± 51.33 ^{c}	266.00 ± 107.77 ^{a}	207.00 ± 52.59 ^{b}	56.02 ± 7.24 ^{b}
P_3	347.00 ± 91.00 ^{bc}	224.75 ± 179.77 ^{a}	179.00 ± 70.58 ^{ab}	50.88 ± 7.76 ^{b}

Description: The numbers that followed by different letters in the same column showed that the results of the test were significantly different ($p < 0.05$).

blood glucose levels of 21.20%. According to Robertson *et al.* (2004), hyperglycemia conditions in diabetic rats tended to increase ROS production. The excessive ROS compounds increased oxidative stress and caused the damage of pancreatic β cells. Therefore, it increased the blood glucose levels of the diabetes control group.

The dose of *A. bisporus* 500 mg/kg BW can provide a comparable effect with the dose of 750 mg/kg BW. This was indicated by the percentage of decrease in blood glucose levels in both groups i.e. 56.02% (500 mg/kg BW) and 50.88% (750 mg/kg BW) which statistically not significantly different. This results showed that the dose of *A. bisporus* 500 mg/kg BW is the effective dose. The similiar results was found in Yamac *et al.* (2010) which stated that the dose of *A. bisporus* 400 mg/kg BW given for 7 days was effective in reducing blood glucose levels in diabetic rats by 29.68%.

This hypoglycemic effect was caused by the active compound of *A. bisporus*. Flavonoids contained in mushroom can reduce blood glucose levels with its ability as an antioxidant. Norshazila *et al.* (2010) and Ramirez-Sanchez *et al.* (2010) stated that flavonoids work by donating H^+ electrons to superoxide anions as a free radical scavenger so that it can protect the lipoprotein, protein, and pancreatic β cells DNA from oxidation. According to Lukacinova (2008), the ability of flavonoid as radical scavenger related to its chemical structure. The presence of -OH in the B rings of flavonoid has the properties of donating H^+ electrons targeting the radical compounds. The presence of -OH and double chains on C rings of flavonoid also play a role in reducing the activity of Fe^{3+} and other metals that can catalyse the oxidation reactions of free radical reduction.

Flavonoid is a strong inhibitor of glucose transporter type 2 (GLUT2) in the intestinal mucosa. Song *et al.* (2002) stated that this condition caused a reduction in the absorption of glucose and fructose from the intestine so that the blood glucose levels will decrease. Flavonoid can increase the insulin secretion of pancreatic β cells by increasing Ca^{2+} metabolism and regenerating pancreatic β cells. The interaction with ATP-sensitive K channels on the membrane of β cells caused depolarization of the membrane and this condition open the Ca channel so that Ca^{2+} ion entered the β cells and then stimulate the granules that contained insulin resulted in insulin secretion (Wibudi *et al.*, 2008; Brahmachari, 2011).

According to Tiong *et al.* (2013), alkaloids have potential as antioxidants by inhibiting the formation of H_2O_2 which can induce the oxidative stress in pancreatic β cells. Nitrogen compounds of alkaloid play a role in the synthesis of proteins needed for the glutathione production, which is the substrate of antioxidant enzyme, GPx. Alkaloid can also increase the glucose absorption in ß cells. Alkaloid is an inhibitor of dypeptidyl peptidase-4 (DPP-4) and protein tyrosine phosphatase-1B (PTP-1B) so GLP-1 can bind back to its receptor and able to increase insulin secretion by pancreatic β cells.

Synergistic with flavonoids, terpenoids and saponins work as antioxidants by act as free radical scavengers. The result of the study by Leyva-Lopez (2017), showed that terpenoids had high antioxidant activity by acting as a radical scavenger and inhibiting the occurrence of prooxidation. According to Elekofehinti *et al.* (2012), saponins can also functionate as metal chelates, which can inhibit the occurrence of excessive Fenton reactions. Koneri (2014), added that saponin group compounds play a role in modulation of Ca^{2+} channels and pancreatic β cells rejuvenation.

Free radicals levels in rats could be seen by measuring the MDA levels. The diabetes

control group had significantly different MDA levels compared to healthy rats, with an increase in MDA levels by 522.86%. The increase of the MDA levels were caused by the high free radicals that induced lipid peroxidation, this could be used as a marker of the level of damage that occured in pancreatic β cells due to free radicals. Lipid peroxidation that occured in the pancreatic β cells phospholipid membrane cause reduction of insulin production as well as its function in decreasing glucose intake into tissues (Robertson *et al.*, 2004; Yuriska, 2009). Therefore, this caused high levels of MDA and increased levels of glucose in the blood.

Table 2. The MDA levels of the six treatment groups

Treatment	MDA (μM/mL)
K_S	0.35± 0.12 [a]
K_D	2.18 ± 1.12 [b]
K_G	1.04 ± 0.20 [a]
P_1	0.87 ± 0.35 [a]
P_2	0.73 ± 0.15 [a]
P_3	0.73 ± 0.31 [a]

Description: The numbers followed by different letters show significantly different result ($p < 0.05$).

Administration of *A. bisporus* extract based on the result of the study was able to reduce the MDA levels of diabetic rats. The MDA measurement result (Table 2) showed that the group with *A. bisporus* extract had MDA levels that were significantly different from diabetes control and not significantly different from the glibenclamide group. The decrease of MDA levels illustrated the decrease activity of lipid peroxidation in cell membranes as well as increase in pancreatic β cells damage, this was confirmed by the blood glucose levels of diabetic rats which decreased after the treatment. This result was supported by the result of Yamac *et al.* (2010) which proved that administration of *A. bisporus* extract affected the increase in the number of pancreatic β cells of diabetic rats induced by streptozotocin.

According to Liu *et al.* (2014), flavonoid is one of the active compounds that plays a role in inhibiting lipid peroxidation, so that, it can help maintaining the membrane integrity. In addition, the fruit body of *A. bisporus* is rich in ascorbic acid (vitamin C) and α tocopherol (vitamin E). The content of vitamin C and vitamin E of *A. bisporus* is 17 mg/100 g and 1-4 mg/100 g dry weight (Muszynska *et al.*, 2017). Vitamin C can protect cell membranes from lipid peroxidation by regenerating oxidized vitamin E, where vitamin E is a chain breaker of lipid peroxidation reactions (Darko *et al.*, 2002).

Agaricus bisporus extract has a better effect on reducing the MDA levels than glibenclamide, although the value is not significantly different. In the glibenclamide group, the MDA levels were still relatively high, which was 1.04 μM/mL. According to Arokiyaraj *et al.* (2008), the work of glibenclamide is more focused on stimulating insulin secretion by pancreatic β cells to reduce the blood glucose levels. In this case, glibenclamide is thought not to be able to provide a good inhibitory effect on the lipid peroxidation. The group with administration of *A. bisporus* 500 and 750 mg/kg BW gave the same MDA value of 0.73μM/mL.

The measurement of SOD activity after 14 days of treatment showed a significant difference between diabetic control rats and *A. bisporus* groups (Table 3). The diabetes control group had a significantly different SOD activity with healthy rats (29.04 μM/mL). The low SOD activity of diabetic rats was related to an increase in the amount of superoxide anion radicals in cells. If the excessive amounts of free radicals are formed, the endogenous antioxidants will decrease their activity (Halliwell and Guteridge, 2003).

Table 3. SOD activities for the six treatment groups

Treatment	SOD (U/mL)
K_S	34.67± 1.26 [a]
K_D	29.04 ± 0.18 [b]
K_G	33.49 ± 1.40 [a]
P_1	32.91 ± 0.53 [a]
P_2	32.99 ± 0.93 [a]
P_3	34.33 ± 0.62 [a]

Description: The numbers followed by different letters show the significantly different results ($p < 0.05$).

Administration with *A. bisporus* extract was proven to increase the SOD activity. The group with *A. bisporus* administration of 250, 500 and 750 mg/kg BW had SOD activity of 32.91 U/mL, 32.99 U/mL and 34.33 U/mL, respectively, which approached the condition of healthy rats (34.67 U/mL). The variety of extract doses showed a different effect that was not statistically significant. *A. bisporus* in this case acted as an exogenous antioxidant which can increase endogenous SOD activity. This was supported by Sikka (2004)

Table 4. The result of TLC test

Compounds	Reagent	Color		Rf
		Visible	UV 366 nm	
Alkaloids	Dragendorff	orange	-	0.75 0.98
Flavonoids	Sitroborat	-	Greenish-yellow, blue	0.13 0.48 0.54 0.73 0.88
Terpenoid	Vanilin-sulfurid acid	purple	-	0.31
Saponin	Libermann-Burchard	pink	-	0.44

which stated that exogenous antioxidants can enhance the defense system of endogenous antioxidants in the body so as to reduce the occurrence of oxidative stress due to excessive free radicals.

Secondary metabolites that play a role in increasing SOD activity in diabetic rats are flavonoids and terpenoids. Liu *et al.* (2014), stated that quercetin can increase the SOD activity. In addition, Huang *et al.* (2013) stated that flavonoid is able to activate SOD by increasing the expression of endogenous antioxidant genes. Leyva-Lopez (2017), stated that terpenoids also play a role in modulation of SOD activity. Moreover, the amino acid ergothionine in mushrooms also play a role in increasing SOD activity in diabetic rats.

A. bisporus has a high content of ergothionin, which is >0.93 mg/g dry weight (Chen *et al.*, 2012). It is needed in GSH formation, and ergothionin which binds to the organic cation *transporter novel type 1* (OCTN1) can protect pancreatic β cells from free radicals damage. The erghotionin-OCTN1 complex is able to pass through the cell membrane and maintain the balance of the oxidation-reduction reaction so that it can degrade free radicals quickly (Markova *et al.*, 2009).

The fruit body of *A. bisporus* has a high content of zinc (Zn). Zn content of *A. bisporus* fruit body is 7.5-15 mg/100 g dry weight (Kalac, 2010; Muszynska *et al.*, 2017). The presence of Zn makes the SOD work perfectly (Winarsi, 2017), so that the SOD activity of the group with administration of *A. bisporus* extract increased. These minerals act as SOD enzyme cofactors so that the endogenous SOD can be active again.

According to Rahmawati *et al.* (2014), vitamin C helps in reducing the amount of superoxide anion radicals and increasing SOD activity by inhibiting the protein glycation process. In addition, vitamin C can act as an exogenous antioxidant which can substitute the role of endogenous antioxidants if they are low in the body. As an antioxidant, vitamin C can directly react with superoxide anions, hydroxyl radicals, singlet oxygen and lipid peroxide. As a reducing agent, vitamin C will donate one electron H^+ to form semi-dehydroascorbate which is not reactive and then reacts to form dehydroascorbate which is unstable. The dehydroascorbate will degrade to form more stable oxalic acid and threonic acid.

The result of TLC test showed that the secondary metabolites contained in ethanol extract of *A. bisporus* mushroom are flavonoids, alkaloids, terpenoids and saponins (Table 4).

This study provides information about the potential of *A. bisporus* as antidiabetic agent, which can decrease the blood glucose and MDA levels as well as increasing SOD activity. The benefits of this reasearch results was the product of *A. bisporus* can be used as an alternative natural antidiabetic agent. Furthermore, this product can be applied in the community with ease and in a more controlled industrial scale.

CONCLUSION

A. bisporus administration at the dose of 250, 500 and 750 mg/kg BW have an effect on decreasing the blood glucose and MDA levels as well as increasing SOD activity in diabetic rats, with the most effective dose is 500 mg/kg BW. Secondary metabolites produced by *A. bisporus* are flavonoids, alkaloids, terpenoids and saponins.

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