Antidiabetic Effects and Antioxidant Properties of the Saggy Ink Cap Medicinal Mushroom, *Coprinus comatus* (Agaricomycetes), in Streptozotocin-Induced Hyperglycemic Rats

Fajar Husen,[a] Hernayanti Hernayanti,[a] Nuraeni Ekowati,[a] Dalia Sukmawati,[b] & Nuniek Ina Ratnaningtyas[a,*]

[a]Biology Department, Faculty of Biology, Universitas Jenderal Soedirman, Karangwangkal, Banyumas 53122, Indonesia; [b]Biology Department, Universitas Negeri Jakarta, Rawamangun, Pulo Gadung, Jakarta 13220, Indonesia

*Address all correspondence to: Nuniek Ina Ratnaningtyas, Biology Department, Faculty of Biology, Universitas Jenderal Soedirman, Jalan Dr. Suparno 63, Karangwangkal, Banyumas 53122, Indonesia; Tel.: +62812-2702-826; Fax: +62 281-638794, E-mail: nuniek165@yahoo.com

ABSTRACT: *Coprinus comatus* is known for its antihyperglycemic benefits. This study aimed to identify the effect of bioactive compounds of *C. comatus* extract as an antidiabetic agent linked to glucagon-like peptide 1 (GLP-1) and antioxidant properties in increasing glutathione (GSH) levels. This study used six groups of Wistar rats (n = 24). Group 1 comprised the healthy control. Groups 2–6 received 45 mg of streptozotocin/kg body weight (BW) once. Group 3 was also given 45 mg of metformin/kg BW, whereas groups 4–6 were also given 250, 500, and 750 mg of *C. comatus* ethyl acetate extract/kg BW for 14 days. Antidiabetic effects of alkaloids and saponin were seen in blood glucose and glycated hemoglobin (HbA1c) degradation, increased insulin, and increased inhibition of GLP-1 through dipeptidyl peptidase-4 activity. Flavonoid antioxidants, ascorbic acid (vitamin C), and α-tocopherol (vitamin E) are useful in protecting pancreatic β cells from free radicals. Data were analyzed using analysis of variance and Duncan's multiple range test. *C. comatus* ethyl acetate extract at doses of 250, 500, and 750 mg/kg BW worked as an antidiabetic and antioxidant agent that contained flavonoids (16.4 mg/L), alkaloids (2.97 mg/L), saponin, rutin (351.13 ppm), vitamin C (132.342 mg/L), and vitamin E (102.320 g/L). The 250-mg dose was effective in increasing insulin (8.11 mlU/mL) and reducing blood glucose (23.92%) and HbA1c (3.775%), whereas the 500-mg dose was effective in increasing levels of GLP-1 (1056.923 ng/L) and GSH (4.62 µmol/L).

KEY WORDS: *Coprinus comatus*, diabetes mellitus, GLP-1, hyperglycemia, medicinal mushrooms

ABBREVIATIONS: AGE, advanced-glycation end product; **DM**, diabetes mellitus; **DPP-4**, dipeptidyl peptidase-4; **ELISA**, enzyme-linked immunosorbent assay; **GLP-1**, glucagon-like peptide 1; **GLUT**, glucose transporter; **GPx**, glutathione peroxidase; **GSH**, glutathione; **GSSG**, glutathione disulfide; **HbA1c**, glycated hemoglobin; **HC**, healthy control; **HPLC**, high-performance liquid chromatography; **iNOS**, inducible nitric oxide synthase; **NC**, negative control; **NEG**, nonenzymatic glycation; **NO**, nitric oxide; **NOS**, nitric oxide synthase; **•OH**, hydroxyl anion; **OH^-**, hydroxyl radical; **$ONOO^-$**, peroxynitrite; **PC**, positive control; **ROS**, reactive oxygen species; **STZ**, streptozotocin; **T1**, treatment group 1 (given 250 mg extract); **T2**, treatment group 2 (given 500 mg extract); **T3**, treatment group 2 (given 750 mg extract); **WHO**, World Health Organization

I. INTRODUCTION

Diabetes mellitus (DM) is a metabolic disorder that is linked to carbohydrate metabolism disorders and to degradation of insulin production and sensitivity, or both.[1] In Indonesia, the number of patients with DM was 7 million in 2009 and the International Diabetes Federation estimates this will reach 12 million in 2030.[2] The worldwide prevalence of DM is continuously increasing. The prevalence of DM in adults aged 20–79 years was 6.4% (285 million) in 2010 and is predicted to increase to 7.7% (429 million) in 2030. The Ministry of Health of the Republic of Indonesia considers DM a disease of public health, and the prevalence of DM is estimated to reach 21.3 million by 2030.[3] This significant increase in prevalence motivates scientists to find an effective option in providing DM treatment and therapy through herbal medicine, as recommended by World Health Organization (WHO).[2] The American Diabetes Association and WHO have

1521-9437/21/$35.00

recommended herbal medication as an alternative to synthetic drugs.[1] In this study, herbal medication refers to extract of the shaggy ink cap mushroom, *Coprinus comatus* (O. F. Mull.) Pers. (Agaricaceae, Agaricomycetes), which is expected to have antihyperglycemic effects by increasing glucagon-like peptide 1 (GLP-1) hormone levels and bioactive compounds such as antioxidants.[4]

C. comatus grows in the tropical forest, utilizing decomposed organic forest wastes (leaves, twigs, and weathered logs) as substrate and available nutrients.[5] This mushroom is also known to have medicinal potential because it contains bioactive compounds (e.g., comatin) that lower blood sugar.[6] A recent study showed that comatin and ergothioneine are found in *C. comatus* ethanol extract, with potential to act as immunomodulatory, hypolipidemic, hypoglycemic, anticancer, and antioxidant agents.[5] Consumption of synthetic drugs such as metformin often causes side effects, especially with long-term use. Insulin is expensive and less effective for long-term use.[7] Thus, the development of herbal medicines (e.g., the use of *C. comatus* here) is essential.[8]

A recent study compared the ethanol extract, alkali-soluble polysaccharide fraction, protein fraction, and crude fiber fraction of the *C. comatus* fruiting body and found that the polysaccharide fraction achieves the best results in lowering glucose blood levels.[8] Several studies developed in China report that *C. comatus* extract can lower blood glucose levels in DM.[9] For example, *C. comatus* ethanol extract at a dose of 500 mg/kg body weight (BW) lowered blood sugar levels from 331.2 to 100.5 mg/dL (69.65%) after 14 treatments.[10] Comatin works as an antidiabetic agent. An experiment using *C. comatus* ethanol extract at a dose of 80 mg/kg BW lowered the blood glucose concentration from 92.52 to 77.04 mg/dL (16.73%) within 3 h.[11]

GLP-1 is incretin hormone in the intestine that works as insulinotropic agent (i.e., stimulates insulin production). In the normal state, GLP-1 is degraded immediately by the dipeptidyl peptidase-4 (DPP-4) enzyme, and only 65% can reach its receptor in pancreatic β cells. Inhibition of DPP-4 is needed to prevent its rapid degradation of GLP-1 in DM conditions. Thus, *C. comatus* extract containing comatin and alkaloids is essential. Comatin can inhibit DPP-4 from degrading GLP-1. Alkaloids can also work as DPP-4 inhibitors to prevent GLP-1 degradation.[11] Hyperglycemia during DM stimulates the formation of reactive oxygen species (ROS), especially in the form of superoxide anions (O_2^-), resulting in a chain reaction of damaged lipid peroxide that begins from cells to tissues and then to organs. *C. comatus* extract containing vitamin C, vitamin E, and flavonoids can resolve pancreatic β-cell damage. Flavonoids in the form of rutin compounds can donate H^+ and neutralize free radicals, and they also stop the lipid peroxidation that damages the pancreas. Vitamins C and E in *C. comatus* ethyl acetate extract work as antioxidants. Vitamin C can donate H^+ to O_2^- and convert it to hydrogen peroxide, nonreactive monodehydroascorbic acid, or dehydroascorbic acid. This change can also be reversed into ascorbic acid by the monodehydroascorbate reductase and dehydroascorbate reductase enzymes. Vitamin E is fat soluble and can maintain cell membrane integrity and complexity in preserving cells from free radical attack. Vitamin E contains an OH group that functions similarly to vitamin C as an H^+ donor.[12] The imbalance between free radicals formed in DM to the small amount of endogenous antioxidants in pancreatic β cells could cause oxidative stress or lead to lipid peroxidation and cell necrosis. Endogenous antioxidants, such as glutathione peroxidase (GPx), cannot handle the formed free radicals. Thus, an exogenous antioxidant in *C. comatus* ethyl acetate extract (e.g., flavonoid, rutin, vitamin C, or vitamin E) is needed to increase protection. As a result, insulin production by pancreatic β cells remains uninterrupted.[13]

Improvement is needed in the development of herbal medicines as an alternative treatment for DM in Indonesia, especially the use of *C. comatus* cultivated in Cianjur, Indonesia. This study aimed to identify the effectiveness of *C. comatus* ethyl acetate extract as an antidiabetic agent by increasing insulin and GLP-1 levels and lowering blood glucose and glycated hemoglobin (HbA1c). This study also aimed to determine the effect of increasing glutathione (GSH) levels as an antioxidant. Studies of *C. comatus* as an antidiabetic agent have been conducted; however, the effects of endogenous antioxidant levels on GLP-1 levels in DM still need to be evaluated. Herbal medicines could be further developed, especially those that act as antioxidant supplements in addition to antidiabetic agents. This study hypothesized that a 500-mg/kg BW dose of

C. comatus ethyl acetate extract could lower blood glucose and HbA1c levels and increase insulin, GLP-1, and GSH levels.

II. MATERIALS AND METHODS

A. Materials

C. comatus mushrooms obtained and utilized in this research were cultivated by commanditaire venootschap (CV), Asa Agro Corporation located in Cianjur, Indonesia, according to a previous study.[10] Wistar rats were obtained from the animal breeding house of usaha dagang (UD) in Bantul, Yogyakarta, Indonesia. The analysis was conducted using a GlucoDr glucometer and an HbA1c kit (BioHermes glycohemoglobin test kit), a plasma insulin kit, and a GLP-1 hormone kit (BT Laboratories, Shanghai, China). This study was conducted in mycology and phytopathology laboratories (Universitas Jenderal Soedirman) and plant physiology laboratory of biology faculty (UNSOED), pharmacology laboratory and veterinary house of sciences faculty, research laboratory of medical faculty, and integrated research laboratory of medical faculty, as well as the Universitas Jenderal Soedirman (UNSOED) in Purwokerto, Indonesia, and the Universitas Diponegoro integrated research laboratory in Semarang, Indonesia.

B. *C. comatus* Ethyl Acetate Extract

Up to 1.5 kg of *C. comatus* fruiting body was cut and dried in an oven at a temperature of 40–45°C. The dried fruiting body was blended and macerated with 1000 mL of absolute ethyl concentration solvent at the ratio of 1:5, 1:3, and 1:2. The macerate was then filtered with a Millipore vacuum and mixed and evaporated with a rotary evaporator vacuum to obtain a thick extract.[10]

C. Qualitative and Quantitative Analysis of Bioactive Compounds of *C. comatus* Ethyl Acetate Extract

Qualitative analysis of total flavonoids was conducted using amyl alcohol reagent Mg + HCl, alkaloids of Dragendorff color reagent, and saponin with aquadest and boiling treatment.[14] For quantitative analysis of total flavonoids and alkaloids, spectrophotometry at a wavelength of 510–530 nm was used.[15] Specific identification with high-performance liquid chromatography (HPLC) was done qualitatively and quantitatively in a phase C-18 column (Develosil ODS-UG-3). 25% acetonitrile was the organic solvent used for mobile phases in HPLC analysis.[16]

D. Streptozotocin Induction Method

The rats were acclimated for 14 days and then their body weight was measured. Food and water were given to rats through *ad libitum* access during the acclimation period. Streptozotocin (STZ) induction was done through intraperitoneal injection by using STZ diluted into a 2.5-mL solution with 0.5 M (pH 4.5) of citrate buffer at a dose of 45 mg/kg BW (based on dose conversion to rats).[17] STZ induction was done on all treatment groups, except the HC group.

E. Animal Treatment

Twenty-four male Wistar rats were divided into six groups: healthy control (HC; without treatment), negative control (NC; induction with 45 mg of STZ), positive control (PC; given 45 mg of metformin/kg BW),

treatment group 1 (T1; given 250 mg of ethyl acetate extract/kg BW), treatment group 2 (T2; given 500 mg of ethyl acetate extract/kg BW), and treatment group 3 (T3; given 750 mg of ethyl acetate extract/kg BW). *C. comatus* ethanol extract and metformin were given orally at day 5 after STZ induction. The extract was given for 15 days.

F. Sample Collection

Before blood samples were collected in the animal cage, all rats were fasted for 8–10 h. Initial blood glucose was taken from the rats' lateral veins at day 4 after STZ induction and directly dripped onto glucose strips. At day 15, blood was taken from the orbital veins for use in the measurement of final glucose, insulin, GLP-1, HbA1c, and GSH levels.

G. Measurement of Main Parameters

Measurement of the main parameters used blood serum samples taken at day 15 after treatment. Blood glucose was measured using a GlucoDr glucometer. Plasma insulin and GLP-1 hormone were measured using the enzyme-linked immunosorbent assay (ELISA; with the rat insulin procedure and ELISA GLP-1 kit from BT Laboratories).[18] HbA1c was measured with the glycohemoglobin analyzer method using the AICEZ 2.0 procedure (BioHermes glycohemoglobin test kit). GSH was measured using the Ellman method based on 5-5′-dithiobis-2-nitrobenzoic acid reaction.

H. Statistical Analysis

Data obtained from all parameters are presented as means ± SD. The statistical comparison was carried out using analysis of variance. Differences among the means were determined through Duncan's multiple range test at a significance level of $P \leq 0.05$.

III. RESULTS AND DISCUSSION

A. Qualitative and Quantitative Analysis of *C. comatus* Extract

Extraction of 500 g of *C. comatus* fruiting body powder with 2000 mL of ethyl acetate solvent resulted in 4.5 g of thick extract. The results of qualitative and quantitative identification with spectrophotometry are shown in Table 1.

Qualitative analysis of the bioactive compound content of *C. comatus* ethyl acetate extract showed similar results to the prior research, which demonstrated the presence of flavonoids, alkaloids, and saponin.[10] The prior study assessed *C. comatus* by using HPLC. The results showed that extract of *C. comatus*

TABLE 1: Analysis and identification of bioactive compounds

No.	Bioactive compound	Qualitative result*	Quantitative result, mg/L (%)
1	Flavonoids	Reddish yellow (++)	16.40 (32.8)
2	Alkaloids	Dark orange (+)	2.97 (5.94)
3	Saponins	Formed foam (+)	—

The dash indicates that it is not quantitatively analyzed.
*Plus signs indicate the following: + (low) and ++ (middle). Tested with spectrophotometry of 510 nm (flavonoid) and 530 nm (alkaloid).

mushroom fruiting body contained 26.80 μg of quercetin flavonoid compounds and 0.77 μg of rutin.[19] Although HPLC results for the *C. comatus* ethyl acetate extract detected rutin compounds (351.133 ppm), quercetin was not detected here. The difference in detected compounds was influenced by the solvent used (Table 2).[10] This study used ethyl acetate with semi-polar solvent properties. Semi-polar solvents can attract polar and slightly nonpolar substances or compounds in the extraction process. The prior study used ethanol solvent, which is included in polar solvent; hence, the affinity to attract polar and nonpolar substances and compounds was larger than that of a semi-polar solvent like ethyl acetate. Ethanol is categorized as a strong polar solvent.[20] The bioactive compounds identified by HPLC are presented in Table 2.

Rutin is a flavonoid (polyphenol) with several OH groups in its chemical structure. The activity and reaction of rutin in neutralizing free radicals is equal to that of quercetin.[21] Rutin works as an ion H^+ donor on free radicals such as quercetin, thus preventing the cell membrane from forming lipid peroxidation reactions. Rutin contains many OH groups and could work as an electron H^+ and quercetin donor.[21] Rutin works as a lipid peroxidation reaction inhibitor of the cell membrane and protects the cell membrane from damage.[22]

B. Blood Glucose Level

In a study by Firdaus et al., male rats weighing 120–140 g and induced with streptozotocin (40 mg/kg BW) were categorized as hyperglycemic and had a fasting blood sugar level > 126 mg/dL within 4 days after induction.[23] Prior studies used male rats weighing 150 g that were induced with streptozotocin (40 mg/kg BW), and their results showed a mean blood glucose level of 189.92 ± 5.27 mg/dL 48 h after STZ induction in all groups.[3] In this study, blood glucose levels with the 45-mg/kg BW dose changed in 4 days after induction with > 150 mg/kg BW, indicating that the rats were hyperglycemic. Blood glucose levels are presented in Table 3.

Blood glucose levels of the NC group increased to 49.91% (Table 3), thus indicating that glucose metabolism was inhibited due to STZ damage of pancreatic β cells. STZ caused pancreatic β-cell damage and hyperglycemia, which increased free radical production. Increased free radicals, especially ROS, damaged the cell membrane, protein, and DNA. As a result, insulin production by Langerhans pancreatic β cells was disturbed.[24] Group T1 had the highest mean initial blood pressure, whereas the HC group had the lowest blood pressure (Table 3). The increased blood glucose level in the NC group was affected because STZ formed highly reactive free radicals that could damage the cell membrane, protein, and DNA, thus disrupting insulin production by pancreatic β cells. Streptozotocin entered the Langerhans pancreatic β cells through glucose transporter 2 (GLUT-2) and caused alkylation. This was initiated through the limitation of adenosine triphosphate formation in mitochondria due to the formation of free radicals, increased xanthine oxidase, and inhibition in the Krebs cycle.[25]

TABLE 2: HPLC analysis results

No.	Target compound	Detected compound	Retention time*	Concentration
1	Flavonoids (quercetin and rutin)	Flavonoids: Rutin and quercetin (ND)	3.517	351.133 ppm
2	Vitamin C	Ascorbic acid	1.856	132.342 mg/L
3	Vitamin E (α-tocopherol)	*α*-tocopherol	2.731	102.320 g/L

ND, not detected.

*Flavonoid standard (quercetin): 4.334 (100 ppm); flavonoids (rutin): 3.682 (100 ppm); vitamin C: 1.839 (500 mg/L); and vitamin E (α-tocopherol): 2.844 (150 g/L).

TABLE 3: Blood glucose levels

No.	Treatment group	Blood glucose level, mg/dL	
		Initial*	Final†
1	HC	96.75 ± 6.49[a]	109.75 ± 4.60[a]
2	NC	157.50 ± 17.75[ab]	236.11 ± 108.05[b]
3	PC	155.75 ± 21.68[ab]	142.75 ± 10.91[a]
4	T1	186.67 ± 40.4[b]	142.00 ± 10.95[a]
5	T2	157.80 ± 6.91[ab]	142.00 ± 6.53[a]
6	T3	159.57 ± 13.08[ab]	140.40 ± 13.69[a]

Data are presented as means ± SD (n = 4). Scores marked with the same letter in the same column are not significantly different ($P \leq 0.05$). HC, healthy control; NC, negative control (induction with 45 mg of STZ); PC, positive control (45 mg of metformin); T1, administration of 250 mg of *Coprinus comatus* ethyl acetate extract; T2, administration of 500 mg of *C. comatus* ethyl acetate extract; and T3, administration of 750 mg of *C. comatus* ethyl acetate extract.

*Blood glucose level by day 4 after STZ induction.

†Blood glucose level by day 15 after STZ induction.

Initial blood glucose levels were higher in the T1 group than in the T2 and T3 groups. Blood glucose was measured by day 4 after STZ induction. Differences in glucose levels occurred due to the differing physiological response of each animal. Although the animals were given the same food and treatment, each animal showed a different response. Some rats showed more active movement and eating responses compared to the others. Responses after STZ induction would go directly to the pancreatic β cells with the GLUT-4 mechanism; STZ led to severe degeneration, necrosis, and degranulation in the β cells of the pancreatic islets, causing an increase in serum glucose levels.[26] The conditions in which STZ affected β-cell damage and caused increased blood pressure in rats depended on the physiology, biochemistry, and immunity of each rat. Biochemical conditions caused by increasing glycoxidation reactions of glucose generate ROS such as superoxide, hydrogen peroxide, and hydroxyl radicals; these accelerate the formation of advanced glycosylation end-products (AGEs), which in turn generate more free radicals and can damage pancreatic β cells.[27] Although this results in the same effect of increasing blood glucose levels, the immunity and antioxidant levels of each rat are different.[28] These factors significantly affected the difference in blood glucose levels between groups T2 and T3. STZ stimulated nitric oxide synthase (NOS) syndrome (i.e., inducible NOS [iNOS]) to form nitric oxide (NO^-) free radicals. The reaction between O_2^- and NO^- formed, and the peroxynitrite ($ONOO^-$) radical was toxic to endothelial NOS. Hyperglycemic rats contained > 310.20 μg/mL NO^- produced by iNOS, compared to 108.2 μg/mL for healthy rats,[29] which caused blood glucose to increase further. Free radical formation occurred continually and caused oxidative stress; therefore, it affected the increase of blood glucose since pancreatic β cells were damaged by lipid peroxide.[24]

A significant decrease in glucose levels occurred in rats given *C. comatus* ethyl acetate extract at all doses. Various doses of *C. comatus* ethyl acetate extract decreased blood glucose levels by 23.92% (250 mg/kg BW), 10.01% (500 mg/kg BW), and 12.01% (750 mg/kg BW). Metformin was used as an oral diabetes treatment in the PC group and decreased blood glucose levels by 9.63% (Table 3). The decrease in blood glucose with the highest presentation in group T1 was affected by the high increase in insulin in that group, which was 8.11 mlU/mL compared to group T1 or T3 (Table 4). This occurred because the insulin hormone in DM tended to be low and caused high blood glucose levels.

C. comatus ethyl acetate extract at a dose of 250 mg/kg BW was given to hyperglycemic rats induced with alloxan, and it decreased blood glucose levels by 17.05% (from 331.25 to 274.75 mg/dL).[10] The decrease in blood glucose levels by *C. comatus* ethyl acetate extract was made possible by bioactive compounds such as alkaloids, which activate in GLUT-4 in peripheral tissue or muscle to maximize glucose

TABLE 4: Insulin hormone levels

No.	Experimental group	Insulin hormone level, mlU/mL
1	HC	8.20 ± 0.463^{b}
2	NC	6.85 ± 0.199^{a}
3	PC	7.96 ± 0.418^{b}
4	T1	8.11 ± 0.774^{b}
5	T2	7.82 ± 0.653^{b}
6	T3	7.59 ± 0.110^{ab}

Data are presented as means ± SD ($n = 4$). Scores marked with the same letter in the same column are not significantly different ($P \leq 0.05$). HC, healthy control; NC, negative control (induction with 45 mg of STZ); PC, positive control (45 mg of metformin); T1, administration of 250 mg of *Coprinus comatus* ethyl acetate extract; T2, administration of 500 mg of *C. comatus* ethyl acetate extract; and T3, administration of 750 mg of *C. comatus* ethyl acetate extract.

conversion. Results of qualitative and quantitative analysis showed that *C. comatus* ethyl acetate extract contained polysaccharides that could decrease blood glucose levels by 42.78%.[30] The extract contained flavonoids that could work as antioxidants to protect pancreatic β cells,[10] and saponin played a role in adenosine monophosphate kinase activity to boost glucose catabolism reaction.[31]

The decrease in blood glucose levels after administration of *C. comatus* ethyl acetate extract was possible because of the role of flavonoids in the form of rutin and quercetin.[19] According to a recent study, quercetin can inhibit the excessive activity of GLUT-2 in absorbing glucose in the intestinal mucosa of experimental animals.[32] Control of GLUT-2 activity by quercetin is essential because it can optimize glucose that is only required by the body; hence, there will not be an excessive buildup that has the potential to increase blood glucose. Polysaccharides from extract of the *C. comatus* fruiting body could inhibit nonenzymatic glycosylation (NEG).[28] NEG is the reaction between glucose and amino groups of protein that produce AGEs. Inhibition of the NEG reaction could decrease AGE formation that might increase toxic dicarbonyl compounds, which leads to free radical formation. A prior study showed that NEG inhibition could decrease the blood glucose concentration.[33] Alkaloids also work as an antidiabetic agent by controlling the mechanism of GLUT-2 in the intestine and preventing excessive glucose absorption. Alkaloids could increase GLUT-4 activity in muscle and in liver tissues. The referred excessive glucose could immediately be stored in other forms (glycogen or fat) in muscle or liver through GLUT-4 regulation, in which performance is improved by alkaloids.[34]

C. Insulin Hormone

The increase of free radicals allows damage and degeneration of pancreatic β cells, leading to decreased insulin levels. Insulin production and release also depends on the stimulation of GLP-1. The NC group had the lowest insulin levels, whereas the HC group had the highest (Table 4). Insulin hormone levels are presented in Table 4.

Membrane lipid peroxidation occurs when ROS react to polyunsaturated fatty acids that might cause cell leakage, necrosis, and cell death.[35] Nitric oxide has a significant effect because of its interaction with other free radicals like superoxide anion (O_2^-), which can form additional peroxynitrite ($ONOO^-$) that is more cytotoxic to the cell membrane. Pancreatic β-cell damage occurs because ROS are formed in a large quantity (not controlled), exceeding the capacity of endogenous antioxidants; thus, the cells could cause necrosis.[36] The pancreatic β-cell membrane will attack easily and has lipid peroxidation capability, which will further interfere with insulin production. This may have occurred in the NC rats, as their insulin level was very low (< 7 mlU/mL).

STZ-induced hyperglycemic rats had the lowest insulin level (< 7 mlU/mL) compared with their normal level of 7 mlU/mL (in the fasting state); the normal insulin level is > 7–10 mlU/mL.[37] Increased levels of insulin in the treatment groups by administration of *C. comatus* ethyl acetate extract could occur due to the presence of flavonoid compounds that donated H^+, thus neutralizing free radicals and stopping the lipid peroxidation that damaged the pancreas.[38]

Flavonoids work as antioxidants, offering protection against free radicals that could attack pancreatic β cells. Some flavonoids act in the modulation and signaling activity of pancreatic β cells in insulin hormone stimulation and release.[39] *C. comatus* polysaccharides could also stimulate insulin release from pancreatic β cells directly with the help of Ca^{2+}.[30] The antioxidative properties of flavonoids, vitamin C, and vitamin E that inhibit polyADP-ribosylation can weaken STZ toxicity on pancreatic β cells; hence, this inhibition prevents DNA damage by STZ.[24]

This study indicates that inactivation and degradation by the DPP-4 enzyme against GLP-1 in DM produces metabolites in the form of GLP-1 (9-36) amide, which cannot stimulate insulin formation and production.[40] GLP-1 that is unbound to the GLP-1 receptor on the surface of pancreatic β cells could cause decreased insulin levels and increased blood glucose levels.[41]

D. GLP-1 Level

The administration of *C. comatus* ethyl acetate extract varied in the experimental groups. The HC and T2 groups had the highest GLP-1 level, whereas the NC group had the lowest (Table 5). GLP-1 levels are presented in Table 5.

All groups of hyperglycemic rats that were given *C. comatus* ethyl acetate extract showed positive results (significantly different), and each group experienced an increase in GLP-1 hormone levels.

GLP-1 was measured in this study to determine 1) the effectiveness of bioactive compounds of *C. comatus* ethyl acetate extract, 2) the regulation of glucose metabolism involving GLP-1 hormone and insulin, and 3) the function of GLP-1 in regulating blood glucose levels of the gastrointestinal tract and its role as an insulinotropic (insulin release stimulator).[40]

GLP-1 hormone degradation by DDP-4 in hyperglycemic rats occurred in < 2 min, which could decrease GLP-1 levels and affect decreased insulin levels and increased blood glucose levels.[42] The GLP-1 level of the NC group was < 450 ng/L (Table 5).

Different results were shown for hyperglycemic rats administered *C. comatus* ethyl acetate extract in groups T1, T2, and T3 where GLP-1 was high. Group T2 had the highest GLP-1 level compared to the

TABLE 5: Glucagon-like peptide 1 hormone levels

No.	Experimental group	GLP-1 hormone level, ng/L
1	HC	1052.360 ± 199.930^{c}
2	NC	404.002 ± 214.345^{a}
3	PC	555.712 ± 341.968^{ab}
4	T1	510.063 ± 249.786^{a}
5	T2	1056.923 ± 176.728^{c}
6	T3	950.649 ± 217.816^{bc}

Data are presented as means ± SD ($n = 4$). Scores marked with the same letter in the same column are not significantly different ($P \leq 0.05$). HC, healthy control; NC, negative control (induction with 45 mg of STZ); PC, positive control (45 mg of metformin); T1, administration of 250 mg of *Coprinus comatus* ethyl acetate extract; T2, administration of 500 mg of *C. comatus* ethyl acetate extract; and T3, administration of 750 mg of *C. comatus* ethyl acetate extract.

others. Although group T3 was given a high dose of *C. comatus* ethyl acetate extract (750 mg), its GLP-1 levels were lower than those of group T2. The difference in GLP-1 hormone levels was highly affected by the rats' physiological response. The alkaloids and comatin in the *C. comatus* ethyl acetate extract worked as DPP-4 inhibitors and prevented GLP-1 acceleration by DPP-4. DPP-4 inhibition by alkaloids affected increased GLP-1 activity.[43] The GLP-1 level of the T3 group was normal (> 450 ng/L), which was slightly lower than that for the T2 group. Dose differences for each experimental animal were significantly affected by various internal factors such as DDP-4 enzyme level, bioactive compound level on each dose, and the animal's immune and physiological responses.[11]

GLP-1 activity occurs when luminal substrate (food) enters the intestine. GLP-1 can directly break down carbohydrates, which might attach to the GLP-1 receptor on the surface of pancreatic β cells to stimulate insulin secretion. GLP-1 activity that is directly degraded by DPP-4 could result in decreased insulin production.[44] GLP-1 decomposition by DPP-4 is inhibited by the comatin compounds in the extract of the *C. comatus* fruiting body.[6]

Comatin binds to DPP-4 and causes DPP-4 to be inactive.[40] Comatin can work as a DPP-4 inhibitor by inhibiting DPP-4 binding to GLP-1. Comatin also binds to the active sides of GLP-1, so DPP-4 cannot further bind GLP-1; thus, the effects of DPP-4 inactivation and degradation on GLP-1 can be minimized.[29]

E. HbA1c

HbA1c is the bond between hemoglobin and glucose, which is also referred to as glycated hemoglobin or hemoglobin A1c, and is the mean blood sugar level bound to hemoglobin. The results of this study showed that the increase in blood glucose levels was related to the increase in HbA1c levels (Fig. 1). HbA1c levels are presented in Fig. 1.

High and low levels of HbA1c also depend on the blood glucose level in the body. Hence, it is crucial to monitor HbA1c parameters in the hyperglycemic state. HbA1c levels in the experimental treatment groups given *C. comatus* ethyl acetate extract (T1, 3.77%; T2, 3.94%; and T3, 3.9%) were similar to the HC group (3.9%). The mean HbA1c rate of the three groups given the extract was 3.85%. The HbA1c level of the NC group was 4.77%, which was the highest among all groups because the rats were not given the extract or metformin (Fig. 1).

HbA1c levels in NC rats were > 4.5%, whereas the normal level was 3.5%–4.5% in the fasting state.[45] The increase in hemoglobin concentration was significantly glycolyzed, along with the increased blood glucose

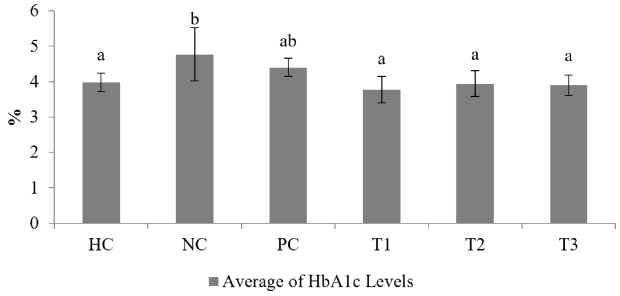

FIG. 1: HbA1cs level. Bars marked with the same letter are not significantly different ($P \leq 0.05$). HC, healthy control; NC, negative control (induction with 45 mg of STZ); PC, positive control (45 mg of metformin); T1, administration of 250 mg of *Coprinus comatus* ethyl acetate extract; T2, administration of 500 mg of *C. comatus* ethyl acetate extract; and T3, administration of 750 mg of *C. comatus* ethyl acetate extract.

concentration in the body of patients with DM.[46] A constantly increasing blood glucose level increases the oxidative reaction of hemoglobin, resulting in increased HbA1c and blood glucose levels of patients with diabetes.[47]

The fungi interconnectedness of *C. comatus* bioactive compounds (e.g., flavonoids, alkaloids, saponin, vitamin C, and vitamin E) worked synergistically in the HbA1c reduction effect that occurred in the treatment groups. STZ-induced rats had hyperglycemia that could increase free radical production. Flavonoids protect pancreatic β cells that contain a few endogenous antioxidants. The decrease in blood glucose levels in treatment groups given *C. comatus* ethyl acetate extract (Table 3) was correlated with the decrease in HbA1c levels. Antioxidants such as flavonoids, vitamin C, and vitamin E can prevent hemoglobin oxidation as blood glucose increases in DM.[13]

Flavonoids protect β cells from free radicals by neutralizing free radicals formed in DM by donating H^+ ions to free radicals and forming a nonreactive flavonoid radical. Flavonoids can prevent lipid peroxidation from pancreatic β-membrane cells and allow β cells to produce the insulin hormone to regulate glucose in the body.

F. GSH

GSH is an intracellular antioxidant and cosubstrate of GPx, and it is instrumental in increasing the body's defenses against the increase of free radicals in DM. GSH blood measurements of hyperglycemic rats given *C. comatus* ethyl acetate extract and metformin showed various results between each treatment group as follows (Fig. 2). GSH levels are presented in Fig. 2.

The NC group had the lowest GSH value (< 4 µmol/L), whereas the HC group had the highest. GSH levels among groups given *C. comatus* ethyl acetate extract and metformin had GSH levels > 4 µmol/L compared with 4.4 µmol/L for group T1, 4.62 µmol/L for T2, and 4.21 µmol/L for T3. The PC group had a GSH value of 5.23 µmol/L (Fig. 2). Based on previous research, the total GSH level in normal rats is 5–6 µmol/L, compared with 3.025 µmol/L (< 5 µmol/L) in diabetic rats.[48] With normal conditions and a level of 5 µmol/L, GSH is capable of working as an antioxidant and is able to reduce free radicals.[21]

The increase of free radicals in DM occurs through three main mechanisms: a cycle of polyol sorbitol (aldose reductase reaction), nonenzymatic glycation, and glucose autoxidation that occurs due to increased blood glucose levels.[49] GSH at a high concentration has the potential to inhibit radical formation (O_2^-, $ONOO^-$, and NO^-), dialuric acid autoxidation, and hydroxyl radical (OH^-) formation. GSH at high concentrations can also inhibit hydroxyl anion (•OH) formation and directly neutralize hydroxyl radicals.[24] The

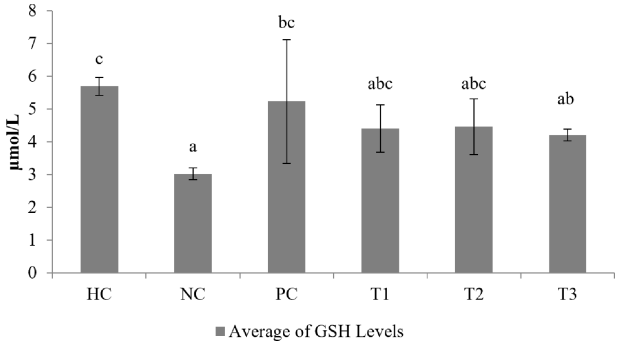

FIG. 2: GSH levels. Bars marked with the same letter are not significantly different ($P \leq 0.05$). HC, healthy control; NC, negative control (induction with 45 mg of STZ); PC, positive control (45 mg of metformin); T1, administration of 250 mg of *Coprinus comatus* ethyl acetate extract; T2, administration of 500 mg of *C. comatus* ethyl acetate extract; and T3, administration of 750 mg of *C. comatus* ethyl acetate extract.

flavonoids in *C. comatus* ethyl acetate extract are the biochemistry agent responsible for protecting pancreatic β cells against the damaging effects of ROS, which can also contribute to their antitumor, anti-inflammatory, and antioxidant activity.[50]

The decrease in GSH levels in the NC group occurred due to increased free radical attack during DM.[51] Hyperglycemia due to STZ induction can cause oxidative stress in β cells due to the increase in free radicals formed.[24] Endogenous antioxidants in pancreatic β cells can resolve this because of the low concentration.[51] This is worsened by high content of the amino acid cysteine, which contains the -SH groups that play a role in GSH production. The -SH group is very vulnerable to ROS attack and can cause GSH to lose its ability to fight free radicals. Diabetogenic (STZ) compounds can release the H^+ cluster from -SH GSH, which can cause the decrease in GSH activity.[52]

GSH is found in almost all tissues of mammals, including in the heart, and it is active as an antioxidant. There are two forms of GSH in the body, which are reduced GSH and oxidized glutathione disulfide (GSSG).[53] GSSG is an active free radical scavenger.[31] Flavonoids, vitamin C, and vitamin E contained in *C. comatus* ethyl acetate extract act as antioxidants to improve defense against ROS.[15] Through a chemical process, GSH is able to react with ROS directly, such as superoxide radicals, hydroxyl radicals, and singlet oxygen, and directly serves as a ROS scavenger.[21]

IV. CONCLUSION

This study demonstrated the effect of ethyl acetate extract of *C. comatus* as an antidiabetic agent and its role in increasing endogenous antioxidant levels. The results showed that *C. comatus* ethyl acetate extract could decrease blood glucose and HbA1c levels, increase the highest insulin level at a dose of 250 mg, and increase GLP-1 and GSH hormone levels at a dose of 500 mg. The qualitative identification results showed that *C. comatus* ethyl acetate extract contains flavonoids, alkaloids, and saponins. The quantitative analysis results with HPLC show that *C. comatus* contains rutin compounds, vitamin E, and vitamin C, which are significantly beneficial as antioxidant supplements.

ACKNOWLEDGMENTS

The researchers express gratitude to Universitas Jenderal Soedirman (UNSOED), which facilitated the research, and to the Institute of Research and Community Service (LPPM), which funded this research through a Public Service Agency (BLU) Research Proposal under contract P/308/UN23/PN/2019.

REFERENCES

1. World Health Organization. Definition, diagnosis and classification of diabetes mellitus and its complications: Report of a WHO consultation. Part 1, Diagnosis and classification of diabetes mellitus. Geneva, Switzerland: WHO; 1999.
2. Unwin N, Gan D, Mbanya JC, Ramachandran A, Roglic G, Shaw J, Soltèsz G, Whiting D, Zgibor J, Zhang P, Zimmet P. IDF Diabetes Atlas, 4th ed. Brussels, Belgium: International Diabetes Federation; 2009.
3. Sulistyorini R, Johan A, Djamiatun K. Effect of ethanol extract of Moringa oleifera leaves on insulin expression and insulitis in diabetes mellitus rats. Maj Kedokt Bandung. 2013;47(2):69–76.
4. Dadich KA. Diabetes mellitus: A guide to patient care, 1st ed. Munden J, editor. Philadelphia, PA: Lippincott Williams & Wilkins; 2007. p. 123–30.
5. Li B, Lu F, Suo X, Nan H, Li B. Antioxidant properties of cap and stipe from Coprinus comatus. Molecules. 2010;15(3):1473–86.
6. Ding Z, Lu Y, Lu Z, Lv F, Wang Y, Bie X, Wang F, Zhang K. Hypoglycaemic effect of comatin, an antidiabetic substance separated from Coprinus comatus broth, on alloxan-induced-diabetic rats. Food Chem. 2010;121(1):39–43.
7. McFarland MS, Cripps R. Diabetes mellitus and increased risk of cancer: Focus on metformin and the insulin analogs. J Hum Pharmacol Drug Ther. 2010;30(11):1159–78.

8. Reyes RG, Lopez LLMA, Kumakura K, Kalaw SP, Kikukawa T, Eguchi F. Coprinus comatus, a newly domesticated wild nutriceutical mushroom in the Philippines. J Agric Technol. 2009;5(2):299–316.
9. Yu J, Cui PJ, Zeng WL, Xie XL, Liang WJ, Lin GB, Zheng L. Protective effect of selenium-polysaccharides from the mycelia of Coprinus comatus on alloxan-induced oxidative stress in mice. Food Chem. 2009;117(1):42–7.
10. Ratnaningtyas NI, Hernayanti H, Ekowati N, Sukmawati D, Widianti H. Chicken drumstick mushroom (Coprinus comatus) ethanol extract exerts a hypoglycaemic effect in the Rattus norvegicus model of diabetes. Biocatal Agric Biotechnol. 2019;19:1–4.
11. Shukla A, Srinivasan BP. 16,17-Dihydro-17b-hydroxy isomitraphylline alkaloid as an inhibitor of DPP-IV, and its effect on incretin hormone and β-cell proliferation in diabetic rat. Eur J Pharm Sci. 2012;47(2):512–9.
12. Gaschler MM, Stockwell BR. Lipid peroxidation in cell death. Biochem Biophys Res Commun. 2017;482(3):419–25.
13. Rock CL, Jacob RA, Bowen PE. Update on the biological characteristics of the antioxidant micronutrients: Vitamin C, vitamin E, and the carotenoids. J Am Diet Assoc. 1996;96(7):693–702.
14. Ergina, Nuryanti S, Pursitasari ID. Qualitative test of secondary metabolites compounds in palado leaves (Agave angustifolia) extracted with water and ethanol. J Akad Kim. 2014;3(3):165–72.
15. Suresh S, Chhipa AS, Gupta M, Lalotra S, Sisodia SS, Baksi R, Nivsarkar M. Phytochemical analysis and pharmacological evaluation of methanolic leaf extract of Moringa oleifera Lam. in ovalbumin induced allergic asthma. S African J Bot. 2020;130:484–93.
16. Meyer VR. Practical high-performance liquid chromatography, 5th ed. Chichester, UK: John Wiley & Sons; 2010.
17. Abeeleh MA, Ismail ZB, Alzaben KR, Abu-Halaweh SA, Al-Essa MK, Abuabeeleh J, Alsmady MM. Induction of diabetes mellitus in rats using intraperitoneal streptozotocin: A comparison between 2 strains of rats. Eur J Sci Res. 2009;32(3):398–402.
18. Bioassay Technology Laboratory. Rat insulin and rat GLP-1 ELISA kit procedure. Shanghai, China: Biotech. Co.; 2018. p. 1–8.
19. Tešanović K, Pejin B, Šibul F, Matavulj M, Rašeta M, Janjušević L, Karaman M. A comparative overview of antioxidative properties and phenolic profiles of different fungal origins: Fruiting bodies and submerged cultures of Coprinus comatus and Coprinellus truncorum. J Food Sci Technol. 2017;54(2):430–8.
20. Tian F, Li B, Ji B, Yang J, Zhang G, Chen Y, Luo Y. Antioxidant and antimicrobial activities of consecutive extracts from Galla chinensis: The polarity affects the bioactivities. Food Chem. 2009;113:173–9.
21. Winarsi H, Wijayanti SPM, Purwanto A. Activity of superoxide dismutase, catalase, and glutathione peroxidase enzymes in women with metabolic syndrome. Maj Kedokt Bandung. 2012;44(1):7–12.
22. Morel I, Lescoat G, Cogrel P, Sergent O, Pasdeloup N, Brissot P, Cillard P, Cillard J. Antioxidant and iron-chelating activities of the flavonoids catechin, quercetin and diosmetin on iron-loaded rat hepatocyte cultures. Biochem Pharmacol. 1993;45(1):13–9.
23. Firdaus F, Rimbawan R, Marliyati SA, Roosita K. Streptozotocin, sucrose-induced diabetic male rats model for research approach of gestational diabetes mellitus. J MKMI. 2016;12(1):29–34.
24. Szkudelski T. The mechanism of alloxan and streptozotocin action in B cells of the rat pancreas. Physiol Res. 2001;50:537–46.
25. Saputra NT, Suartha IN, Dharmayudha AAGO. Diabetagonic agent streptozocin to make white rats male diabetes mellitus. Bul Vet Udayana. 2018;10(2):116.
26. Kanter M, Yoruk M, Koc A, Meral I, Karaca T. Effects of cadmium exposure on morphological aspects of pancreas, weights of fetus and placenta in streptozotocin-induced diabetic pregnant rats. Biol Trace Elem Res. 2003;93(1-3):189–200.
27. Ahmed R. The physiological and biochemical effects of diabetes on the balance between oxidative stress and antioxidant defense system. Med J Islam World Acad Sci. 2005;15(1):31–42.
28. Houcher Z, Boudiaf K, Benboubetra M, Houcher B. Effects of methanolic extract and commercial oil of Nigella sativa L. on blood glucose and antioxidant capacity in alloxan-induced diabetic rats. Pteridines. 2007;18(1):8–18.
29. Ji W, Huang H, Chao J, Lu W, Guo J. Protective effect of agaricus brasiliensis on STZ-induced diabetic neuropathic pain in rats. Evid Based Complement Altern Med. 2014;2014:679259
30. Yamac M, Zeytinoglu M, Kanbak G, Bayramoglu G, Senturk H. Hypoglycemic effect of crude exopolysaccharides produced by Cerrena unicolor, Coprinus comatus, and Lenzites betulina isolates in streptozotocin-induced diabetic rats. Pharm Biol. 2009;47(2):168–74.
31. Zhang ZM, Wu WW, Li GK. A GC-MS study of the volatile organic composition of straw and oyster mushrooms during maturity and its relation to antioxidant activity. J Chromatogr Sci. 2008;46(8):690–6.
32. Gutierrez MRP, Gomez YG, Ramirez EB. Nephroprotective activity of prosthechea michuacana against cisplatin-induced acute renal failure in rats. J Med Food. 2010;13(4):911–6.
33. Ding Z, Wang W, Wang F, Wang Q, Zhang K. Polysaccharides production by submerged fermentation of Coprinus comatus and their inhibitory effects on non-enzymatic glycosylation. J Med Plants Res. 2012;6(7):1375–81.

34. Sharma B, Salunke R, Balomajumder C, Daniel S, Roy P. Anti-diabetic potential of alkaloid rich fraction from Capparis decidua on diabetic mice. J Ethnopharmacol. 2010;127(2):457–62.
35. Ahmad P, Sarwat M, Sharma S. Reactive oxygen species, antioxidants and signaling in plants. J Plant Biol. 2008;5(3):167–73.
36. Rees D, Alcolado J. Animal models of diabetes mellitus. Diabet Med. 2005;22(4):359–70.
37. Yuniastuti A, Susanti R, Iswari R. Effect of garut tuber infusion (Marantha arundinacea L.) on glucose and insulin levels in streptozotocyn-induced rat plasma. Indones J Math Nat Sci. 2018;41(1):34–9.
38. Cai R, Li X, Chen B, Xie Y, Xie H, Chen D. Antioxidant change in biosynthesis from naringenin chalcone to flavonoid apingenin. Chem Select. 2019;4(17):5155–9.
39. Vinayagam R, Xu B. Antidiabetic properties of dietary flavonoids: A cellular mechanism review. Nutr Metab. 2015;12(1):1–20.
40. Drucker DJ. Dipeptidyl peptidase-4 inhibition and the treatment of type 2 diabetes: Preclinical biology and mechanisms of action. Diabetes Care. 2007;30:1335–43.
41. Stehouwer CDA, Schaper NC, editors. Therapeutic strategies in diabetes, 1st ed. Oxford, UK: Atlas Medical Publishing; 2009. p. 250–62.
42. Padidela R, Patterson M, Sharief N, Ghatei M, Hussain K. Elevated basal and post-feed glucagon-like peptide 1 (GLP-1) concentrations in the neonatal period. Eur J Endocrinol. 2009;160(1):53–8.
43. Bharti SK, Krishnan S, Kumar A, Rajak KK, Murari K, Bharti BK, Gupta AK. Antihyperglycemic activity with DPP-IV inhibition of alkaloids from seed extract of Castanospermum australe: Investigation by experimental validation and molecular docking. Phytomedicine. 2012;20(1):24–31.
44. Augustyns K, Veken P, Senten K, Haemers A. The therapeutic potential of inhibitors of dipeptidyl peptidase IV (DPP IV) and related proline-specific dipeptidyl aminopeptidases. Curr Med Chem. 2005;12(8):971–98.
45. Ramachandran V, Saravanan R. Efficacy of asiatic acid, a pentacyclic triterpene on attenuating the key enzymes activities of carbohydrate metabolism in streptozotocin-induced diabetic rats. Phytomedicine. 2012;20(3-4):230–6.
46. Kim D-H, Yang B-K, Hur N-J, Das S, Yun J-W, Choi Y-S, Song C-H. Hypoglycemic effects of mycelia produced from a submerged culture of Phellinus linteus (Berk. et Curt.) Teng (Aphyllophoromycetidae) in streptozotocin-lnduced diabetic rats. Int J Med Mushrooms. 2001;3(1):1–6.
47. Fatimah RN. Type 2 diabetes mellitus. J Major. 2015;4(5):93–101.
48. Mclennan S V, Heffernan S, Wright L, Rae C, Fisher E, Yue DK, Turtle JR. Changes in hepatic glutathione metabolism in diabetes. Diabetes. 2006;40(3):344–8.
49. Wang Y, Branicky R, Noë A, Hekimi S. Superoxide dismutases: Dual roles in controlling ROS damage and regulating ROS signaling. J Cell Biol. 2014;217(6):1915–28.
50. Wu T, Xu B. Antidiabetic and antioxidant activities of eight medicinal mushroom species from China. Int J Med Mushrooms. 2015;17(2):129–40.
51. Suarsana IN, Utama IH, Agung IG, Suartini A. Hyperglycemic and vitamin E effect on malondialdehyde and antioxidant intracellular enzyme in rat pancreatic tissue. Maj Kedokt Bandung. 2011;43(2):72–6.
52. Prameswari OM, Widjanarko SB. The effect of water extract of pandan wangi leaf to decrease blood glucose levels and pancreas histopathology at diabetes mellitus rats. J Pangan dan Agroindustr. 2014;2(2):16–27.
53. Heisterkamp N, Groffen J, Warburton D, Sneddon TP. The human gamma-glutamyltransferase gene family. Hum Genet. 2008;123(4):321–32.