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Journal of Biology & Biology Education

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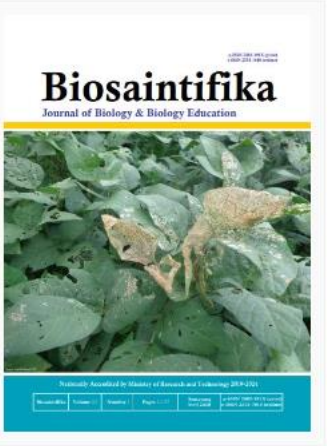

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Nuniek Ina Ratnaningtyas, Hernayanti Hernayanti, Nuraeni Ekowati, Fajar Husen, Isnaini Maulida, Riama Kustianingrum, Vika Vidiyanti

Abstract

Coprinus comatus, well-known as Shaggy Ink Cap mushroom, is potential herbal medicine. Synthetic medicines sometimes cause side effects; therefore, it is necessary to innovate with herbal medications with minimal side effects. The study evaluated *in vitro* and *in vivo* treatments to evaluate the antioxidant effect and activity of *C. comatus*. The *in vivo* treatment was conducted using six groups of Wistar rats (n = 24). Group 1 healthy control (HC), groups 2–6 received 45 mg/Kg BW of streptozotocin once, group 2 just streptozotocin-induced (NC), group 3 was given 45 mg/kg BW of metformin (PC), groups 4–6 were given 250 (T1), 500 (T2), and 750 mg (T3) of *C. comatus* extract for 14 days, and the *in vitro* was conducted using an antioxidant oxidant assay. Data were analyzed using analysis of variance and Duncan's multiple range tests. Based on qualitative analysis, *C. comatus* mycelium extract contained polyphenol, flavonoids, terpenoids, and fruiting body extract had flavonoids, alkaloids, and saponins. The *in vitro* analysis showed that the mycelium extract had an antioxidant activity by inhibiting free radicals up to 58.51% with an IC_{50} value of 72.77 mg/L. The *in vivo* treatment using *C. comatus* fruiting body extract showed that it could increase the endogenous antioxidant levels of GPx, SOD, catalase and reduce MDA levels ($p < 0.05$). The most effective dose of *C. comatus* extract is 500 mg. This research has shown the potential of mycelium and *C. comatus* fruitbody extract as an antioxidant supplement in a diabetic rat model.

Keywords

antioxidant, bioactive compound, coprinus comatus, medicinal mushrooms, reactive oxygen species

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Antioxidant Activities and Properties of Coprinus comatus Mushroom Both Mycelium and Fruiting Body Extracts In Streptozotocin-Induced Hyperglycemic Rats Model

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Abstract. *Coprinus comatus*, well-known as Shaggy Ink Cap mushroom, is potential herbal medicine. Synthetic medicines sometimes cause side effects; therefore, it is necessary to innovate with herbal medications with minimal side effects. The study evaluated *in vitro* and *in vivo* treatments to evaluate the antioxidant effect and activity of *C. comatus*. The *in vivo* treatment was conducted using six groups of Wistar rats (n = 24). Group 1 healthy control (HC), groups 2–6 received 45 mg/Kg BW of streptozotocin once, group 2 just streptozotocin-induced (NC), group 3 was given 45 mg/kg BW of metformin (PC), groups 4–6 were given 250 (T1), 500 (T2), and 750 mg (T3) of *C. comatus* extract for 14 days, and the *in vitro* was conducted using an antioxidant oxidant assay. Data were analyzed using analysis of variance and Duncan's multiple range tests. Based on qualitative analysis, *C. comatus* mycelium extract contained polyphenol, flavonoids, terpenoids, and fruiting body extract had flavonoids, alkaloids, and saponins. The *in vitro* analysis showed that the mycelium extract had an antioxidant activity by inhibiting free radicals up to 58.51% with an IC_{50} value of 72.77 mg/L. The *in vivo* treatment using *C. comatus* fruiting body extract showed that it could increase the endogenous antioxidant levels of GPx, SOD, catalase and reduce MDA levels ($p < 0.05$). The most effective dose of *C. comatus* extract is 500 mg. This research has shown the potential of mycelium and *C. comatus* fruitbody extract as an antioxidant supplement in a diabetic rat model.

Key words: antioxidant, bioactive compound, *coprinus comatus*, medicinal mushrooms, reactive oxygen species

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INTRODUCTION

Diabetes mellitus (DM) is a metabolic disorder caused by the degradation of insulin production, sensitivity, or both (Husen et al., 2021). More than 100 million people worldwide were affected by DM, and in the following decade, the incidence was increased (Ding et al. 2010). World Health Organization predicts that there will be an increase in the number of people with diabetes in Indonesia from 8.4 million in 2000 to approximately 21.3 million in 2030 (Saeedi et al. 2019). In 2009, the International Diabetes Federation (IDF) predicted that there would be an increase in the number of people with DM from 7.0 million in 2009 to 12.0 million in 2030 (Saeedi et al. 2019). Diabetes mellitus is characterized by hyperglycemia. The disease triggers an increase in free radicals in pancreatic β cells, which causes oxidative stress and impairs insulin secretion that, causes abnormalities in carbohydrate, lipid, and protein metabolism that can damage organs such as the pancreas, liver, and kidneys. In contrast, long-term DM treatment using synthetic drugs causes side effects, so it is necessary to develop herbal medicines from natural resources (e.g., mushrooms) (Husen et al., 2021).

Indonesia is well-known for its rich biological resources with high potential as a raw material of medicines. One of the natural resources widely used as medicinal ingredients is mushrooms. Mushrooms have been widely used as a natural medicine in China and Japan. In contrast, locally cultivated mushrooms in Indonesia still need further exploration and research because there have not been any significant use and development as medicines, especially for *Coprinus comatus* mushroom that well-known as the Shaggy Ink Cap Mushroom or Chicken Drumstick Mushroom (Ratnaningtyas et al., 2019). The potential of the *C. comatus* as herbal medicine for diabetes has been widely reported (Husen et al., 2021). Therefore, it is necessary to conduct a study on the

development and application of DM co-medications containing antioxidant supplements to protect pancreatic β cells of DM patients from free radical attack and improve the medicines' effectiveness (Ratnaningtyas et al., 2019).

The *C. comatus* extracts have antioxidants and hypoglycemic activities, potentially an alternative medicine for DM. Exogenous antioxidants are required to overcome enzymatic antioxidant deficiency, including the exogenous antioxidants of *C. comatus* extract. The bioactive compounds in the *C. comatus* are flavonoids, which can stabilize free radicals. The scavenging impact on the superoxide anion could reach 95% (Cao et al., 2019). Flavonoids have antioxidant activity that can protect the body against damage caused by ROS (reactive oxygen species) by donating their hydrogen atoms, binding with free radicals, and forming flavonoids radicals that are not reactive.

In this research, we focused on *in vitro* and *in vivo* combining and applying ethyl acetate to both extracts of mycelium cultures and the fruiting body of *C. comatus. In vitro* treatment with mycelium extract was carried out using the DPPH test to determine the scavenging ability of free radicals and their inhibition effectiveness. *In vivo* treatment using the fruiting body extracts were carried out to hyperglycemic rats induced by STZ to test the efficacy of the bioactive compounds of *C. comatus* in increasing the endogenous or enzymatic antioxidant levels. This study offered ethyl acetate solvent for extraction because the solvent is more likely to extract the compounds better than others and draw more samples from the aqueous solvent than the organic layer. It has two chemical and biological characteristics: medium polarity and minimum toxicity. This study aimed to identify the bioactive compounds of ethyl acetate extract of *C. comatus* mycelium culture and fruiting body and investigate its benefits as antioxidants based on *in vitro* and *in vivo* evaluation. This study was expected to provide *in vitro* and *in vivo* data about the antioxidant activity of *C. comatus* bioactive as a candidate of antioxidant supplement for patients with diabetes mellitus.

METHODS

Reagents

The reagents used in the study included ethyl acetate (pro-analysis), potato dextrose agar (PDA) media, and potato dextrose yeast broth (PDYB) media. Other reagents were ethanol, alcohol 70%, tissue paper, aluminium foil, blue tip, and dimethyl sulfoxide. Additional chemicals utilized were ascorbic acid, streptozotocin (STZ), metformin, and more.

Mycelium culture

Mushroom mycelium on media PDA was sliced using a cork drill and then inoculated in the Erlenmeyer flask containing media PDYB. Each Erlenmeyer flask filled with 100 mL liquid medium and six plugs of mycelial slices with a diameter of 5 mm, were inserted into the flask. Subsequently, the inoculated media was incubated for 28 days at room temperature and lightly shaken daily.

Mycelium and fruiting body extraction of *C. comatus*

Mycelium extract

For about a day, the dried mycelium was mashed up using mortar and pestle soaked in ethyl acetate solvent. The main solution was made for treatment concentration test in three repetitions using DMSO solvents (40, 60, 80, and 100 ppm) with the following formula:

V1 (main stock) x M1(dilute solution) = V2 (main stock solution) x M2 (dilute solution)

Fruiting body extract

The *C. comatus* mushrooms were cut into small pieces, oven-dried at 80ºC, and ground into powder. Ethyl acetate solvent was added at a 1:5 ratio of Simplicia and ethyl acetate. Then it was left to settle for 24 hours, where it was filtered using filter paper afterwards, and the fibre was obtained and stored in a clean bottle. Finally, evaporation was carried out to get thick extract using a rotary vacuum evaporator at the temperature of 77ºC.

Induction preparation of streptozotocin (STZ)

The STZ was induced in an *in vivo* treatment using 24 male rats of Wistar strain (*Rattus norvegicus*) aged 3-4 months, weighed 180-200 g, and the rats were acclimatized for seven days.

The rats were diabetic because of the induction using 45 mg STZ suspended using 2.5 ml of citrate buffer. The STZ compound was applied using an intraperitoneal injection of 0.5 mL. This study measured blood glucose levels in the lateral vein. The rats in fasting condition and whose blood glucose levels were more than 150 mg/dL were categorized as hyperglycemia and ready for application.

Animals' treatment

The rats were reared in a plastic cage with dimensions of 60 x 40 x 20 cm, and the bottom of the cage was covered using straw, while the upper part was covered using woven wire. The straws were replaced after every three days. Each cage contained five white rats and was fed twice a day in the morning and the evening. This study applied ethyl acetate extract of *C. comatus* fruiting body to rat groups (T1: 250 mg/kg BW, T2: 500 mg/kg BW, and T3: 750 mg/kg BW). This study used 45 mg/kg BW metformin as a positive control (PC). Rat fed without any extract supplementation was referred to as negative control (NC). The treatment group of T1, T2, T3, PC, and HC has received 45 mg of streptozotocin/kg body weight (BW) once, while the healthy rats (HC) were fed only with distilled water. The study applied the extract in the morning before feeding for 14 days.

Qualitative analysis and identification of bioactive compound

Flavonoids test was carried out by steaming 2 mL of sample extract for 5 minutes. Then, 0.1 g of HCl was added to each concentration. Suppose yellow colour (+), orange colour (++), and red colour (+++) were observed (Ergina et al. 2014). The polyphenol test was carried out by adding 5 mL of distilled water to each sample extract, steaming it for ± 5 minutes, and adding two drops of $FeCl_3$. The terpenoid test was carried out by adding three drops of strong acid HCl and a drop of H_2SO_4 to 2 mL of sample extract. Saponins were identified by boiling distilled water and adding 2 mL of methanol. The sample was then cooled and shaken for about 10 seconds (Ratnaningtyas et al., 2019).

Antioxidant content analysis of hyperglycemia rats' glutathione peroxidase (GPx)

Five hundred µL of PBS, 850 µL of sterile distilled water, and 50 µL of homogenized CTNB were put into a cuvette. Subsequently, 50 µL of plasma samples were added to the cuvette. Absorbance was read using spectrophotometry at the wavelength of 340 nm in the first minute and the second minute. The absorbance results were calculated using the following formula (Khalid et al., 2015):

GPx = (Abs 1st minute – Abs 2nd minute) x 450 µmol/L

Superoxide dismutase (SOD) analysis

A thousand µL of SOD buffer solution and 100 µL of xanthine oxidase solution were piped into a blank tube. A standard solution of 20 µL SOD plus 1000 µL of SOD buffer solution and 100 µL of xanthine oxidase solution was put into a standard tube using a pipette. Twenty µL of plasma samples plus 1000 µl of SOD buffer solution and 100 µL of xanthine oxidase solution were piped into a sample tube and measured using a UV-V is spectrophotometer at the wavelength of 520 nm. The sample's SOD level was calculated using the following formula (Jeane et al., 2018): SOD = (Abs Sample / Abs Standard) x Std. Concentration (30.65 U/mL).

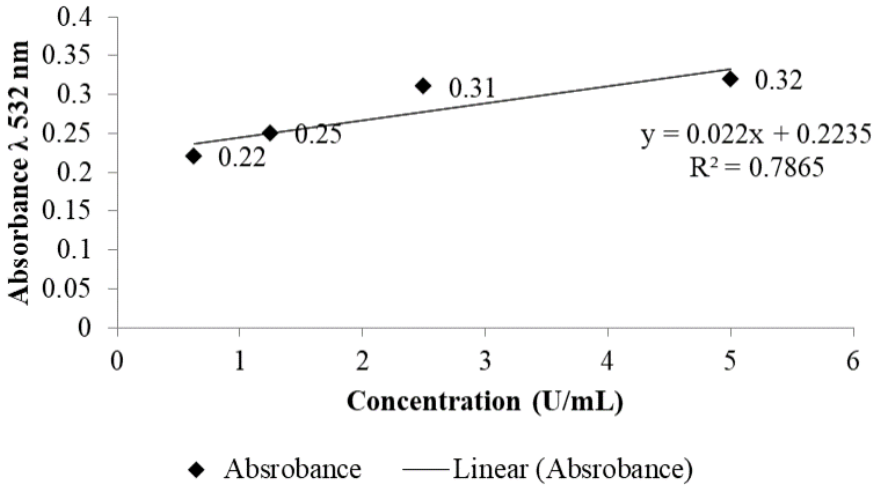

Figure 1. The standard calibration curve of tetra ethoxy propane (TEP)

Catalase analysis

Standards and samples were measured in duplicate. Fifty µL of PBS and 2000 µL of H_20_2 were homogenized and put into a cuvette as control or standard solutions. Two thousand µL of H_20_2 and 100 µL of plasma samples were put into the cuvette. Then absorbance was read using spectrophotometry at the wavelength of 240 nm

and calculated using the following formula (Puspitaningrum et al., 2014):

Catalase = ((Abs 1st minute – Abs 2nd minute) / Molarity (27.2)) x 2000 µmol/L

Malondialdehyde (MDA) analysis

Four hundred µL of plasma sample plus 400 µL of 20% TCA solution was taken and then vortexed. After that, the solution was centrifuged for 10 minutes at 4000 rpm. Four hundred µL of the resulting supernatant was taken and added with 1 mL of TBA 0.67%. Afterwards, the solution was put into a water bath for 10 minutes. The absorbance was read at the wavelength of 532 nm. The MDA activity could be determined by drawing a standard calibration curve of the stock solution of Tetra Ethoxy Propane (TEP) (Vitriyaturrida et al., 2019):

Antioxidant activity evaluation of mycelium extract using DPPH method

The ethyl acetate extract of *C. comatus* mycelium was prepared by homogenizing 2 mL of each concentration (40 ppm, 60 ppm, 80 ppm, and 100 ppm) and 2 mL of liquid DPPH with vortex and then storing it for 30 minutes at 37ºC. After that, absorbance was read using spectrophotometry at the wavelength of 517 nm. Both extract and vitamin C were calculated using the following formula (Jami'ah et al., 2018). IC_{50} (Inhibition Concentration) was estimated to get the minimum concentration of the extract using the regression formula:

$$IC_{50} = (a + b)\ .\ x$$

Description = a (constant); b (regression coefficient); x (independent variable)

Statistical analysis

This research uses One-way analysis of variance (ANOVA), Duncan's multiple range tests, and correlation tests using the SPSS statistical package (v.20.0) to compare the main parameters. The GPx, catalase, SOD, and MDA parameters were expressed as mean±standard error (SE) and independent sample groups. The P values were indicative of statistical significance.

Ethical approval

All treatments upon the experimental animals have received ethical approval from the Health Research Ethics Commission of Faculty of Medicine, Universitas Jenderal Soedirman, Purwokerto (approval number Ref.3797/KEPK/VIII/2019). The termination process was carried out under the Institutional Animal Care and Use Committee (IACUC) to minimize or even eliminate the suffering of the animals.

RESULTS AND DISCUSSION

Bioactive identification

A total of 200 g of dry cultured *C. comatus* mycelium powder was extracted with ethyl acetate and made at 40, 60, 80, and 100 ppm with DMSO. Qualitative identification of mycelium extract using Thin Layer Chromatography (TLC) for each concentration performed showed that at a concentration of 100 ppm, there was a change in colour to dark orange, red, and pink. Meanwhile, at concentrations of 40, 60, and 80 ppm, the colour changed to dark orange and red, but there was no change in colour to pink (Table. 1). Based on Susanto et al. (2018), the ethanol extract of the fruiting body of *C. comatus* analyzed by the TLC method showed an orange colour change which indicated that the extract contained flavonoids besides that the extract also showed a pink colour change which means that the extract had terpenoids. The results of the qualitative analysis of mycelium extract are summarized in Table 1.

Table 1. The bioactive compounds of the mycelium ethyl acetate extract of *C. comatus*

Bioactive Compounds	Ethyl Acetate Concentrations (ppm)			
	100	80	60	40
Flavonoids	+++	+++	+++	++
Polyphenol	+++	+++	+++	+++
Terpenoid	+	-	-	-

Note: Qualitative test results: + (low), ++ (medium), +++ (high/strong).

The dry powder of the fruiting body of *C. comatus* as much as 2000 g, which was extracted with ethyl acetate with two macerations, resulted in 4.2 g of thick extract after going through the evaporation process. The results showed that the fruiting body extract of *C. comatus*, which was analyzed qualitatively with the addition of HCl,

showed a colour change to reddish-yellow. In contrast, after adding HCl and H_2SO_4, it showed a colour change to dark orange, and in the heated extract, there was foam formation that lasted for 2 minutes after adding the methanol (Table 2). According to Husen et al. (2021), qualitative and quantitative analysis of bioactive compounds in the ethyl acetate extract of the fruiting body of *C. comatus* showed that the extract contains flavonoids, alkaloids, and the formation of foam after boiling, where the foam lasted 1-2 minutes indicated the extract had saponins. In addition, the results of the quantitative analysis also showed that the ethyl acetate extract of *C. comatus* contained 16.4 mg/L of flavonoids and 2.97 mg/L of alkaloids. Based on Ratnaningtyas et al. (2019), the ethanol extract of *C. comatus* fruiting body contains flavonoids (red colour), triterpenoids (dark purple colour), alkaloids (yellow-brown colour), and saponins (formation of stable foam).

The flavonoids have an unsubstituted hydroxyl group, a polar compound generally soluble in certain solvents such as ethanol, methanol, butanol, water, and ethyl acetate (Wati et al., 2017). The polyphenols test of the *C. comatus* also indicative of a positive colour change, meaning that the species contained many polyphenols (Susanto et al., 2018). Generally, the results of the qualitative analysis of the bioactive compound of the ethyl acetate extract of the *C. comatus* are consistent with those of the prior study showing that there are flavonoids, alkaloids, and saponins compounds found in the ethanol extract of *C. comatus* fruiting body (Ratnaningtyas et al., 2019).

The flavonoids found in medicinal plant species were also tested in medicinal mushrooms, such as *Ganoderma* sp. (Ratnaningtyas et al., 2018). They could function as antioxidants by acting as electron donors to stabilize free radicals, which inhibit possible metabolic damage caused by free radicals accumulated in the body. The HOO· radical scavenging of the selected flavonoids was also examined through the potential energy surface, natural bond orbitals, and kinetic calculations. The favoured radical scavenging mechanism of the flavonoids is hydrogen atom transfer (Vo et al., 2019). Flavonoids and tannins are phenolic compounds, and phenolics are, in general, a group of

Table 2. The bioactive compounds of the ethyl acetate extract of *C. comatus* fruiting body with quantitative analysis

Bioactive Compounds	Results (Qualitative)	Level
Flavonoids	Reddish Yellow	++
Alkaloids	Dark Orange	+
Saponins	Formed Foam	+

Note: Qualitative test results: + (low), ++ (medium), +++ (high/strong).

mycelium was positive because of the reduction reaction. The polyphenols test of the fourth concentration sample showed a strong result. Tannins are responsible as antitumor and antioxidative (Yilmaz et al., 2017) and stabilize free radicals such as Fe^{3+} (Patay et al., 2016). The result of the terpenoid compound test of the mycelium extract was positive (Table 1), while another concentration sample was negative, as indicated by the lack of colour change. Most fragile compounds are non-polar, so they are readily soluble in non-polar solvents such as N-Hexsan and Toluene. Other terpenoid compounds are fat-soluble and usually extracted using ether or chloroform (Widyawati et al., 2014).

The study showed that the ethyl acetate extract of the *C. comatus* fruiting body had flavonoids (Table 2). The identification of the polyphenol compound of the fruiting body of *C. comatus* was compounds that act as primary antioxidants (Zarta et al., 2018). The bioactive compounds in *C. comatus* mushrooms are flavonoids in rutin compounds that can ward off free radicals (Zhao et al., 2018). Flavonoids have an antioxidant activity that could protect the body against damage caused by ROS by donating their hydrogen atoms and forming more stable flavonoid radicals that could inhibit lipid peroxidation that damaged cells (Sasmita et al., 2017).

Glutathione peroxidase (GPx)

The results of the GPx measurement showed a significant value ($p<0.05$). The administration of *C. comatus* ethyl acetate extract in the DM rats model increased GPx content after 14 days of treatment. Meanwhile, the NC group showed a decrease in GPx levels. The GPx level in the NC group was 8.82 ± 3.3 U/mL and was the group with the lowest GPx level compared to the other

five groups. Meanwhile, the highest GPx levels were in the T1 group with 39 ± 4.9 U/mL, the T2 group with 38.5 ± 16.5 U/mL, and the T3 group with 22.19 ± 8.1 U/mL. GPx levels in the HC group (healthy) was 23 ± 5.9 U/mL, and in the group receiving metformin (PC) was 33 ±16.7 U/mL (Figure 2). In general, the group given the extract showed an increase in GPx compared to NC, with the percentage increases in the T1, T2, T3, and PC were 77.35%, 77.06%, 60.02%, and 73.24%, respectively (Figure 2). Previous research by Stilinović et al. (2020) showed that intraperitoneal induction of CCl_4 caused a decrease in GPx levels, where the GPx level of the negative group was 0.76 ± 0.01, while the CCl_4 induction group given *C. comatus* ethyl acetate extract was 0.91 ± 0.04 and the group that given *C. comatus* extract without induction of CCl_4 was 1.48 ± 0.07, measured after 42 days of rearing and caring for experimental animals (Stilinović et al., 2020). This data shows that the ethyl acetate extract of *C. comatus* can increase the endogenous antioxidant levels of rats.

The *C. comatus* extract at the dose of 500 mg/kg BW effectively decreases blood glucose, MDA, and HbA1c levels and increases SOD and plasma insulin levels. The endogenous antioxidants can increase SOD levels (Ratnaningtyas et al., 2019). Also, it raises plasma insulin levels, which indicates the improvement of pancreatic β cells. *Phellinus rimosus* mushroom at 50 mg/kg, 140 mg/kg, and 250 mg/kg has been tested in hyperglycemic rats induced by alloxan at the dose of 250 mg/kg BW, and *P. rimosus* mushrooms contain antioxidant compounds such as flavonoids that role as antidiabetic and antioxidant agents (Rony et al., 2013). The *C. comatus* mushroom also contains an ergothioneine (EGT) compound similar to methionine (Ding et al. 2010). The methionine is a substrate of GPx. The presence of EGT results in the formation of the GSH substrate, and the GPx activity becomes normal (Asahi et al., 2016).

The ethyl acetate extract of *C. comatus* applied to diabetic rats at the doses of 250 and 500 mg/kg BW could increase the activity of the GPx enzyme. The group of rats treated with the ethyl acetate extract of *C. comatus* at the doses of 250 and 500 mg/kg BW had the GPx activities higher than those of the NC group. The low GPx activity in the NC group is related to increased H_2O_2 free radicals in cells. SOD enzyme is an endogenous antioxidant found in cells that plays an essential role as the front line of defence against free radicals. In contrast, the GPx enzyme continues the SOD activity by cleaning free radicals with enzymatic reactions and converting them into more stable forms. The hyperglycemic condition ROS. The ROS can be overcome using an antioxidant from the mushroom extract. Catalase can neutralize and accelerate the degradation of H_2O_2. This peroxide may cause damage to macromolecular components of cells (Kurniawaty & Lestari, 2016). At the same time, the GPx enzyme will convert H_2O_2 to H_2O and O_2 supported by Se^{2-} as a cofactor. *C. comatus* mushrooms contain Se^{2-} and also Fe^{2+} representing catalase enzyme cofactor. The catalase enzyme activity converts H_2O_2 to H_2O and O_2 (Winarsi et al., 2012). The enzymatic activity of endogenous

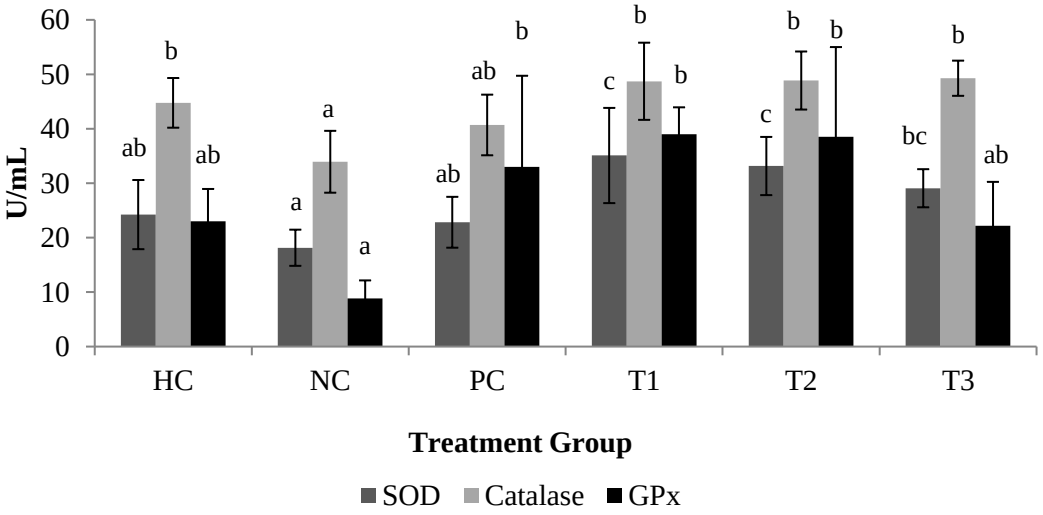

Figure 2. The antioxidant levels of hyperglycemic rats treated using the ethyl acetate extract of C. comatus fruit body

antioxidants can decrease excessive free radicals (Suarsana et al., 2011).

Hyperglycemia is a condition in which blood glucose levels remain high because the glucose could not enter cells, which caused, in turn, the formation of free radicals (Afsari et al., 2016). Pancreatic β cells have fewer antioxidants than those of other organs sensitive to the ROS attack. Excessive free radicals will oxidize and attack the lipid components of cell constituents composed of polyunsaturated fatty acid and MDA as the end product of lipid. When free radicals enter the body, they will naturally be controlled by enzymatic defence systems such as SOD, catalase, and GPx (Kristina et al., 2015). GPx and catalase will convert hydrogen peroxide (H_2O_2) into H_2O and O_2 (Ramadhan et al., 2019). The endogenous or enzymatic antioxidants increase after applying the ethyl acetate extract (Figure 2).

Catalase

The results of the catalase measurement showed a significant value ($p<0.05$). The results showed that the administration of ethyl acetate extract of *C. comatus* fruiting bodies increased serum catalase levels. The NC group was the group with the lowest catalase level with 33.94 ± 5.7 U/mL, and the T3 group was the group with the highest catalase level with 49.28 ± 3.2 U/mL, while the catalase levels of T2 and T1 groups were 48.86 ± 5.3 U/mL and 48.72 ± 7.1 U/mL respectively. All groups given the extract showed higher catalase levels than the NC and HC groups (23 ± 4.6 U/mL), while the PC group (40.7 ± 5.6 U/mL) was slightly lower than HC. The percentage increase in catalase levels in the extract and metformin group compared to NC were 43.55% (T1), 43.95% (T2), and 45.19% (T3), while the PC group increased by 19.91% (Figure 2). Previous studies by Hamdy & Taha (2009) have shown that induction of hyperglycemic rats by STZ at a dose of 60 mg increased the catalase by 4.2 ± 0.1 nmol/mg protein. Meanwhile, the catalase level in the DM rat group treated with *Nigella sativa* oil was 12 ± 0.2 nmol/mg protein, while in healthy rats, it was 12.2 ± 0.4 nmol/mg protein (Hamdy & Taha, 2009).

The catalase is a homotetramer enzyme-containing ferriheme with Fe^{2+} that served as its cofactor. It plays an important role in converting H_2O_2 to H_2O and O_2 (Zainuri & Wanandi, 2012). The supplementation of the ethyl acetate extract of *C. comatus* shows a significant increase in catalase activity in rats compared to the NC group. It has been proven that the ethyl acetate extract of *C. comatus* can increase the catalase enzyme activity. The treated rat group of rats had the catalase of >48 U/mL (Figure 2).

The increase in catalase levels is related to the decrease in the number of free radicals contained in the cells. It proves that the extract could increase endogenous antioxidant activity in the body to prevent oxidative stress. The presence of tocopherol can break the lipid peroxidation chain reaction. Flavonoids with many OH groups can directly counteract free radicals by donating H^+ to free radicals (Li et al., 2010). The *C. comatus* mushrooms also contain Fe^{2+} representing a catalase enzyme cofactor (Khatua & Acharya, 2020). The catalase enzyme reduces peroxide compounds in cells such as H_2O_2 and converts them into H_2O and O_2 (Winarsi et al., 2012).

Superoxide dismutase (SOD)

Measurement of SOD in the experimental group after administration of the extract also showed a significant value ($p<0.05$). The lowest SOD level was in the NC group with 18.15 ± 3.3 U/mL, while the HC group (healthy) was 24.24 ± 6.4 U/mL. The group with the extract also showed a higher value than NC, where the T1, T2 and T3 group had SOD levels of 35.09 ± 8.7 U/mL, 33.16 ± 5.3 U/mL, and 29.08 ± 4.5 U/mL, respectively. Meanwhile, the SOD level of the PC group with metformin was 22.83 ± 4.7 U/mL. The percentage increase in SOD levels in both the extract and metformin group compared to NC showed a significant value, where the T1, T2, and T3 group were increased by 48.27%, 45.26%, and 37.58%, respectively, while the PC group showed an increase of 20.49%. All groups given extract and metformin showed higher SOD values than HC and NC. These results can provide good information that the increase in endogenous antioxidants is expected to suppress free radicals in DM conditions (Figure 2). Previous research by Ratnaningtyas et al. (2019) showed that the 250 mg dose of ethanol extract of the fruiting body of *C. comatus* increased SOD levels by 40.56 ± 7.04 U/mL. In contrast, the negative group (without extract) had low SOD levels of 29/17 ± 1.0 U/mL (Ratnaningtyas et al., 2019).

The extract of *C. comatus* also contains compounds that act as antioxidants such as flavonoids, vitamin E, and vitamin C (Tešanović et al., 2017). The antioxidants such as flavonoids can donate H^+ electrons to ROS such as O^{2-} and OH that the ROS becomes neutral and stop the lipid peroxidation chain reactions. The flavonoids can stimulate the activity of the SOD enzyme. The

SOD in the body captures O^{2-} and with the help of H^+ ions, and converts them into H_2O_2. The process inhibits lipid peroxidation that causes damage to pancreatic β cells (Retnaningsih et al., 2013).

Superoxide dismutase is a primary enzyme that protects cells from free radical attack. In DM conditions, free radicals can lead to oxidative stress, and SOD is an enzymatic antioxidant that acts as the first line of defence against free radical attack (Ratnaningtyas et al., 2021). The SOD enzymes can catalyze superoxide anion (O^{2-}) to H_2O_2. It works perfectly in the presence of minerals such as copper (Cu^{2+}), zinc (Zn^{2+}), and manganese (Mn^{2+}) that are abundant in *C. comatus* (Retnaningsih et al., 2013) or with herbs containing flavonoids (Tiwari et al., 2013). The increase in the SOD enzyme activity indicates the ability of the ethyl acetate extract of *C. comatus* to improve antioxidant function in diabetic rats. It is consistent with the prior study results showing that applying the ethanol extract of *C. comatus* at 500, 750, and 1000 mg/kg BW can increase the SOD levels in diabetic rats (Ratnaningtyas et al., 2019).

According to Li et al. (2010), the low levels of SOD activity indicate that oxidative stress is caused by an imbalance between antioxidants and free radicals. Streptozotocin-induced rats cause the increase in the production of O^{2-} in the mitochondria that subsequently activate protein kinase C (PKC) and result in the formation of AGEs, both of which will interfere with the function of pancreatic β cells in producing insulin (Retnaningsih et al., 2013). The group of rats induced with streptozotocin and treated with ethyl acetate extract of the *C. comatus* have lower final blood glucose levels than initial blood glucose levels (Ratnaningtyas et al., 2019).

Malondialdehyde (MDA) levels

The measurement results of MDA levels in the experimental group showed a significant value (*p<0.05)*. The highest MDA level was found in the NC group with 3.32 ± 0.02 mol/L, while in the HC group (healthy), it was 1.12 ± 0.33 mol/L. The increase in MDA levels indicated the occurrence of lipid peroxidation reactions and increased ROS. The extract and metformin group showed other interesting results, where the MDA level was < 1.5 mol/L, where in the T1, T2, and T3 group they were 1.35 ± 0.1 mol/L, 1.15 ± 0.11 mol/L, and 0.96 ± 0.07 mol/L respectively. Moreover, the MDA level of PC was 1.37 ± 0.06 mol/L. In general, the results showed that the group with the extract showed a decrease compared to the NC group. The percentage decrease in MDA levels in the T1, T2, T3, and PC groups were 59.33%, 65.36%, 71.08%, and 58.73%, respectively (Figure 3). Previous research by Ratnaningtyas et al. (2019) showed that Wistar rats induced by alloxan 200 mg showed an increase in MDA levels by 1.38 ± 0.34 nmol/mL, while the group given the ethanol extract of the fruiting body of *C. comatus* at a dose of 1.38 ± 0.34 nmol/mL was 750 mg 0.82 ± 0.22 nmol/mL.

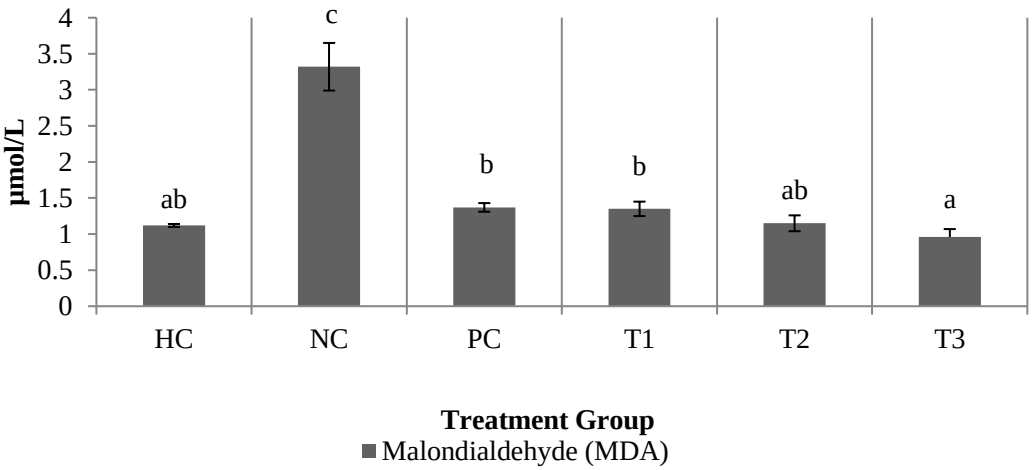

Figure 3. The MDA levels of the hyperglycemia rats treated with ethyl acetate extract of *C. comatus* fruiting body

Malondialdehyde is a biomarker of free radicals that enter the body (Permatasari et al., 2017). MDA is the end product of lipid peroxidation, which was toxic to cells. It also represents a dialdehyde compound with three carbon chains (Miletić et al., 2018). The lipid peroxidation occurs in the pancreatic β cells because pancreatic β cells contain low enzymatic antioxidant concentration. It has a significant impact on MDA levels because the peroxidation reaction continued. MDA is an indicator of lipid

peroxidation, representing a key event in liver cell destruction caused by oxidative stress (Stilinović et al., 2020b). The highest mean MDA level was found in the NC group (Figure 3), indicating that the rats experienced oxidative stress.

The MDA levels of the diabetic rats treated using the ethanol extract of *C. comatus* at the doses of 500, 750, and 1000 mg/kg BW decreased (Ratnaningtyas et al., 2019). It indicated the decrease in lipid peroxidation activity in cell membranes and the recovery of pancreatic β cell damage. MDA has been widely used to detect oxidative stress status (Zhao et al., 2018), as SOD activity. The antioxidants compounds of the extract, such as flavonoids compounds, play an essential role in neutralizing lipid peroxidation that subsequently causes the decrease in MDA levels (Ratnaningtyas et al., 2018). It is an indication of the improvement of the health of the rats and the pancreatic β cells. It is corroborated by the data of the decrease in the blood glucose levels and the weight gain in the diabetic group of rats treated with *C. comatus* fruit body extracts (Ratnaningtyas et al., 2019).

Antioxidant oxidant activity assay

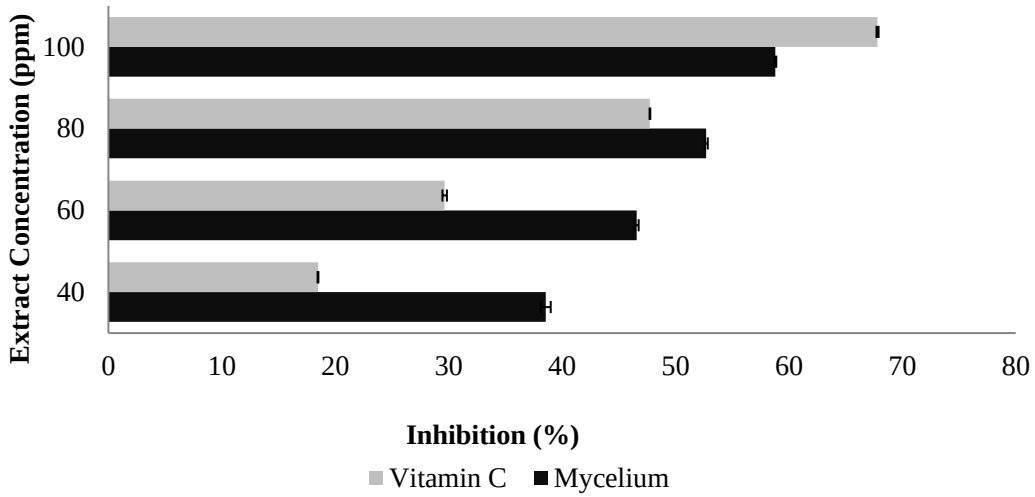

Figure 4. The inhibition percentage of the ethyl acetate extract of *C. comatus* mycelium

shown by the increase in the activity of the SOD enzyme in diabetic rats after applying the extract. The decrease in the MDA levels can result from the presence of the flavonoids, which can donate H^+ ions (Figure 3).

According to Permatasari et al. (2017), the presence of free radicals triggers lipid peroxidation in cell membranes. The end product of lipid peroxidation chain reactions is MDA. The results of MDA measurements showed that the ethyl acetate extract of *C. comatus* and metformin showed that MDA levels of the treatment group were statistically almost the same as the MDA levels of the healthy group. The extract dosage variations of 250, 500, and 750 mg/kg BW caused a significant difference in reducing MDA levels of diabetic rats. The ethyl acetate extract of *C. comatus* at the dose of 500 mg/kg BW effectively reduced the MDA levels of diabetic rats close to normal (Figure 3).

The free radicals of O^{2-} and peroxynitrite ($ONOO^-$) become neutral, and the minerals of Cu^{2+}, Zn^{2+}, and Mn^{2+} in the extract can increase

The results of the *in vitro* test against DPPH radical inhibition using *C. comatus* mycelium ethyl acetate extract showed a significant value ($p<0.05$) compared to vitamin C. The concentrations of *C. comatus* mycelium ethyl acetate extract and vitamin C were 40, 60, 80, and 100 ppm. The results showed that the inhibition of mycelium extract at 40, 60, 80, and 100 ppm were 38.56 ± 0.44%, 46.50 ± 0.17 %, 52.72 ± 0.13 %, and 58.81 ± 0.1% respectively. Meanwhile, vitamin C showed inhibition value against DPPH radicals at a concentration of 40, 60, 80, and 100 ppm by 18.48 ± 0.03 %, 29.65 ± 0.19 %, 47.75 ± 0.03 %, and 67.82 ± 0.09% respectively. The results of the antioxidant assay are illustrated in Figure 4. Previous research showed that the administration of 2000 ppm ethanol extract and n-hexane of *C. comatus* fruiting bodies could inhibit DPPH radicals. In contrast, ethanol extracts inhibited 70% of DPPH radicals, higher than n-hexane extracts (Susanto et al., 2018).

The antioxidant extract trapped the free radical, and its concentration becomes stable, and the more

effective the antioxidant extract would be in inhibiting the free radical, as indicated by the inhibition percentage of each concentration. The ethyl acetate extract at the concentration of 100 ppm showed the highest percentage, while the mean percentage in blocking the free radical was 58.51% (Figure 4). The ethyl acetate extract of the cap of the fruiting body showed the scavenging ability of 57.9% at one ppm, while the scavenging capacity of the ethyl acetate extract of the stipe was 22.7% at one ppm. Thus, the cap extract showed better scavenging ability in the antioxidant oxidant assay than extracts of the stipe (Li et al., 2010).

Antioxidants are beneficial compounds able to recover oxidative damage (Handayani et al., 2014). The study used *C. comatus* fungal mycelium extracted using the maceration method representing one of the cold extraction methods (Ibrahim & Rusli, 2010). The maceration method used in this study was ethyl acetate solvent. The advantage of the technique is that its procedure is simple, and the required are were easy to obtain (Handayani et al., 2014).

Figure 5 also illustrates the ability of the antioxidant compound of *C. comatus* and the vitamin C extracts to block free radicals. The ethyl acetate is a solvent easily applied in polyphenol extraction with a low toxicity level (Wissam et al., 2012). The value of IC_{50} is important to evaluate in determining the ability of the ethyl acetate fraction to eradicate free radicals (El Ouadi et al., 2017). The ethyl acetate solvent is a semi-polar one that could attract semi-polar antioxidant compounds such as flavonoids (Tanaya & Retnowati, 2015).

We established the IC_{50} value by substituting the concentration of the extract into the x-axis and the mean inhibition percentage into the y-axis of a potential linear regression application (Figure 5). The antioxidant properties resulting from the assay were summarized in Table 3 and then normalized and expressed as IC_{50} values (mg/L). The table shows that the IC_{50} value of the mycelium extracts is considered to have vigorous antioxidant activity. The effectiveness of the antioxidant properties is inversely correlated to their IC_{50} values (Li et al., 2010). The IC_{50} value is the value of the antioxidant concentration in blocking 50% of free radical activity (Sami & Rahimah, 2016). This study has identified polyphenols, flavonoids, alkaloids, and terpenoids as the bioactive compounds in ethyl acetate extract of *C. comatus* of mycelium cultures and fruiting body. Mycelium ethyl acetate extract has vigorous activity as an antioxidant with a high IC_{50} value. Fruiting body extract administered to hyperglycemia rats increase the levels of GPx, SOD and reduce the MDA levels. Increasing antioxidant levels is especially important in hyperglycemia conditions because it can prevent a free radical attack on pancreatic β cells. The information demonstrates the potential of *C. comatus* as a candidate for an antioxidant supplement for DM sufferers. However, further research on the bioactive compounds of *C. comatus* through compound fractionation is needed, and the development of *C. comatus* cultivation needs to be increased. Aside from that, the consumption of antioxidant supplements, which can increase the endogenous antioxidant content in the body, is helpful to minimize free radicals attack to the body. This research provides scientific information regarding the bioactive compounds of *C. comatus*, which can benefit the development of *C. comatus* as a potential candidate of antioxidant supplement that can scavenge free radicals and increase endogenous antioxidants.

CONCLUSION

This study identified bioactive compounds from the mycelium and the fruiting body of the *C. comatus* ethyl acetate extract using a qualitative identifying method that involved polyphenols, flavonoids, terpenoids, alkaloids, and saponins. Endogenous glutathione peroxidase, catalase, and superoxide dismutase were significantly increased after applying *C. comatus* extracts. *C. comatus* extracts had also decreased the malondialdehyde (MDA) levels. The results of the *in vivo* evaluation corroborated those of the *in vitro* evaluation that the ethyl acetate extract of the *C. comatus* mycelium could significantly inhibit the free radicals in the antioxidant oxidant assay and had vigorous antioxidant activity.

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