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Influence of ultrasound on *Pleurotus pulmonarius* (Fr.) Quél. (Grey Oyster Mushroom) – Protective Effects Against Metabolic Syndrome in Rats Fed with a High-fat Diet

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ABSTRACT

The *Pleurotus pulmonarius* (Fr.) Quel. (Grey Oyster mushroom) is one of the most widely consumed mushroom species worldwide, and it possesses various medicinal properties. The application of ultrasound resulted in *P. pulmonarius* extracts with improved bioactivities both in cellular and animal models. However, no studies have investigated the preventive effects of ultrasound extract of *P. pulmonarius* in animal models. In this study, Wistar-Kyoto rats were pre-treated with the ultrasound extract of *P. pulmonarius* at high (500 mg/kg bw) and low (200 mg/kg bw) doses for 15 days. The rats were subsequently fed with a high-fat diet (HFD) for another 15 days. Rats pre-treated with *P. pulmonarius* extract from a high-dose ultrasound showed lower levels of serum cholesterol (1.50 mmol/L), triglyceride (0.60 mmol/L), and LDL (0.29 mmol/L). These rats also had lower oxidative stress, with MDA levels of only 10.81 µg/mg protein. The ultrasound extract of *P. pulmonarius* also exhibited anti-hyperglycaemic effects, whereby rats had lower blood glucose levels. Rats pre-treated with the ultrasound extract of *P. pulmonarius* also had inhibited inflammatory biomarkers CREB1 (2.85 pmol/mL), NF-κB2 (0.81 ng/mL), and STAT3 (0.48 ng/mL), proving the anti-inflammatory activity

of the ultrasound extract. This study is the first to show the ability of *P. pulmonarius* extract prepared using ultrasound to prevent developing metabolic syndrome such as hyperlipidaemia, hyperglycaemia, oxidative stress, and inflammation in rats fed with HFD.

Keywords: cardiovascular, fungal metabolites, green, extraction, ultrasound, mushroom, natural products

INTRODUCTION

Metabolic syndrome is a cluster of metabolic abnormalities, and it is correlated with type II diabetes mellitus (T2D) and cardiovascular diseases (CVD) (Xu et al., 2019). Some of the most prominent elements of metabolic syndrome include obesity (especially central adiposity), hyperglycaemia, hypercholesterolaemia, increased triglyceride levels, low high-density lipoprotein (HDL)-cholesterol, and hypertension (Dommermuth & Ewing, 2018; Sperling et al., 2015). It is also associated with other co-morbidities such as proinflammatory and prothrombotic conditions, as well as nonalcoholic fatty liver disease (NAFLD) (Dommermuth & Ewing, 2018; Wang et al., 2020). The development of metabolic syndrome involved several complex biochemical pathways, resulting in excess reactive oxygen species (ROS), endothelial dysfunction, atherosclerosis (Van Gaal et al., 2006), dysregulated glucose and lipid metabolisms (Hajer et al., 2008; Van Gaal et al., 2006), inflammation (Dandona et al., 2006), and the activation of transcription factors like activator protein-1 (AP1) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Haddad, 2002; Sukkar & Rossi, 2004).

Crude extracts or isolated compounds obtained from edible mushrooms have been shown to improve cardiovascular and metabolic conditions with their anti-diabetic, anti-inflammatory, cardioprotective, and hypoglycaemic activities (Tung et al., 2020). The *Pleurotus pulmonarius* (Fr.) Quél (Pleurotaceae, Agaricomycetes) is the most cultivated mushroom species from the genus Pleurotus (dos Santos Bazanella et al., 2013). Various studies have proven that *P. pulmonarius* has medicinal properties, including anti-diabetic, anti-inflammatory, antioxidative, and hypolipidaemic (Balaji et al., 2020; Zainal Abidin et al., 2016a, 2018). Our previous investigations showed that *P. pulmonarius* extracts obtained using ultrasound had improved composition and in vitro bioactivities. The ultrasound extracts from *P. pulmonarius* had more varieties of organic acids and phenolic compounds, and hydroxybenzoic acid compounds were only detected in ultrasound extracts, but not in the extract prepared using the conventional method (Soxhlet) (Amirullah et al., 2020). The ultrasound extract of *P. pulmonarius* was also shown to decrease metabolic syndrome in rats fed with a high-fat diet (HFD) (Amirullah et al., 2021). Ultrasound-assisted extraction (UAE) involves cavitation that induces physical and chemical changes, which facilitates the diffusion of biocompounds into the liquid solvent (Tao et al., 2019). Ultrasound shortens extraction time and requires fewer solvents; thus, it is deemed a more rapid, efficient, economical, and eco-friendly method than most conventional extraction techniques (Awad et al., 2012; Tao et al., 2019).

No studies have investigated the preventive activities of the *P. pulmonarius* extract. To our knowledge, only one study has investigated the preventive effects of *P. pulmonarius* in an animal model (Lavi et al., 2010). However, Lavi et al. (2010) studied the preventive effects of *P. pulmonarius* for a model of dextran sulfate sodium (DSS)-induced colitis in mice, not for metabolic syndrome. Therefore, this study aims to determine the potential of the ultrasound extract of *P. pulmonarius* to prevent the development of metabolic syndrome in rats. The rats were pre-treated with the ultrasound extract, followed by high-fat diet (HFD) treatment. The lipid and glucose levels, oxidative stress, and inflammatory biomarkers of the rats were evaluated to determine whether the ultrasonic extract of *P. pulmonarius* could prevent the development of metabolic syndrome in rats fed with HFD.

MATERIALS AND METHODS

Mushroom Material

The fruiting bodies of *P. pulmonarius* were procured from Vita Agrotech Sdn. Bhd., Malaysia. The species were ascertained based on its morphology and DNA sequence. A mushroom culture was submitted to the Mycology Laboratory, Universiti Malaya (registration number: KUM61119). A specimen voucher was given to the Universiti Malaya Herbarium (registration number: KLU-M1234).

Preparation of Extract

Fruiting bodies of *P. pulmonarius* were dried (40°C) and ground into a powder form. Ultrasound-assisted extraction (UAE) (140 W, 50 min) was carried out based on a previous study (Amirullah et al., 2020) using an ultrasonic homogeniser (Sonopuls HD 3400, VS 200 T probe, 25 mm diameter, Bandelin, Berlin, Germany). The extract was rotary-evaporated (BUCHI) and stored at 4°C.

Animal Studies Using Wistar-Kyoto Rats

Seven-week-old male Wistar-Kyoto (WKY) rats were obtained from the Animal Experimental Unit, Faculty of Medicine, Universiti Malaya. The rats were acclimatised in the Laboratory Animal Centre (LAC) housing site for 14 days. The LAC was maintained at 24±2°C with 12 h of light and 12 h of the dark cycle, and food and water were given ad libitum. All procedures were approved by the Universiti Malaya Institutional of Animal Care and Use Committee (UM IACUC). The ethics number is S/04012018/30082017-01/R.

Animal Diet

The Wistar-Kyoto rats were provided with a 1324 diet (10 mm pellet), cereal-based standard diet (soy, wheat, and corn) (Altromin, Germany) (this formula did not include nitrosamines). The C 1090 – 60 variation was used for the high-fat diet (Altromin, Germany).

Experimental Design

Nine weeks male Wistar-Kyoto rats were divided into five groups, with six rats in each group. Rats were administered various treatments of distilled water (normal and hyperlipidaemia control (HC) groups), 10 mg/kg bw simvastatin, 500 mg/kg bw of *P. pulmonarius* extract (high dose group), and 200 mg/kg bw of *P. pulmonarius* extract (low dose group) from 1 to 15 days while they were fed with the normal diet. The distilled water, simvastatin, and ultrasound extract were given through oral gavage using a curved feeding needle (G18 x 2"). The normal control (NC) rats were fed with a normal diet from days 1 to 30. From day 16 until day 30, hyperlipidaemia was induced in rats in the HC, simvastatin, high-dose, and low-dose groups by feeding the rats with HFD. From days 16 to 30, rats in the HC, simvastatin, high dose, and low dose groups were not given any more treatments. Water was provided ad libitum throughout the experiment. On day 31, all rats were euthanized. The preventive experimental design is shown in Figure 1. The groups and their respective treatments are as follows:

Group I (Normal control, NC):
Standard diet + distilled water (days 1 – 30)
Group II (Hyperlipidaemia control, HC):
Normal diet + distilled water (days 1 – 15)
High-fat diet (days 16 – 30)
Group III (Simvastatin):
Normal diet + 10 mg/kg bw simvastatin (days 1 – 15)
High-fat diet (days 16 – 30)
Group IV (High dose *P. pulmonarius* extract):
Normal diet + 500 mg/kg bw of *P. pulmonarius* extract (days 1 – 15)
High-fat diet (days 16 – 30)
Group V (Low dose *P. pulmonarius* extract):
Normal diet + 200 mg/kg bw of *P. pulmonarius* extract (days 1 – 15)
High-fat diet (days 16 – 30)

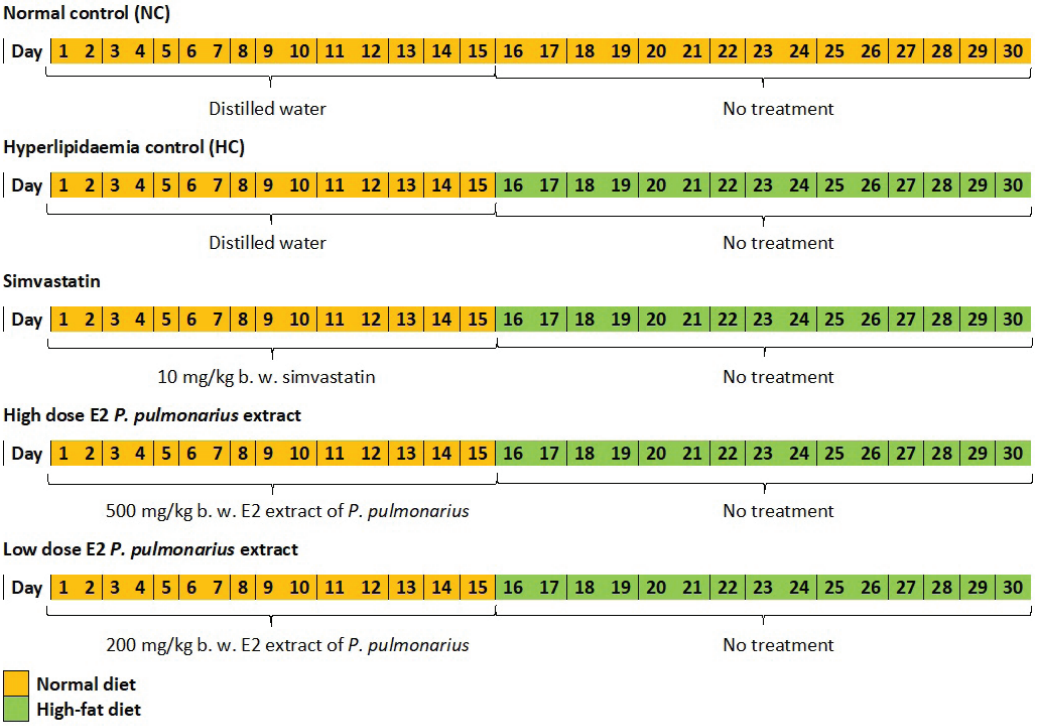

Figure 1 The experimental prevention setup. From days 1 until 15, the rats were fed with a normal diet and distilled water (normal control group (NC) and high-fat diet control (HC) group), normal diet and simvastatin (simvastatin control group), normal diet and 200 mg/kg bw of E2 *Pleurotus pulmonarius* extract (low dose group), or 500 mg/kg bw of E2 *P. pulmonarius* extract (high dose group). From days 16 until 30, the NC group received a normal diet, while rats in the HC, simvastatin, low-dose and high-dose groups received a high-fat diet—abbreviations: body weight, bw; high-fat diet control, HC; normal control, NC.

Euthanization

The rats were euthanized based on previous methods (Amirullah et al., 2021). Briefly, the rats were anaesthetized using ketamine/xylazine, then a cardiac puncture was performed to obtain 3 mL of blood. After a cardiac puncture, the rats were euthanized by carbon dioxide (CO_2) asphyxiation. The blood was allowed to clot for 10 min, then it was centrifuged at 1400 rpm (KUBOTA) for 10 min to obtain the serum. The serum was stored at 20 °C.

Determination of Serum Total Cholesterol, Triglycerides and LDL

Serum samples were submitted to the haematology laboratory, Veterinary Laboratory Service Unit (VLSU), Universiti Putra Malaysia, for analyses, as was previously described (Amirullah et al., 2021). The analysed parameters were the total cholesterol, triglycerides, and low-density lipoprotein (LDL)-cholesterol.

Determination of Fasting Blood Glucose

Blood glucose was determined using a glucometer and glucose strips (ACCU-Chek® Active). The rats were fasted overnight, but water was made available. Readings were taken at the beginning of the experiment (Week 0) and continued every week (Week 1, Week 2, Week 3, and Week 4).

Preparation of Liver Tissue

An estimated 100 mg of liver were homogenised in 2.5 mL of cold PBS (pH 7.4) using a cold tissue homogeniser. The mixture was transferred into a 2 mL tube and was centrifuged at 5000 rpm (10 min). The supernatant was kept in a 500 µL tube while the precipitate was disposed of. Liver homogenates were freshly prepared for each analysis.

Measurement of Lipid Peroxidation

The level of lipid peroxidation was determined using a previous method with modifications (Rahman et al., 2014). Trichloroacetic acid (TCA) (500 µL, 15% (w/v)) and 1 mL of 1% (w/v) thiobarbituric acid (TBA) were added into 1 mL liver homogenate, and the mixture was heated in a water bath at 95°C for 30 min. The absorbance values of the cooled sample (300 µL) were read at 532 nm. A standard curve of malondialdehyde bis-(dimethyl acetal) (Merckmillipore) was used.

Detection of Inflammatory Markers Using Enzyme-Linked Immunosorbent Assay (ELISA)

Transcription factors CAMP responsive element binding protein 1 (CREB1), NF-κB2, and signal transducer and activator of transcription 3 (STAT3) were detected using enzyme-linked immunosorbent assay (ELISA) kits from Elabscience. The used kits were: rat CREB1 kit (catalogue number E-EL-R0289), rat NF-κB2 kit (catalogue number E-EL-R2397), and rat STAT3 kit (catalogue number E-EL-0118). The methods of detection were as previously described (Amirullah et al., 2021).

Histology Analysis

Rat organs were collected and washed in a cold saline solution. Histology preparations were made according to previous studies (Amirullah et al., 2021; Razali et al., 2016). The organs were stored in 10% formalin and were fixed for 24 h. The organs were dehydrated using 70%, 75%, 85%, and 95% ethanol (30 min at each concentration). Then the organs were soaked in terpineol for 30 min, twice. The organs were transferred into terpineolparaffin solution (1:1) (60°C, 30 min). Next, the organs were submerged in paraffin (60°C, 1 h). This step was repeated twice. Once the paraffin had cooled, the

paraffin blocks were sliced into 5 µm thick sections. Mayer's albumin was spread on slides, and thin organ sections were appended on top. The organ sections were stained with hematoxylin and eosin (H&E), and the tissues were observed.

Statistical Analyses

All data are expressed as the standard deviation (SD) of triplicates from three independent experiments. Data from the assays were analysed using a one-way analysis of variance (ANOVA). Significant differences were determined using Tukey's honestly significant difference (HSD) at a 95% confidence interval (p-value < 0.05) using the RStudio Version (2015) (R Development Core Team, 2008). Graphs were created using GraphPad Prism version 5.00 for Windows (GraphPad Prism, 2010).

RESULTS AND DISCUSSION

According to Grundy (2016), metabolic syndrome constitutes a complex set of risk factors contributing to the development of atherosclerotic cardiovascular disease (ASCVD) and type II diabetes mellitus. The metabolic syndrome encompasses five components related to ASCVD: atherogenic dyslipidemia, increased blood pressure, dysglycemia, a pro-thrombotic condition, and a pro-inflammatory condition (Grundy, 2016). In addition to that, oxidative stress has been associated with both diabetes and atherosclerosis (Petrie et al., 2018). Thus, this study was conducted to observe the effects of pretreatment of *P. pulmonarius* on the body weight, glucose levels, lipid levels, oxidative stress, and pro-inflammatory biomarkers of rats fed with HFD.

Previously we investigated the bioactivities of *P. pulmonarius* extracts prepared using ultrasound and conventional (Soxhlet) extraction techniques in a cellular model (murine macrophage cells, RAW264.7 cells). The ultrasound extracts showed better anti-inflammatory and antioxidant activities in the RAW264.7 cells compared to the Soxhlet extract (Amirullah et al., 2020). Based on these findings, we further investigated the therapeutic effects of the ultrasound extract in an animal model (WKY rats) (Amirullah et al., 2021). Prior to this, no study had investigated the bioactivities of ultrasound extract of *P. pulmonarius* on an animal model.

Several studies have shown the effects of *P. pulmonarius* extracts in alleviating aspects of metabolic syndrome (Balaji et al., 2020; Nguyen et al., 2016). However, in these studies, conventional extraction methods were used. Balaji et al. (2020) used the Soxhlet technique with acetone and water to obtain extracts of *P. pulmonarius* (the extraction time was not specified). Nguyen et al. (2016) used the maceration technique with 80% methanol and 3 days of extraction to obtain the extracts of *P. pulmonarius*. In comparison to these methods, the ultrasound extraction used in the current study only took 50 min to obtain the *P. pulmonarius* extract.

Currently, there is interest in utilising greener extraction techniques to obtain bioactive compounds from natural sources. These alternative techniques are more cost-effective and can shorten extraction time, with lowered extraction temperature (Vinatoru

et al., 2017). One research utilised ultrasound to obtain extracts of *P. pulmonarius*, but the bioactivities examined were limited to in vitro studies only (Milovanovic et al., 2021). Other studies used microwave-assisted extraction (MAE) to obtain *P. pulmonarius* extracts, but again, the studies were limited to in vitro models only (Elfirta et al., 2023; Gil-Ramírez et al., 2019).

In our previous study, results from liquid chromatography-tandem mass spectrometry (LCMS/MS) analyses showed that the extract prepared using these ultrasound parameters contained ascorbic acid, malic acid, pyroglutamic acid, cinnamic acid and its hydroxycinnamic acid derivatives (coumaric acid derivatives, p-hydroxycinnamoyl derivative, and caffeic acid derivatives), and gallic acid (hydroxybenzoic acid). Interestingly, hydroxybenzoic acid and its derivates were only found in the ultrasound extracts (Amirullah et al., 2020).

Ascorbic acid is recognised for its antioxidant properties. It functions as an effective scavenger or neutraliser for various types of oxygen and nitrogen-reactive species (Carr & Frei, 1999). Malic acid, a dicarboxylic acid synthesised by all living organisms, occurs naturally in fruits and vegetables (Barros et al., 2013). Malic acid has also been detected in certain oyster mushroom species, including *Pleurotus eryngii* (DC.) Quél and *Pleurotus ostreatus* (Barros et al., 2013). Numerous hydroxycinnamic compounds have been identified as having antioxidant, anti-inflammatory, and anti-cancer properties (Razzaghi-Asl et al., 2013), while hydroxybenzoic acid compounds are recognised for their antioxidant and anti-inflammatory properties (Masella et al., 2012; Velika & Kron, 2012). The presence of these organic acids and phenolic compounds could potentially explain the therapeutic effects of *P. pulmonarius* extract.

Our previous investigation also proved the ultrasound extract had a lethal dose (LD_{50}) of more than 2,000 mg/kg bw, as per the Organisation for Economic Co-operation and Development (OECD) Test No. 425: Acute Oral Toxicity: Up-and-Down Procedure ("Test No. 425: Acute Oral Toxicity: Up-and-Down Procedure," 2008). This shows that the ultrasound extract was not toxic towards the animal model used in this study (Amirullah et al., 2021).

We previously investigated the treatment effects of *P. pulmonarius* ultrasound extract on WKY rats treated with HFD. In the treatment model, the rats were concurrently fed with HFD and the *P. pulmonarius* extract. Rats treated with the *P. pulmonarius* extract had reduced lipid levels, as demonstrated through their serum lipid profiles and histological analyses. Additionally, these rats exhibited lowered lipid peroxidation and inflammatory biomarkers (Amirullah et al., 2021). In this current investigation, we were interested to see the preventive potential of *P. pulmonarius* extract. In the prevention model, the animal models were fed with the compound of interest first, followed by the induction of disease conditions.

Very little research has been conducted to investigate the preventive potential of *P. pulmonarius* extracts. As far as we are aware, only one study has used the preventive experiment in an animal model (Lavi et al., 2010). In one of the animal groups, Lavi et al. (2010) pre-treated female BALB/c mice with glucans of *P. pulmonarius* for 11 days, then induced colitis in the mice by administering DSS for the next 18 days. The glucans extract prevented the development of symptoms associated with DSS-

induced colitis in these mice, such as colonic shortening, increased myeloperoxidase (MPO), tumour necrosis factor α (TNF-α), and interleukin 1β (IL-1β). The study by Lavi et al. (2010) proved that *P. pulmonarius* glucans had the potential to prevent colitis development. However, no other studies have employed the preventive model to investigate the potential of *P. pulmonarius* extracts to prevent metabolic disorders such as hyperlipidaemia and hyperglycaemia.

As far as we are aware, this current study is the first to investigate the preventive potential of the ultrasound extract of *P. pulmonarius*. In the prevention experiment, the rats in the hyperlipidaemia control (HC), simvastatin, high dose, and low dose ultrasound extract groups received their treatments (water, simvastatin, and ultrasound extracts) along with normal diet from days 1 until 15. Then these rats were fed with HFD from days 16 until 30, by which time their treatments were stopped (Figure 1). This model was created to observe if the pre-treatment of the ultrasound extract could prevent metabolic disorder conditions from developing (or worsening), even when the rats had stopped receiving the mushroom extract and started HFD. By Week 4, the rats in the NC, simvastatin, high-dose, and low-dose ultrasound groups exhibited significantly lower body weights than the rats in the HC group (p-values < 0.05) (Figure 2). This proved that the pre-treatment of ultrasound extracts prevented the rats from gaining significantly higher weight, even when they stopped receiving the extract treatment.

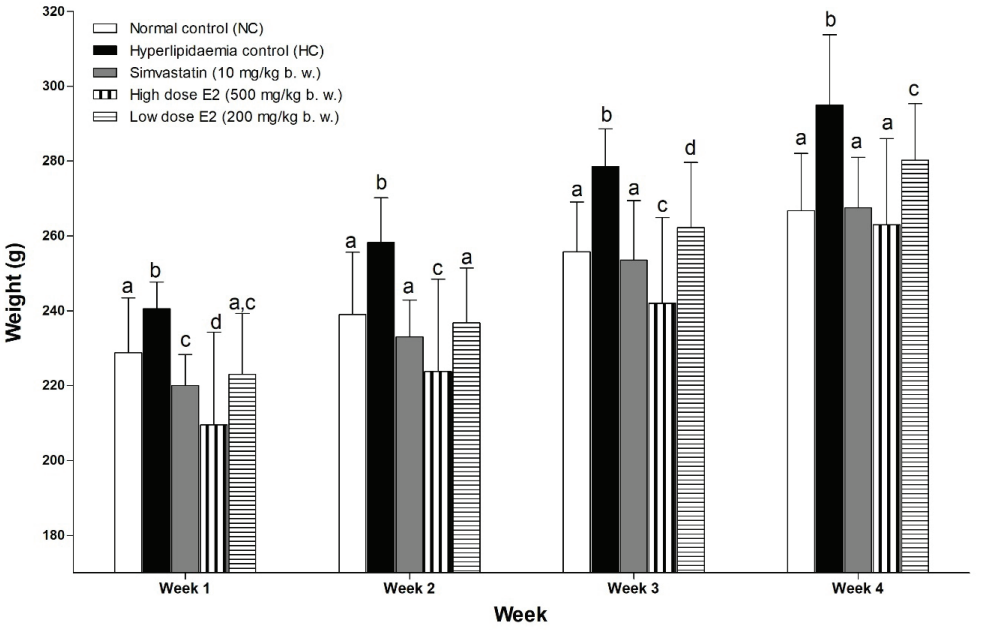

Figure 2 Rat body weights in the prevention experiment. Different letters indicate statistically significant differences between groups (analysis using one-way ANOVA with Tukey's HSD post-test, p-value<0.05).

Earlier research illustrated a similar observation: by 2 weeks, the rats fed with only HFD showed significantly higher body weight than rats fed with a normal diet (Huang et al., 2014). Likewise, another study also found that by 2 weeks, rats treated with atorvastatin and P. eryngii extracts had significantly lower body weights than rats which received only HFD (Huang et al., 2020). This proves that by 2 weeks of HFD treatment, the body weights of rats would be significantly increased, and 2 weeks of statin and mushroom treatments would be sufficient to lower body weights. Despite this, there were no significant differences in the weights of the hearts and livers of the rats in all the groups. This was probably due to the short HFD treatment in the prevention model (from days 16 until 30), thus not providing sufficient time for the organs to retain lipids (Table 1).

Table 1 The organ weights of rats in the prevention experiment

Group	Heart (g)	Liver (g)
Normal control (NC)	1.39 ± 0.34^{a}	10.21 ± 0.51^{b}
Hyper-lipidaemia control (HC)	1.24 ± 0.09^{a}	10.85 ± 2.50^{b}
Simvastatin (10 mg/kg bw)	1.31 ± 0.12^{a}	9.84 ± 1.93^{b}
High-dose extract (500 mg/kg bw)	1.08 ± 0.07^{a}	9.19 ± 0.96^{b}
Low-dose extract (200 mg/kg bw)	1.57 ± 0.38^{a}	10.84 ± 2.07^{b}

The difference was based on one-way ANOVA with Tukey's HSD post-test (p-value < 0.05). Different superscript letters denote significant differences for each column.

The results on body weight from this study prove the preventive effects of *P. pulmonarius*. This shows that *P. pulmonarius* could be consumed as a functional food for its health benefits (Zainal Abidin et al., 2016b). Pleurotus, commonly known as oyster mushrooms, is a genus of edible fungi highly valued for their flavour and texture (Ro et al., 2007). These mushrooms are rich in protein, carbohydrates, and minerals while being low in fat. They have a short life cycle and are cultivated extensively due to their adaptability and health benefits (Yildiz et al., 2002). Various species within the Pleurotus genus, including *P. eryngii*, *P. florida*, *P. ostreatus*, *P. pulmonarius*, and *P. tuber-regium* possess pharmacological properties (Ragunathan et al., 1996). This makes Pleurotus a sought-after choice in mushroom cultivation, offering a range of nutritional and health advantages.

In this investigation, hyperlipidaemia was induced by feeding rats with HFD from days 16 until 30. Three parameters of serum lipid profiles were measured: total cholesterol, triglycerides, and LDL (Table 2). Elevated serum cholesterol, triglycerides, and LDL concentrations are associated with an increased risk of CVD (Podszun et al., 2014; Reckless & Lawrence, 2003).

Rats in the hyperlipidaemia control (HC) group exhibited significantly higher levels of total cholesterol, triglyceride, and LDL compared to rats in the normal control (NC) group (p-values < 0.05) (Table 2). This proves that 2 weeks of HFD treatment were sufficient to elevate serum lipid levels in Wistar-Kyoto rats. Rats with a high dose of *P. pulmonarius* extract exhibited lower serum cholesterol, triglyceride, and

LDL concentrations than HC rats (p-values < 0.05). Compared with simvastatin, the mushroom extract obtained using ultrasound showed better cholesterol and LDL-lowering effects in HFD-fed rats (Table 2). Previous research findings had similar observations (Flamment et al., 2012). It was discovered that 2 weeks of HFD treatment were sufficient to increase the body weight and lipid levels of rats. The results from our study show that pre-treatment of Wistar-Kyoto rats with simvastatin or with the ultrasound extracts of *P. pulmonarius* could confer protective hypolipidaemic effects on these rats. The *P. pulmonarius* extract exerted its cholesterol-, triglyceride-, and LDL-lowering effects even after the rats stopped receiving the extract and began consuming HFD. These observations proved that the ultrasound extract had excellent preventive activities, especially at high doses. To our knowledge, no studies have investigated the hyperlipidaemic preventive effects of *P. pulmonarius* in HFD-treated rats.

Table 2 The serum lipid profiles (total cholesterol, triglycerides, and low-density lipoprotein (LDL)), liver peroxidation levels (MDA), and inflammatory biomarkers (CREB1, NF-κB2, and STAT3) of rats. Different superscript letters indicate statistically significant differences between groups in each column. The difference was based on one-way ANOVA with Tukey's HSD post-test (p-value < 0.05). Abbreviations: body weight, bw; malondialdehyde, MDA.

Group	Total cholesterol (mmol/L)	Triglyceride (mmol/L)	Low-density lipoprotein (LDL) (mmol/L)	MDA (μg/mg protein)	CREB1 (pmol/mL)	NF-κB2 (ng/mL)	STAT3 (ng/mL)
Normal control (NC)	1.63 ± 0.45^{a}	0.39 ± 0.15^{a}	0.25 ± 0.07^{a}	10.07 ± 0.85^{a}	1.87 ± 0.10^{a}	0.42 ± 0.01^{a}	1.19 ± 0.16^{a}
Hyper-lipidaemia control (HC)	3.40 ± 0.50^{b}	1.41 ± 0.46^{b}	0.49 ± 0.10^{b}	17.64 ± 1.68^{b}	8.47 ± 1.17^{b}	1.46 ± 0.09^{b}	1.58 ± 0.08^{b}
Simvastatin (10 mg/kg bw)	2.27 ± 0.61^{b}	0.70 ± 0.04^{a}	0.37 ± 0.04^{b}	6.91 ± 2.36^{a}	4.48 ± 0.78^{c}	0.88 ± 0.01^{a}	0.75 ± 0.23^{a}
High-dose extract (500 mg/kg bw)	1.50 ± 0.70^{a}	0.60 ± 0.04^{a}	0.29 ± 0.02^{a}	10.81 ± 1.41^{a}	2.85 ± 1.32^{a,c}	0.81 ± 0.01^{a}	0.48 ± 0.05^{a}
Low-dose extract (200 mg/kg bw)	1.57 ±0.25^{a}	0.55 ± 0.08^{a}	0.37 ± 0.04^{b}	6.19 ± 1.47^{a}	3.71 ± 0.45^{a,c}	0.75 ±0.04^{a}	0.57 ± 0.08^{a}

Elevated plasma glucose has been associated with metabolic syndrome. According to Grundy (2016), hyperglycaemia could lead to microvascular conditions, and chronic hyperglycaemia is also a risk factor for atherogenesis (Aronson & Rayfield, 2002; Grundy, 2016). In this experiment, the glucose levels of the rats were monitored every week, beginning at Week 0 before the rats were given any treatments until Week 4. All the rats in all groups (NC, HC, simvastatin, high dose extract, and low dose extract groups) had similar blood glucose levels from Week 0 until Week 2. By Week 3, only rats in the NC group had significantly lower glucose levels (4.20 mmol/L) compared to HC rats (33 mmol/L) (Figure 3). By Week 4, rats in the NC and the high ultrasound *P. pulmonarius* extract dose and low ultrasound *P. pulmonarius* extract dose groups had significantly lower glucose levels compared to rats in the HC group (p-values < 0.05) (Figure 3). This shows that the *P. pulmonarius* extract possesses glucose-lowering or anti-hyperglycaemic potential. A previous study suggested that mushroom polyphenols

could exert their protective antioxidative and anti-inflammatory effects on the liver, which might also affect glucose metabolism (Jeong et al., 2010; Thompson et al., 1984). However, further research still needs to be done to examine the preventive effects of mushroom extracts (Jeong et al., 2010).

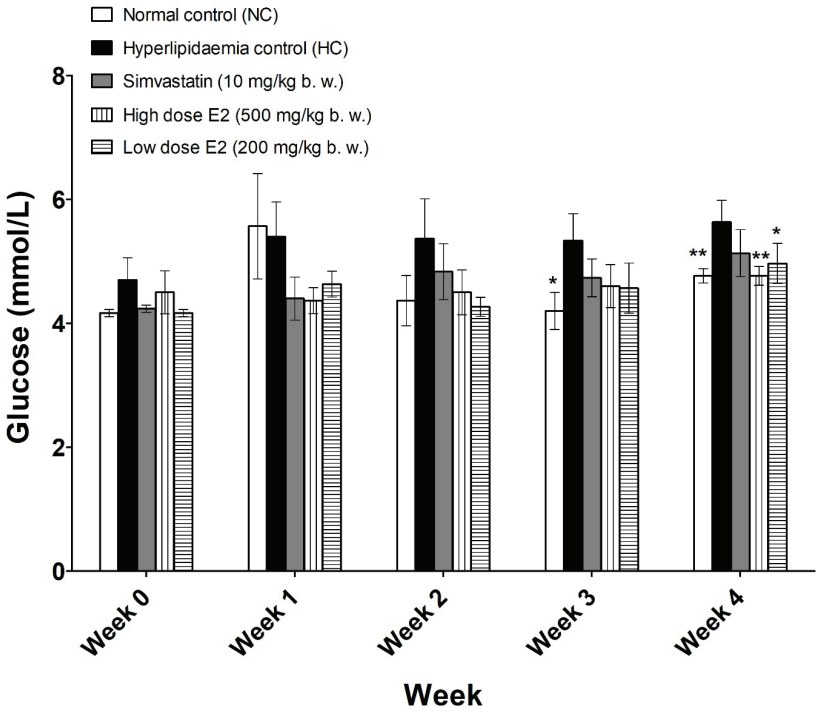

Figure 3 Serum fasting glucose levels of rats from Week 0 until Week 4. Asterisks indicate significant differences in pvalue (* pvalue < 0.05; ** pvalue < 0.01; *** pvalue < 0.001) compared to the hyperlipidaemia control (HC) group. The difference was based on one-way ANOVA with Tukey's HSD post-test (p-value < 0.05).

Histological analyses were done to examine the preventive effects of *P. pulmonarius* on the liver of the Wistar-Kyoto rats. A normal, healthy liver has an architecture in which the hepatocytes form a cord-like pattern that radiates outwards from the hepatic central vein (yellow arrow), as seen in Figure 4. Normal hepatocytes are polygonal in shape (black arrow), with obvious boundaries between cells and intertwining sinusoids among the cells. These structures could be seen in the liver tissue of the normal control (NC) group at 400X magnification. The HC group also had a radiation arrangement of the hepatocytes (yellow arrow), but obvious vacuolisation could also be seen at 100X magnification (white arrows). Vacuolisation of hepatocytes occurs due to lipid accumulation in the cells (Lefkowitch, 2020). At 400X magnification, the liver tissues of rats in the HC group had more prominent vacuolisation of the hepatocytes (white arrows) and inflammation (dashed arrows). At 100X magnification, the livers of rats in the simvastatin, high-dose extract, and low-dose extract groups had

similar radiation patterns as that of the NC group (yellow arrows). At 400X magnification, the livers of rats in the simvastatin, high-dose, and low-dose groups had less hepatocyte vacuolisation (white arrows), and more normal cells were observed (black arrows).

Steatosis (fatty liver disease) could develop from a high-fat diet, and it is characterised by an abnormal buildup of lipids in hepatocytes. Lipids can accrue in hepatocytes due to high triglyceride intake/synthesis, or due to diminished triglyceride removal (Lefkowitch, 2020). A diet rich in fat could induce multiple types of vacuolisation in hepatocytes (Hassan et al., 2018). These vacuolated cells contribute to distorted hepatocyte structures. Histological analyses of our study showed that even after 2 weeks of HFD treatment, vacuolisation of hepatocytes had occurred. The histology analyses proved that HFD treatment affected the cellular structures of hepatocytes in the HC rats. The serum lipid assays showed that the HFD did cause an increase in lipid profiles (Table 2), and the histological analyses confirmed the results of the lipid profiles (Figure 4). A previous study also showed that 2 weeks of HFD was adequate to induce vacuolisation of hepatocytes (Flamment et al., 2012).

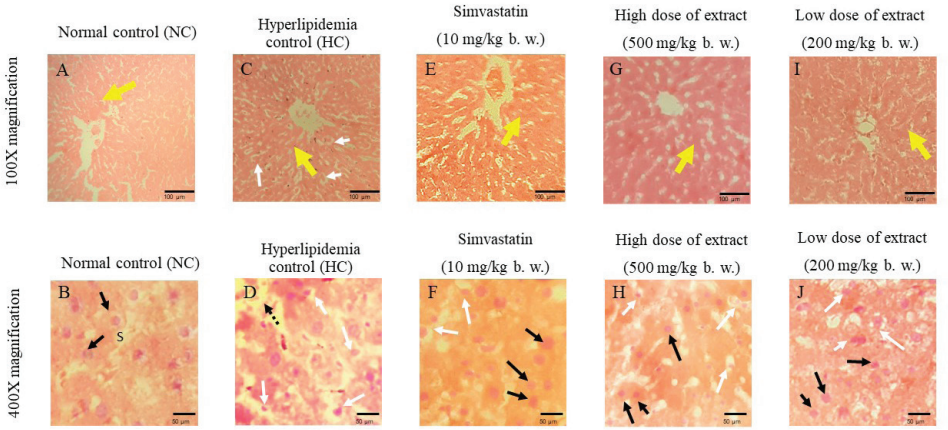

Figure 4 The cross-section of livers of rats (100× and 400× magnification; scales 100 µm and 50 µm respectively). Symbols: radiating pattern (yellow arrows); vacuolated hepatocytes (white arrows); sinusoids (S); normal hepatocytes (black arrows); vacuolated hepatocytes (white arrows); inflammatory foci (dashed arrows). Abbreviation: body weight, bw.

The rats in the simvastatin, *P. pulmonarius* high dose and low dose groups exhibited radial-shaped hepatocyte arrangements. However, some vacuolisation could still be seen. This shows that the simvastatin and *P. pulmonarius* extracts could limit lipid uptake by the hepatocytes. The hepatocytes in these groups also showed lower levels of vacuolisation compared to the liver structures of rats in the HC group. This might be the first study to show the preventive effects of *P. pulmonarius* in reducing lipid uptake in hepatocytes.

Oxidative stress has been associated with glucotoxicity in diabetes (Petrie et al., 2018), as well as atherogenesis (Steinberg, 2009). Lipid peroxidation is a process where reactive oxygen species (ROS) react with unsaturated lipids, generating various oxidation products. One significant outcome is the production of lipid hydroperoxides

(LOOH) (Ayala et al., 2014). Malondialdehyde (MDA) is a prominent aldehyde by-product of this process, widely used as an indicator of oxidative stress in diverse samples like human and animal tissues, fluids, drugs, foods, and natural products (Boligon, 2014). MDA reacts with thiobarbituric acid (TBA) to form thiobarbituric acid reactive species (TBARs), a product with intense pink or red colour. MDA is one of the biomarkers often used to measure oxidative stress in various biological samples (Boligon, 2014; Giera et al., 2012). The rats in the NC, simvastatin, high *P. pulmonarius* extract dose, and low *P. pulmonarius* extract dose groups exhibited significantly decreased MDA levels compared to rats in the HC group (p-values < 0.05) (Table 2). These results proved that simvastatin and ultrasound extract of *P. pulmonarius* possess protective antioxidant effects on the rats. HFD treatment induces oxidative stress while reducing antioxidant defences (Kakimoto & Kowaltowski, 2016). A previous study also showed that oral administration of polysaccharides from *P. pulmonarius* for ten days exhibited a protective effect on BALB/c mice, even after the mice stopped receiving polysaccharide treatment (Lavi et al., 2010).

Extensive experimental evidence substantiates the connections between inflammation, immune system responses, metabolic dysfunction, elevated blood pressure, and cardiovascular health issues (Petrie et al., 2018). Inflammation could also be induced by a diet high in fat content, which could potentially develop through cholesterol crystals. These cholesterol crystals in atherosclerotic lesions can enhance IL-1β secretion by macrophages, thus associating cholesterol metabolism with inflammation (Rajamäki et al., 2010). Among phosphorylation-dependent transcription factors, the cAMP-response binding element protein (CREB) has been extensively investigated (Wen et al., 2010). There is consistent upregulation in hepatic CREB activity in diabetic conditions, which plays a role in the development of hyperglycaemia and insulin resistance (Qi et al., 2009). Besides CREB activity, irregular NF-κB activity also plays a role in various inflammatory, autoimmune, and cancer-related conditions such as rheumatoid arthritis, atherosclerosis, multiple sclerosis, and inflammatory bowel disease (Karin et al., 2002; Tak & Firestein, 2001). NF-κB is a crucial transcription factor that governs the crosstalk between cytokines, adhesion molecules, and growth factors (Pamukcu et al., 2011). Frequently, STAT3 is associated with cardiovascular disorders. A study involving network analysis discovered that both STAT3 and NF-κB play essential roles in signalling pathways in the development of coronary artery disease (CAD) (Nair et al., 2014). Due to these factors, in this study, the transcription factors CREB1, NF-κB2, and STAT3 were measured.

The HC rats had significantly higher CREB1, NF-κB2, and STAT3 concentrations than the NC rats (Table 2). This shows that 2 weeks of HFD treatment increased inflammatory biomarkers in the rats. Simvastatin and *P. pulmonarius* extracts suppressed CREB1, NF-κB2, and STAT3 in HFD-fed rats. These results display that *P. pulmonarius* ultrasound extracts possess preventive effects like simvastatin. One study proved that the oral administration of *P. pulmonarius* polysaccharide could protect mice from secreting inflammatory biomarkers such as IL-1β and TNF-α (Lavi et al., 2010). Considering that the *P. pulmonarius* extract in this study also displayed remarkable antioxidant activities in suppressing MDA, it is unsurprising that it also demonstrated

anti-inflammatory activities. Preceding research suggested that a compound's anti-inflammatory activities might be partially due to its antioxidative properties (Kroes et al., 1992).

Despite these findings, the exact mechanisms that were affected by *P. pulmonarius* extract could not be elucidated. The transcription factors CREB1, NF-κB, and STAT3 are involved in multiple pathways related to several biological processes. There are also crosstalks between the activation pathways of these transcription factors (Grivennikov & Karin, 2010; Ruiz et al., 2016). Thus, further studies need to be done to further understand what mechanisms were affected at the molecular levels.

CONCLUSION

This study investigated the potential of *P. pulmonarius* extract to prevent metabolic disorders in HFD-treated rats. Rats pre-treated with the ultrasound extract had lower total cholesterol, triglyceride, and LDL concentrations, decreased blood glucose levels, suppressed MDA formation, and lowered CREB1, NF-κB2, and STAT3 levels. Even though the rats received HFD after stopping the treatment with the mushroom extracts, the rats still benefitted from the health properties of the extract. Atherosclerotic factors such as oxidative stress, inflammation, and hyperlipidaemia might be connected or interact with each other. One factor could trigger or worsen another atherogenic factor. Reactive oxygen species could directly cause endothelial injury while simultaneously triggering a chronic inflammatory state (Steinberg, 2009). Hyperlipidaemia can also contribute to an inflammatory condition (Björkbacka et al., 2004). Having shown to be on par or better than simvastatin, the hypolipidaemic, hypoglycaemic, antioxidative and anti-inflammatory capabilities of *P. pulmonarius* ultrasound extracts make the mushroom a worthy therapeutic candidate for the prevention of metabolic disorders. Additionally, the results from this study could hopefully advocate for future researchers to explore and apply alternative extraction methods (such as ultrasound) in their work. These alternative extraction techniques are cost-effective, efficient, and environmentally friendly.

However, it should be noted that the experimental period of this investigation is relatively short. As metabolic syndrome often develops due to chronic disease conditions (such as chronic hyperglycaemia and atherosclerosis), further studies need to be conducted to discover the long-term preventive effects of *P. pulmonarius* extract.

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AUTHORS CONTRIBUTION

Nur Amalina Amirullah carried out the experiment and did the data acquisition and analysis for the article, and wrote the main manuscript. Nur Amalina Amirullah, Nurhayati Zainal Abidin, Noorlidah Abdullah, and Sivakumar Manickam contributed to the conceptualisation of the experiment. Nurhayati Zainal Abidin, Noorlidah Abdullah, and Sivakumar Manickam reviewed and edited the manuscript draft before submission. Nurhayati Zainal Abidin was the main supervisor for the experiment and provided funding support.

Conflict of Interest Statement

The authors declare no conflict of interest.

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