

Effectuation of *Pleurotus pulmonarius* on Hypercholesterolemic Wistar-Kyoto Rats: Analysis on Liver and Sera

(Kesan *Pleurotus pulmonarius* terhadap Tikus Wistar-Kyoto Hiperkolesterolemik: Analisis ke atas Hati dan Sera)

NOOR FAZILA MOHD YAHAYA, NORHANIZA AMINUDIN* & NOORLIDAH ABDULLAH

Mushroom Research Centre, Institute of Biological Sciences, Faculty of Science, Universiti Malaya, 50603 Kuala Lumpur, Federal Territory, Malaysia

Received: 30 September 2021/Accepted: 20 June 2022

ABSTRACT

Hypercholesterolemia is one of the potential risks of cardiovascular heart disease (CHD). An early diagnosis and treatment attenuate the risk of CHD. Besides statin prescription for hypercholesterolemia, functional food has an added value to ameliorate the risk. This study was conducted to analyse the significant effect of grey oyster mushroom (*Pleurotus pulmonarius*) on hypercholesterolemia. Thirty-six Wistar-Kyoto rats were assigned into six groups, consisted of normal (N), hypercholesterolemia (H), two groups for prevention (P1 and P2), and two groups for treatment (T1 and T2). For prevention purpose, P1 and P2 were concomitantly induced to be hypercholesterolemic and fed with either crude aqueous extract of *P. pulmonarius* (CA) or simvastatin for 45 days. Following 45 days of hypercholesterolemic-induction, T1 and T2 rats were then orally fed with either CA or simvastatin for another 30 days. On day 45, groups N, H, P1, and P2 were sacrificed, whereas groups T1 and T2 were sacrificed on day 75. Histopathology examination showed the conditions of liver tissues. Fat droplets were observed in hypercholesterolemic hepatic tissues and were also remained in the hepatic tissues of rats belonged to the treatment groups. The tissues' viability was better in prevention groups suggesting the compound(s) present in CA might be able to protect them from further damage. Metabolomic analysis of the sera from P1 and T1 rats showed altered regulation of several metabolites such as pantothenic acid, N-carbamylglutamate, serotonin and ceramide against the control group.

Keywords: Hepatic cells; histopathology; hypercholesterolemia; metabolomic

ABSTRAK

Hiperkolesterolemia adalah salah satu potensi risiko penyakit jantung kardiovaskular (CVD). Diagnosis dan rawatan awal mengurangkan risiko CVD. Selain statin untuk merawat hiperkolesterolemia, makanan berfungsi mempunyai nilai tambah untuk mengurangkan risiko. Kajian ini dilakukan untuk menganalisis pengaruh signifikan cendawan tiram kelabu (*Pleurotus pulmonarius*) terhadap hiperkolesterolemia. Tiga puluh enam tikus Wistar-Kyoto dibahagikan kepada enam kumpulan, yang terdiri daripada normal (N), hiperkolesterolemia (H), dua kumpulan untuk pencegahan (P1 dan P2) dan dua kumpulan untuk rawatan (T1 dan T2). Untuk tujuan pencegahan, P1 dan P2 diaruh menjadi hiperkolesterolemia dan pada masa yang sama diberi ekstrak akues mentah *P. pulmonarius* (CA) atau simvastatin selama 45 hari. Manakala tikus T1 dan T2 pula diberi makan secara oral dengan CA atau simvastatin selama 30 hari lagi selepas 45 hari diaruh menjadi hiperkolesterolemia. Pada hari ke-45, kumpulan N, H, P1 dan P2 dikorbankan manakala kumpulan T1 dan T2 dikorbankan pada hari ke-75. Pemeriksaan histopatologi menunjukkan keadaan tisu hati. Titisan lemak diperhatikan pada tisu hepatik hiperkolesterolemik dan juga tetap berada di dalam tisu hati tikus tergolong dalam kelompok perawatan. Kelangsungan tisu lebih baik pada kumpulan pencegahan yang menunjukkan bahawa sebatian yang terdapat di CA mungkin dapat melindungi mereka daripada kerosakan selanjutnya. Analisis metabolik sera daripada tikus P1 dan T1 mendedahkan perubahan beberapa metabolit seperti asid pantotenat, N-karbamilglutamat, serotonin dan ceramida apabila dibandingkan dengan kumpulan kawalan.

Kata kunci: Hiperkolesterolemia; histopatologi; metabolomik; sel hepatik

INTRODUCTION

World Health Organization (WHO) reported noncommunicable diseases (NCDs) will be the leading mortality factor in the world. Approximately 15 million people aged between 39 and 69 years are succumbed to NCDs with more than 80% of the mortality happens in developing countries. Cardiovascular diseases (CVDs) are one of the NCDs and hypercholesterolemia are among the risk factor of CVDs (Ramírez et al. 2020). In Malaysia, CVD is also ranked as the first reason for mortality. Hypercholesterolemia contributed 38.3% of the disease risk prevalence as reported in 2019 (Thangiah et al. 2021).

Hypercholesterolemia or also known as dyslipidaemia is a disorder caused by the increased level of total cholesterol in the bloodstream. The pathogenesis of hypercholesterolemia is also linked to hypertension and a prolonged accumulation of plaque, which leads to severe diseases including atherosclerosis and heart diseases. Sedentary lifestyle and imbalanced diet are among the core factors that lead to the increment of total cholesterol amount in bloodstream (Warnholtz et al. 2001). Early diagnosis is of paramount importance to reduce the risk of other cholesterol-related diseases. Statins are widely prescribed once the balanced diet and ideal lifestyle are unable to significantly reduce the serum total cholesterol.

An additional effort by consuming nutritional mushrooms is highly recommended as they are fortified with countless benefits with potent compounds (Carrasco-Gonzalez et al. 2017). Specifically, *P. pulmonarius*, or locally known as grey oyster mushroom contains many beneficial constituents including ergothioneine, tryptophan, and ellagic acid which were found to significantly reduce serum total cholesterol. These constituents have been scientifically proven to pose positive impact to reduce cholesterol level and promote cell viability (Zainal Abidin et al. 2016). *In vivo* observation of the augmenting effects of the mushroom on blood cholesterol level and reducing risks of other diseases is crucial to validate its capability as a functional food. Metabolomic quantification of the sera serves as the key component of the disease biomarker to highlight *P. pulmonarius* effects by analysing the altered metabolite regulations (McGarrah et al. 2018). The aim of this study was to explore the potent effect of *P. pulmonarius* on rats' hepatic tissues, metabolomic properties in rat sera samples and their functions in hypercholesterolemia regulations.

MATERIALS AND METHODS

CRUDE AQUEOUS EXTRACT

Pleurotus pulmonarius was locally produced by Agro-

tech Sdn. Bhd. in Selangor, Malaysia. DNA sequencing and morphological characteristics of this particular species has been previously compiled with registration number KUM 61119 by Mycological Laboratory, Mushroom Research Centre, Universiti Malaya.

The fruiting body of *P. pulmonarius* was freshly purchased and shredded into fine pieces for freeze-drying. After a week of freeze-drying, the dried fruiting body was ground into powder. Later, the powder was soaked in distilled water with the ratio of 1:10 (w/v) overnight at 2 to 8 °C. Then, the solution was sieved and the collected liquid was centrifuged at 5000 rpm for 20 min. The supernatant was collected, freeze-dried and kept refrigerated until further use (Zainal Abidin et al. 2016). CA was administered orally by diluting the powder with distilled water. CA was weighted based on the dosage given (2.0 g) and dissolved in distilled water. Distilled water volume was 10 mL/kg body weight. Later, oral gavage of the dissolved CA (18 g curved stainless needle) was carried out for the rats.

RAT GROUPING AND ORAL FED

The procedure has received approval from University of Malaya Institutional Animal Care and Use Committee (UM IACUC) (UM IACUC No: 2015-181006/IBS/R/NFMY). Maximum dosage of CA for rats' oral gavage was determined by using AOT425 Statistic Programme. Simvastatin 10 mg is the commercial drug with active compound of statin (weight of 10 mg) in each tablet. The dosage is according to the prescription by the medical practitioner or pharmacist to the hypercholesterolemic patients.

Thirty-six Wistar-Kyoto male rats were purchased from Animal Experimental Unit (AEU), University of Malaya aged six weeks (~200 g) and were divided into six groups (six rats per group) as recommended by UM IACUC and as referred to work done on hypercholesterolemic rats by Rajput et al. (2014). The weights were recorded on day 1, day 45, and day 75. They were divided into normal (N): healthy rats fed with normal food; hypercholesterolemic (H): rats fed with cholesterol powder* [200 mg/kg body weight (BW)] (day 1-45); prevention 1 (P1): rats fed with cholesterol powder* (200 mg/kg BW) + CA (2.0 g/kg BW) (simultaneously) (day 1-45); prevention 2 (P2): rats fed with cholesterol powder* (200 mg/kg BW) + simvastatin 10 mg/kg BW (simultaneously) (day 1-45); treatment 1 (T1): rats fed with cholesterol powder* (200 mg/kg BW) (day 1-45) + CA (2.0 g/kg BW) (day 46-75); treatment 2 (T2): rats fed with cholesterol powder* (200 mg/kg BW) (day 1-45) + simvastatin 10 mg/kg BW (day 46-75) (*95% stabilized cholesterol powder). Simultaneous feeding of CA and cholesterol powder was a novel observation to

mimic human habit of taking high fat diet and supplement at the same time.

QUANTIFICATION OF SERUM TOTAL CHOLESTEROL

Blood was taken on Day 1 for all the groups utilising lateral tail veins (hypodermic needle 26 G) after anaesthesia [ketamine:xylazine; 80:10 (mg/kg)] and through intracardiac puncture (3 mL; 23 G) on the final day of experiments. The whole blood was centrifuged at 3000 rpm for 10 min and the sera were sent to Faculty of Veterinary, UPM for serum total cholesterol quantification (Automatic Chemistry Analyser: Siemens Dimension Xpand Plus). The liver was harvested and preserved in formalin prior to processing.

HISTOPATHOLOGY

The liver segments were preserved overnight in formalin followed by dehydration process in ethanol (80% for 2 h, 95% for 2 h and ended with 100% for 3 h). The liver segments were later blocked with heated paraffin and sliced into thin layer to fit the microscopic slides. The Haematoxylin and Eosin (H&E) staining took place by soaking the slides into Haematoxylin for 5 min, rinsed and later with Eosin for another one minute. The slides were finally sprayed with 95% of alcohol and left to air dry (Slaoui & Fiette 2011).

SERA METABOLOMIC ANALYSIS

On the basis of histopathological observation, serum sample from four groups were selected; N, H, P1, and T1. A total of 200 μ L of serum were transferred to 1.5 mL Eppendorf tube and added with 400 μ L methanol. Methanol was used to precipitate protein in the serum sample. The mixtures were then vortexed and centrifuged at 12,500 g for 15 min. Supernatant was collected and re-centrifuged prior to drying up and stored at -80 °C (Yang et al. 2013).

LIQUID CHROMATOGRAPHY AND MASS SPECTROMETRY

The serum samples were analysed with Agilent 1290 Infinity LC System coupled to Agilent 6520 Accurate-Mass Q-TOF. The HPLC column used for the analysis was ZORBAX Eclipse Plus C18 column (100 MM \times 2.1 mm \times 1.8 μ m, Agilent Technologies, SA, USA). Two mobile phases were used: 0.1% formic acid in Milli-Q water (A): 0.1% formic acid in acetonitrile (ACN) (B). The gradients of both mobile phases were 5%: 95% (A:B/B:A) with time range from 0 to 48 min and constant flow (mL/min) at 0.25. Dual Agilent Jet Stream ESI was used to perform ElectroSpray Ionization (ESI) under positive mode with the following parameters:

a) Capillary voltage: 4000 V, b) Nozzle voltage: 750 V, c) Fragmentor voltage: 175 V, d) Nebulizer pressure: 30 psi, e) Gas temperature: 325 °C, f) Gas flow: 10 L/min, g) Reference masses: 121.050873, 922.009798, h) MS data range: 100 - 1000 m/z

Data was processed with Agilent Mass Hunter Qualitative Analysis B.05.00. Later, the data were exported to Mass Profiler Professional software to run statistical analysis and metabolites identification via ID browser and METLIN metabolite PCDL database.

At least three runs of each treatment were prerequisite to detect the frequency of occurrence of each compound prior to filter and data normalization. Significant analysis via one-way ANOVA ($p < 0.01$) and fold change analysis ($FC \geq 2$) was conducted using Mass Profiler Professional. Benjamin-Hochberg Procedure was applied for multiple testing corrections to comply with false discovery rate (FDR) $\leq 1\%$. KEGG ID was used to confirm the presence of the compound in *Rattus norvegicus* via KEGG Pathway Database (<https://www.genome.jp/kegg/pathway>). Once confirmed, MetaboAnalyst software 4.0 (<http://www.metaboanalyst.ca/>) platform was used to run pathway analysis. Both sites are free online platforms specifically for metabolomic analysis (Manoharan et al. 2018; Yang et al. 2013).

STATISTICAL ANALYSIS

Overall, SPSS Software Package ver. 24 was used to interpret the significant difference for all the recorded data. The rats' body weight was analysed using One Way ANOVA ($p < 0.05$). Total serum cholesterol was analysed using Paired Sample-Test.

RESULTS AND DISCUSSION

BODY WEIGHT OF THE RATS

Table 1 shows the average of the body weight (BW) throughout the experiment. There were significant differences within the groups of rats and between the rat groups. The variation in the body weight among the groups occurred due to different experimental design for each of rats' group. None of them showed reduction in body weight as the treatment did not involve induction of any severe infection or disease. Simvastatin reduces leptin expression, a crucial hormone in satiety factor, thus increased the calorie intake thus contributed to weight gain in rats prescribed with simvastatin. No data was reported on *Pleurotus pulmonarius* contribution in weight gain or particular CA, however, the weight gain in P1 and T1 could be a normal physiological factor due to aging. In addition, the weight gain in H group can be related to high cholesterol diet which indirect contributed to increase fat intake in the diet (Singh et al. 2018).

TABLE 1. Body weight (BW) for the rats which were divided into six groups

Group	Rat body weight (g)					
	Day 0	Day 15	Day 30	Day 45	Day 60	Day 75
N	199.3 ± 2.5	218.0 ± 1.7	239.7 ± 1.4	259.3 ± 1.2 ^{a,w}	N/A	N/A
H	197.8 ± 2.6	227.5 ± 3.4	247.8 ± 2.1	276.2 ± 3.6 ^{b,w}	N/A	N/A
P1	196.7 ± 1.4	219.3 ± 1.0	241.8 ± 1.7	260.0 ± 1.9 ^{c,w}	N/A	N/A
P2	199.8 ± 2.0	221.0 ± 2.3	241.5 ± 1.9	262.8 ± 1.7 ^{d,w}	N/A	N/A
T1	200.2 ± 2.4	227.7 ± 2.3	245.7 ± 1.0	271.3 ± 2.1	294.0 ± 1.5	315.7 ± 2.2 ^{e,w}
T2	199.5 ± 2.3	225.8 ± 1.3	245.5 ± 1.0	272.2 ± 1.7	295.5 ± 1.4	319.0 ± 1.4 ^{f,w}

The letters a,b,c,d : significant differences (p<0.05) within the groups; The 'w' letter: significant differences (p<0.05) between the groups

QUANTIFICATION OF SERUM TOTAL CHOLESTEROL
Table 2 shows the total cholesterol values of rats in all of the groups. The initial total cholesterol ranges of the groups were 1.80 - 1.95 mmol/L. The total cholesterol values increased proportionally with the increment of age for normal group (2.50 mmol/L) (p<0.05) (Gälman et al. 2007), whereas for hypercholesterolemic-induced rats

(group: H, T1, T2), the serum total cholesterol increased as expected (> 3.00 mmol/L) (p<0.05) (Harini & Astirin 2009). The average value recorded for these groups (H, T1, T2) was ≥ 3.70 mmol/L (p<0.05). In addition, treated groups (T1 and T2) managed to reduce its cholesterol level following the treatment, whereas for the prevention groups (P1 and P2), cholesterol level was maintained within the normal range (p<0.05) (Table 2).

TABLE 2. Serum total cholesterol quantification in, A: Normal (N) and Hypercholesterolemic (H); B: Prevention groups (P1 and P2); C: Treatment groups (T1 and T2)

A		Serum total cholesterol (mmol/L)		
		Day 1	Day 45	
N (Normal)		1.90 ± 0.02	2.53 ± 0.67 ^a	
H (Hypercholesterol)		1.89 ± 0.6	3.83 ± 1.03 ^b	

B		Serum total cholesterol (mmol/L)		
		Day 1	Day 45	
Prevention Groups	P1	1.89 ± 0.07	2.78 ± 0.97 ^c	
	P2	1.90 ± 0.07	2.68 ± 0.06 ^d	

C		Serum total cholesterol (mmol/L)		
		Day 1	Day 45	Day 75
Treatment Groups	T1	1.89 ± 0.67	3.86 ± 0.97 ^{e,f}	2.95 ± 0.09
	T2	1.90 ± 0.70	3.92 ± 0.10 ^{g,h}	2.81 ± 0.98

Letter a-h represents significant differences (p < 0.05) between day 0 and day 45/ day 45 and day 75 when analysed using Paired Sample T-test. The values are expressed in mean ± SD; SD: standard deviation

LIVER HISTOPATHOLOGICAL OBSERVATION

The cross-section of liver tissues showed the differences between both normal and cholesterol-induced rats. Liver tissue in Figure 1(a) appeared normal with tangible nucleus, intact with cells, while the presence of numerous lipid vacuoles which confirmed the condition of hypercholesterolemia in cholesterol fed rats observed

in Figure 1(b). Previous researchers also reported their observations of lipid vacuoles in hypercholesterolemic liver tissues due to cholesterol accumulation in cytoplasm (Makni et al. 2008). Excessive ingested cholesterol had caused imbalance between free radicals' activation and antioxidant defence system. Oxidized LDL had triggered the expression of scavenger receptor on

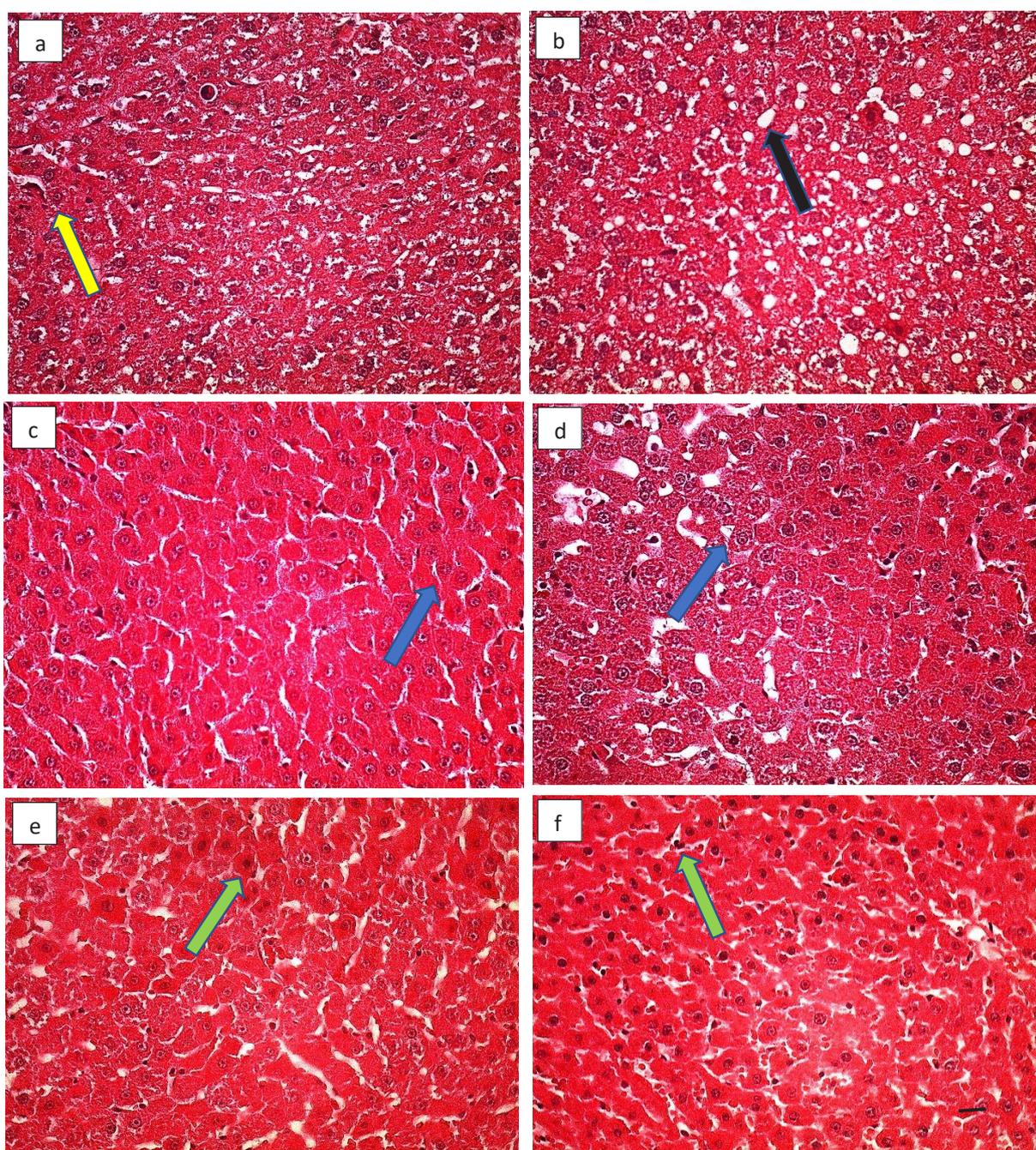


FIGURE 1. Cross section of liver; (a) normal rat (yellow arrow showed the normal nucleus shape); (b) cholesterol fed rat (black arrows show the lipid vacuoles existed in the liver tissues and blue arrow pointing at necrosis); (c and d) The nuclei of cells were intact (blue arrows) and not many necrosis cells (c: P1; d: P2); (e and f) the green arrows pointing at hepatocellular necrosis (e: T1, f: T2) (magnification: 40x) (scale bar: 200 μ m)

the macrophage, thus, causing atheroma or the formation of lipid vacuoles in hepatic cells. Necrotic hepatocytes were also detected as in Figure 1(b) (Alam et al. 2011).

However, the conditions of the hepatic cells were improved as seen in Figure 1(c) and 1(d) as compared to Figure 1(e) and 1(f). Prevention groups showed a better recovery of the hepatic cell conditions compared to hypercholesterolemic and treatment groups with no major necrotic cells observed. An almost similar observation was reported for other *Pleurotus* spp. studied. *Pleurotus eringii* increased the cells viability when it was simultaneously fed with cholesterol to the rats. Besides lowering the serum total cholesterol in rats, *P. eringii* was also found to demonstrate the ability to protect the hepatic cells from further damage when compared with hepatic cells belonged to hypercholesterolemic rats (Alam et al. 2011). A similar pattern of the protective capability of the mushroom was also reported by another finding. The authors recorded that the lipid vacuoles were vastly distributed in hypercholesterolemic rats' liver tissue and less lipid vacuoles were found in liver cells of rats fed with cholesterol and 5% seaweed powder. The liver tissue segment was comparable to normal liver cells in rats fed with cholesterol and 10% of seaweed powder (Wresdiyati et al. 2008).

In addition, an observation by Nežic et al. (2019) also cited an improved hepatic cell condition when treated with simvastatin. Simvastatin was found to enhance cell survival markers such as Bcl-XL and survivin. Activation of NF-kB/p65 also exerted anti-apoptotic effect of simvastatin on the injured cells. Microscopic observation found the hepatic cells survival after treated with simvastatin.

METABOLOMIC ANALYSIS

On the basis of histopathological observations, P1 & P2 showed almost similar cell condition. Identical cell condition also observed in T1&T2. Since P1 & T1 belonged to the group treated with CA (compound of interest in this research), thus only those samples selected for metabolomic analysis.

Based on the generated KEGG code and related metabolites with hypercholesterolemia, the involved pathways for metabolites of interest were detected by using KEGG Database (www.genome.jp/kegg/). MetaboAnalyst 4.0 (www.metaboanalyst.ca/) favoured in order to analyse the pathways impact through the bubble map that was generated by the system (Figure 2). Metabolites are involved either as part of the cholesterol regulation or contribute to cell repair or defence mechanism.

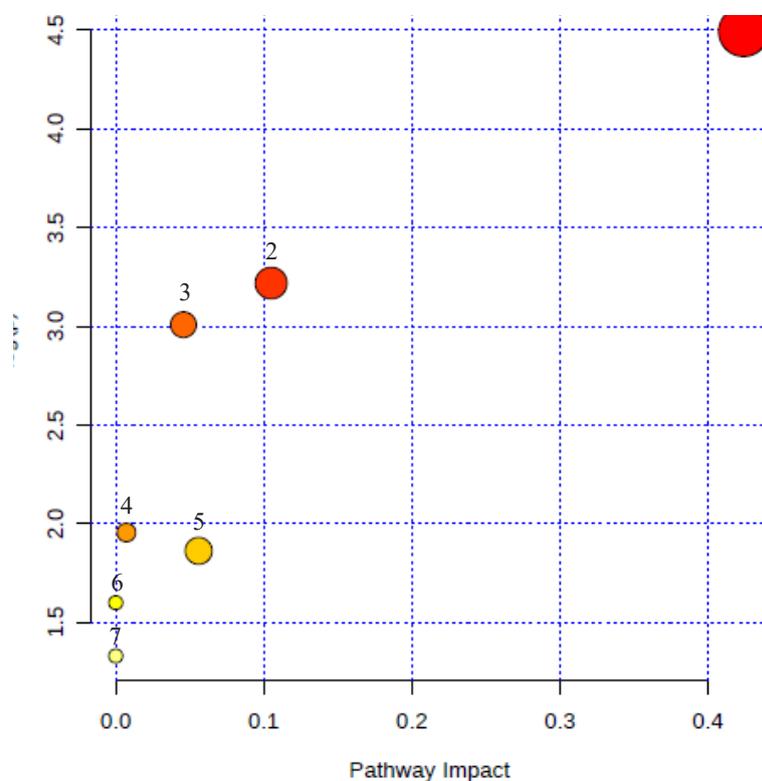


FIGURE 2. Summary of pathways impact analysis relates to hypercholesterolemia. (1) Sphingolipid metabolism; (2) tryptophan metabolism; (3) primary bile acid synthesis; (4) pantothenate and CoA biosynthesis; (5) beta-alanine metabolism; (6) glutathione metabolism; (7) arganine and proline metabolism (colour indication: light yellow - red: represents the severity of the hypercholesterolemia impact on the involved pathways)

Based on established findings, a few metabolites can be grouped as cholesterol regulator; ouabain, taurochenodeoxycholic acid, and glycocholic acid. In addition, pantothenic acid, phosphodimethethanolamine, N-carbamylglutamate, L-arginine, chloranil, serotonin,

sphingolipid, ceramide, isatin, kynurenine, polyamines, and 5-hydroxyquinoline were found to contribute towards cell repair and defence against further deterioration due disease development (McGarrah et al. 2018).

TABLE 3. Upregulation and downregulation of selected metabolites in three comparative groups; hypercholesterolemia group, prevention group (P1) and treatment group (T1)

Metabolites	KEGG ID	Log FC (cholesterol fed)	Log FC (prevention)	Log FC (treatment)	Retention time	p-value	Pathway
Pantothenic acid	C00864	-4.619034	6.4275074	-14.784496	2.7104	6.87E-09	beta-Alanine metabolism; Pantothenate and CoA biosynthesis
Ouabain	C01443	3.5537	0.208642	3.101871	10.9129	4.05E-07	Bile secretion
Glycocholic acid	C01921	0.053475887	0.20864195	16.48431	15.2556	9.15E-06	Bile secretion, Primary bile acid biosynthesis, Cholesterol metabolism
Taurochenodeoxycholic acid	C05465	7.477525	14.799491	14.9162655	11.8906	2.42E-04	Bile secretion, Primary bile acid biosynthesis, Cholesterol metabolism
Phosphodimethylethanolamine	C13482	-17.138626	0.30876923	-0.5167084	14.1146	4.96E-09	Glycerophospholipid metabolism
N-Carbamylglutamate	C05829	0.053475887	15.594496	0.08586981	11.2980	3.81E-39	Histidine metabolism
Chloranil	C18933	0.053475887	15.172274	3.6469731	0.7804	3.31E-07	Metabolomic pathway
Serotonin	C00780	18.515701	0.56415176	0.65921974	2.0665	7.23E-07	Tryptophan metabolism
Spinganine	C00836	1.3905106	-17.831316	-13.973977	22.4375	1.993-05	Sphingolipid metabolism
Ceramide	C00195	11.493731	0.20864195	0.08586981	10.2018	2.53E-09	Sphingolipid signalling pathway
5-Hydroxyquinoline	C05639	-7.770853	8.027875	-7.738459	3.7964	3.10E-04	Tryptophan metabolism
N-Acetylisatin	C02172	0.053475887	8.633935	0.08586981	6.3745	5.53E-06	Tryptophan metabolism
L-Formylkynurenine	C02700	17.905008	0.20864195	0.08586981	8.8158	0	Tryptophan metabolism
L-Arginine phosphate	C05945	0.20864195	3.9286823	0.08586981	3.7658	5.83E-04	Arginine and proline metabolism
Spermine	C00750	0.20864195	15.424756	0.08586981	0.8090	3.53E-04	Arginine and proline metabolism

Fifteen metabolites were selected based on the two platforms; KEGG ID & MetaboAnalyst 4.0

Taurochenodeoxycholic acid was found to be a part of the three pathways; bile secretion, primary bile secretion, and cholesterol metabolism. It is highly upregulated in all the analysed rats' sera. Taurochenodeoxycholic acid is also a conjugated primary bile acid of chenodeoxycholic acid with taurine during enterohepatic circulation of bile acid. Chenodeoxycholic acid has more binding affinity towards taurine instead of glycine. Homeostasis will automatically take place when cholesterol level increased above the normal range as the cholesterol is the precursor for bile acid synthesis. Thus, taurochenodeoxycholic acid contributed in increasing bile acid flow and lipid solubility (Feher 2012).

N-carbamylglutamate (NCG) and L-arginine (Arg) were to be part of the histidine, arginine proline metabolism, respectively. Both metabolites were upregulated in all of the analysed sera. However, the upregulation in prevention group P1 sera was significantly higher as compared to other groups. Previous findings had reported the ability of these metabolites to play the role as anti-oxidant and ROS scavenger (Mo et al. 2018). Perhaps these roles were highly executed in prevention group P1 as part of the cell repair and survival. Real-time PCR analysis on total RNA in rats' spleen has showed that NCG and Arg were able to upregulate the expression of some of the antioxidant genes including superoxide dismutase (SOD), glutathione peroxidase 1 (GPx1), glutathione reductase (GR), and catalase activities. These enzymatic antioxidants were ROS scavengers, alleviated the free radicals' activities and subsequently mitigate the cells damage.

In addition, Arg is also known as the precursor for nitric oxide (NO) synthesis. Besides of its important role in vessel relaxation, Arg is known as angiotensin-converting enzyme inhibitor thus modulates blood pressure. In this study, the metabolomic analysis observed the upregulation of Arg in hypercholesterolemic group as well. Arginine concentration usually increased to reduce the oxidative stress by interacting with H_2O_2 . The concentration of H_2O_2 was found escalated due to cytokine activation which usually occurred when atheroma plaque was present as part of the auto-recovery process (Tousoulis et al. 2002).

Serotonin was detected as part of the tryptophan metabolism and it was upregulated in hypercholesterolemia group. Serotonin was basically stored in platelets and its stimulation is usually due to vascular lesion and during vasoconstriction phase. The increased serotonin level in the blood was one of the biomarkers associated with cardiovascular risk.

It contributed to development and progression of atherosclerotic plaques and also linked to endothelial damage in blood vessels. In this study, it was found that serotonin was upregulated in hypercholesterolemia group as compared to other groups. The escalated level of serotonin was in accordance with previous findings, wherein endothelial dysfunction, serotonin exerted thrombus formation, and causes vasoconstriction; subsequently increased the risk of atherosclerosis (Figueras et al. 2005; Hara et al. 2004; Sugiura et al. 2016).

Sphingolipid and its metabolism pathway are vital for cell protection and cellular signals transmission, however, it also contributed to pathogenesis of major cardiovascular risk including hypercholesterolemia. An observation on sphinganine; one of the compounds in the sphingolipid pathway, found that the hamsters fed to be induced with cholesterol showed an increment in hepatic sphinganine level. In accordance with this finding, sphinganine was detected to be upregulated in hypercholesterolemia group but downregulated in both prevention group P1 and treatment group T1 (Dekker et al. 2015).

Isatin (1H-indole-2,3-dione) is synthesized endogenously via tryptophan pathway. Isatin is widely known as small and potent metabolites with tremendous benefits pharmacologically and its derivatives were synthesized for various medical applications such as antioxidant, antitumor, antimicrobial, and anti-inflammatory. N-acetyl isatin which was found in the rats' sera in this study mainly used as substrate in synthesizing isatin derivatives. N-acetyl isatin was upregulated in all the analysed sera. However, the highest upregulation was found for prevention group P1 as the rats were simultaneously fed with CA extract and induced with hypercholesterolemia. Isatin and its derivatives demonstrated a chelating activity when evaluated using 1,1-diphenyl-2-picryl-hydrazyl (DPPH) and hydrogen peroxide (H_2O_2) (Grewal 2014; Silva 2013; Souza & Chattree 2015).

Kynurenine was highly upregulated in hypercholesterolemia group as compared to the others. Further breakdown of tryptophan produces kynurenine; by the action of enzymes tryptophan dioxygenase and indoleamine 2,3-dioxygenase. Another study had demonstrated an increased in kynurenine and tryptophan ratio (kyn trp^{-1}) in patients with coronary heart diseases (Wirleitner et al. 2003). Besides, Wang et al. (2016) also reported that L-3-Hydroxy- kynurenine can cause oxidative injury to the cells as it is known as an ROS generator.

In this study, it was observed that many metabolites were upregulated to aid in cholesterol breakdown in three groups of the rats, namely the hypercholesterolemia (H), prevention group (P1) and treatment (T1). On the other hand, metabolites that involved in cell repair and survival were highly upregulated in P1 only, for example pantothenic acid, N-carbamylglutamate, chloranil, and spermine. Although the same upregulation of N-carbamylglutamate, chloranil, and spermine were recorded in the hypercholesterolemia and T1 groups, presumably as part of the self-recovery system, their upregulation was not as significant as seen in prevention group P1. Therefore, to maintain cell viability, early prevention was found to be significantly beneficial and effective as compared to treatment after the disease has taken place (Table 3).

CONCLUSIONS

The extract of interest, CA executed the ability to reduce serum total cholesterol in hypercholesterolemic-induced rats. The hepatic tissues conditions were also improved in the prevention and treatment groups compared to hypercholesterolemia group with the increment of essential metabolites in the analysed sera. Thus, CA is corroborated as one of the potential mushroom extracts in minimizing the plethora of hypercholesterolemia risks.

ACKNOWLEDGEMENTS

The authors would like to thank Universiti Malaya (Grants No.: PG109-2014B and BKS015-2018) for the financial support in order to complete this study.

REFERENCES

- Alam, N., Yoon, K.N., Lee, J.S., Cho, H.J., Shim, M.J. & Lee, T.S. 2011. Dietary effect of *Pleurotus eryngii* on biochemical function and histology in hypercholesterolemic rats. *Saudi Journal of Biological Sciences* 18: 403-409.
- Carrasco-Gonzalez, J.A., Serna-Saldivar, S.O. & Gutiérrez-Urbe, J.A. 2017. Nutritional composition and nutraceutical properties of the *Pleurotus* fruiting bodies: Potential use as food ingredient. *Journal of Food Composition and Analysis* 58: 69-81.
- Dekker, M.J., Baker, C., Naples, M., Samsouandar, J., Zhang, R., Qiu, W., Sacco, J. & Adeli, K. 2015. Inhibition of sphingolipid synthesis improves dyslipidemia in the diet-induced hamster model of insulin resistance: Evidence for the role of spingosine and sphinganine in hepatic VLDL-apoB100 overproduction. *Atherosclerosis* 228: 98-109.
- Feher, J. 2012. Pancreatic and biliary secretion. In *Quantitative Human Physiology: An Introduction*, edited by Feher, J. Elsevier, Amsterdam. pp. 721-730.
- Figueras, J., Domingo, E., Cortadellas, J., Padilla, F., Darado, D.G., Segura, R., Galard, R. & Soler, J.S. 2005. Comparison of plasma serotonin level in patients with variant angina pectoris versus healed myocardial infarction. *The American Journal of Cardiology* 96: 204-207.
- Gälman, C., Matasconi, M., Persson, L., Parini, P., Angelin, B. & Rudling, M. 2007. Age-induced hypercholesterolemia in the rat relates to reduced elimination but not increased intestinal absorption of cholesterol. *The American Journal of Physiology - Endocrinology and Metabolism* 293: 737-742.
- Grewal, A.S. 2014. Isatin derivatives with several biological activities. *International Journal of Pharmaceutical Sciences and Research* 6: 1-7.
- Hara, K., Hirowatari, Y., Yoshika, M., Kamiyama, Y., Tsuka, Y. & Takahashi, H. 2004. The ratio of plasma to whole-blood serotonin may be a novel marker of atherosclerotic cardiovascular disease. *Journal of Laboratory and Clinical Medicine* 144: 31-37.
- Harini, M. & Astirin, O. P. 2009. Blood cholesterol levels of hypercholesterolemic rat (*Rattus norvegicus*) after VCO treatment. *Nusantara Bioscience* 1(2): 53-58.
- Makni, M., Fetoui, H., Gargouri, N.K., Garoui, E.M., Jaber, H., Makni, J., Boudawara, T. & Zeghal, N. 2008. Hypolipidemic and hepatoprotective effect of flax and pumpkin seed mixture rich in omega-3 and omega-6 fatty acids in hypercholesterolemic rats. *Food and Chemical Toxicology* 46: 3714-3720.
- Manoharan, S., Shuib, A.S., Abdullah, N., Ashrafzadeh, A. & Kabir, N. 2018. Gly-Val-Arg, an angiotensin-1-converting enzyme inhibitory tripeptide ameliorates hypertension on spontaneously hypertensive rats. *Process Biochemistry* 69: 224-232.
- McGarrah, R.W., Crown, S.B., Zhang, G-F., Shah, S.H. & Newgard, C.B. 2018. Cardiovascular metabolomics. *Circular Research* 122: 1238-1258.
- Mo, W., Wu, X., Jia, G., Zhao, H., Chen, X., Tang, J., Wu, C., Cai, J., Tian, G., Wang, J. & Liu, G. 2018. Role of dietary supplementation with arganine or N-carbamylglutamate in modulating the inflammation, antioxidant property, and mRNA expression of antioxidant-relative signalling molecules in the spleen of rats under oxidative stress. *Animal Nutrition* 4: 322-328.
- Nežić, L., Amidžić, L., Škrbic, R., Gajanin, R., Nepovimova, E., Vališ, M., Kuča, K. & Jačević, V. 2019. Simvastatin inhibits endotoxin induced apoptosis in liver and spleen through up-regulation of Survivin/NF-kB/p65 expression. *Frontiers in Pharmacology* 10: 1-13.
- Rajput, M.S., Balekar, N. & Jain, D.K. 2014. *Lagenaria siceraria* ameliorates atheromatous lesions by modulating HMG-CoA reductase and lipoprotein lipase enzymes activity in hypercholesterolemic rats. *Journal of Acute Disease* 2014: 14-21.
- Ramírez, A.A., Jessica Lopez Peláez, J.L., Bermúdez, I.M. & Botero, J.Y. 2020. Prevalence of hyperlipidemia and its associated factors in university students in Colombia. *Heliyon* 6: e05417.

- Silva, B.V. 2013. Isatin, a versatile molecule: Studies in Brazil. *Journal of the Brazilian Chemical Society* 24: 707-720.
- Singh, P., Zhang, Y., Sharma, P., Covassin, N., Soucek, F., Friedman, P.A. & Somers, V.K. 2018. Statins decrease leptin expression in human white adipocytes. *Physiological Reports* 6(2): e13566.
- Slaoui, M. & Fiette, L. 2011. Chap 4: Histopathology procedures: From tissue to histopathological evaluation. In *Drug Safety Evaluation: Methods and Protocols, Methods in Molecular Biology*, Vol. 691, edited by Gautier, J.C. Springer Science+Business Media, LLC. pp. 69-82.
- Souza, R.D. & Chattree, A. 2015. Design, synthesis and biological activities of Isatin derivatives. *Chemical Science Transactions* 4: 208-212.
- Sugiura, T., Dohi, Y., Yamashita, S., Hirowatari, Y., Fujii, S. & Ohte, N. 2016. Serotonin in peripheral blood reflects oxidative stress and plays a crucial role in atherosclerosis: Novel insights towards holistic anti-atherothrombotic strategy. *Atherosclerosis* 246: 157-160.
- Thangiah, N., Su, T.T., Chinna, K., Jalaludin, M.Y., Mohamed, M.N.A. & Abdul Majid, H. 2021. Longitudinal assessment between lifestyle-related risk factors and a composite cardiovascular disease (CVD) risk index among adolescents in Malaysia. *Scientific Reports* 11: 19135-19143.
- Tousoulis, D., Antoniadis, C., Tentolouris, C., Goumas, G., Stefanad, C. & Pavlos, T. 2002. L-arginine in cardiovascular disease: Dream or reality? *Vascular Medicine* 7: 203-211.
- Wang, Q., Danxia Liu, D., Song, P. & Zou, M-H. 2016. Deregulated tryptophan-kynurenine pathway is linked to inflammation, oxidative stress, and immune activation pathway in cardiovascular diseases. *Frontiers in Bioscience* 20: 1116-1142.
- Warnholtz, A., Mollnou, H., Oelze, M., Wendt, M. & Munzel, T. 2001. Antioxidants and endothelial dysfunction in hyperlipidemia. *Current Hypertension Report* 3: 53-60.
- Wirleitner, B., Neurauter, G., Schrocksnadel, K., Frick, B. & Fuchs, D. 2003. Interferon- γ -Induced conversion of tryptophan: Immunologic and neuropsychiatric aspects. *Current Medicinal Chemistry* 10: 1581-1591.
- Wresdiyati, T., Hartanta, A.B. & Astawan, M. 2008. The effect of seaweed *Eucheuma cottonii* on superoxide dismutase (SOD) liver of hypercholesterolemic rats. *HAYATI Journal of Biosciences* 15: 105-110.
- Yang, H., Hwang, I., Kim, S., Hong, E. & Jeung, E. 2013. *Lentinus edodes* promotes fat removal in hypercholesterolemic mice. *Experimental and Therapeutic Medicine* 6: 1409-1413.
- Zainal Abidin, M.H., Abdullah, N. & Zainal Abidin, N. 2016. Protective effect of antioxidant extracts from grey oyster mushroom, *Pleurotus pulmonarius* (Agaricomycetes) against human low-density lipoprotein oxidation and aortic endothelial cell damage. *International Journal of Medicinal Mushrooms* 18: 109-121.

*Corresponding author; email: hanizaaminudin@um.edu.my