EFFECT OF *PLEUROTUS SAJOR-CAJU* EXTRACT ON LIVER ENZYMES AND HISTOLOGICAL CHANGES IN HYPERCHOLESTEROLEMIC-INDUCED RATS

by

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LIST OF ABBREVIATIONS

Alb	Albumin
Atorva	Atorvastatin
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
A/G	Albumin/Globulin ratio
Ca	Calcium
CHD	Coronary heart disease
CMC	Carboxymethylcellulose
CO_2	Carbon dioxide
CoQ_{10}	Coenzyme Q_{10}
CPD	Critical point drying
Cu	Copper
dH_2O	Distilled water
Fe	Ferum
FH	Familial hypercholesterolemia
GGT	Gamma-glutamyltransferase
Glo	Globulin
HDL	High density lipoprotein
HMG-CoA	HMG-CoA reductase inhibitors/statin
HPC	Hypercholesterolemic
H&E	Haematoxylin and eosin
IFCC	Intrenational Federation of Chemistry
Ile	Isoleucine
K	Potassium
LARUSM	Laboratory Animal Unit Universiti Sains Malaysia
LDL-C	Low density lipoprotein-cholesterol
Leu	Leucine
LFT	Liver function test
Lys	Lysine
M	Molar
Means ± SEM	Means ± standard error of the mean
Met	Methionine
Mg	Magnesium
Mn	Manganese
Na	Sodium
NAFLD	Non-alcoholic fatty liver disease
NC	Normal control
NCEP	National Cholesterol Education Program
ND	Not detected
Phe	Phenylalanine

PSC	*Pleurotus sajor-caju*
Se	Selenium
SEM	Scanning electron microscope
SGOT	Serum glutamate oxaloacetate transaminase
SGPT	Serum glutamate pyruvate transaminase
sp.	Species
spp.	species (plural)
TB	Total bilirubin
TEM	Transmission electron microscope
TG	Triglyceride
Thr	Threonine
TP	Total protein
Tyr	Tyrosine
UPMS	Unit Pengurusan Makmal Sains
USA	*United States of America*
Val	Valine
WHO	World Health Organization
Zn	Zinc

LIST OF SYMBOLS AND UNITS

g	Gram
mbar	millibar
mg	milligram
mg/dL	milligram per decilitre
mg/g	milligram per gram
mg/kg	milligram per kilogram
min	minute
mL	millilitre
mm^3	cubic millimetre
mmol/L	millimol per litre
psi	pound per square inch
%	percent
°C	Degree Celsius

ABSTRAK

Hiperkolesterolemia merupakan keadaan di mana kandungan kolesterol dalam darah melebihi paras normal. Keadaan ini berhubung kait dengan pengumpulan lemak dalam hati yang mana boleh melarat menjadi sirosis dan penyakit hati berlemak. Kajian epidemiologi menunjukkan bahawa 60 peratus daripada penduduk Malaysia yang mempunyai masalah tinggi paras kolesterol turut mempunyai masalah penyakit hati berlemak non-alkohol (NAFLD). Oleh kerana masalah hiperkolesterolemia dan lain-lain penyakit yang berhubung kait dengannya semakin meningkat di seluruh dunia termasuk Malaysia, kajian permulaan ini bertujuan untuk mencari rawatan alternatif dengan menggunakan bahan semulajadi yang dikenali sebagai *Pleurotus sajor-caju* (PSC). PSC merupakan sejenis cendawan tiram spesies *Pleurotus* yang dilaporkan mempunyai kandungan kalori dan lemak yang rendah, kaya protein dan serat serta telah terbukti mempunyai ciri-ciri antihiperlipidemia dan perlindungan terhadap organ hati. Dalam kajian ini, rawatan 100 mg/kg PSC terhadap tikus hiperkolesterolemia selama sebulan terbukti mempunyai kesan terhadap aktiviti enzim hati. Ini dibuktikan dengan penurunan signifikan ($P < 0.05$) enzim alkali fosfatase (ALP) dan tinggi peratusan penurunannya (66.01%) berbanding dos 20 mg/kg and 200 mg/kg PSC. Walau bagaimanapun, penemuan ini bukanlah penemuan yang signifikan disebabkan kontrol tikus hiperkolesterolemia turut mengalami penurunan enzim ALP. Plasma aspartat aminotransferase (AST) dan alanina aminotransferase (ALT) menunjukkan sedikit peningkatan iaitu sebanyak 35.04% and 15.00% masing-masing dan tiada peningkatan signifikan ($P > 0.05$) dalam kedua-dua enzim tersebut. Rawatan atorvastatin juga menunjukkan penurunan enzim ALP tetapi tiada penurun signifikan ($P > 0.05$) jika dibandingkan dengan 100 mg/kg rawatan PSC. Dalam pada itu, rawatan 20 mg/kg atorvastatin menunjukkan peningkatan plasma AST dan ALT dengan masing-masing

meningkat sebanyak 275.97% dan 112.50% dan ini boleh dilihat sebagai salah satu kesan sampingan penggunaan ubat atorvastatin. Dalam kajian histologi, mikrograf hati tikus hiperkolesterolemia yang telah dirawat dengan 100 mg/kg PSC menunjukkan permukaan hepatosit yang jelas kelihatan berbanding tikus hyperkolesterolemia yang tidak dirawat. Selain itu, kajian ini juga telah berjaya mencipta satu kaedah yang praktikal, mudah dam murah untuk menghasilkan tikus hiperkolesterolemia iaitu dengan hanya menggunakan 32 g minyak sapi bagi setiap 68 g pelet tikus.

ABSTRACT

Hypercholesterolemia is a condition where high cholesterol levels are present in the blood. It has been associated with fatty infiltration in the liver which may progress to cirrhosis and hepatic steatosis. Epidemiology study demonstrated that 60% of Malaysians with slightly high in cholesterol levels have NAFLD. Therefore, as the incidence of hypercholesterolemia and other related health problems has been increasing around the world including Malaysia, this preliminary study aimed to find the alternative treatment by using natural substances known as *Pleurotus sajor-caju* (PSC). PSC is one of the *Pleurotus* spp. which has been reported to have low calories and fat but rich in proteins and dietary fibers and was proven to have antihyperlipidemic and hepatoprotective activity. In the present study, rats treated with 100 mg/kg of PSC for a month was found to have an effect on the liver enzymes activities since plasma ALP concentration in this group showed a significant reduction ($P < 0.05$) and a higher percentage reduction (66.01%) as compared to 20 mg/kg-PSC and 200 mg/kg-PSC treatment groups. However, this finding cannot be categorized as a significant finding as ALP reduction was also observed in HPC control group. Plasma AST and ALT only showed a mild increased by 35.04% and 15.00% respectively in 100 mg/kg-PSC treatment group and there was no significant increased ($P > 0.05$) found in these both AST and ALT concentrations. Atorvastatin treatment also showed reduction in ALP enzymes but no significant reduction ($P > 0.05$) as compared to 100 mg/kg-PSC treatment group. On the other hand, plasma AST and ALT in 20 mg/kg of atorvastatin treatment were increased in percentage by 275.97% and 112.50% respectively and this may indicate the adverse effects of statin in term of elevation of plasma enzymes activities. On the histological part, there was no significant finding in the micrograph study between treatment and HPC liver rat. The micrograph of HPC rat liver treated

with 100 mg/kg PSC only showed smooth and clear surface of hepatocytes compared to HPC group. In addition, a practical, simple and cheap method of dietatical model for inducing hypercholesterolemia in rats has been successfully developed by using 32 g of ghee per 68 g of rat pellet.

CHAPTER 1

INTRODUCTION

Hypercholesterolemia is a condition characterized by high cholesterol levels in the blood. It is also generally defined as low density lipoprotein cholesterol (LDL-C) level greater than 160 mg/dL or 4.1mmol/L based on National Cholesterol Education Program (NCEP) guidelines (prolipid.com). According to the World Health Organization's (WHO) classification, hypercholesterolemia has emerged as a major risk factor for cardiovascular diseases (CVDs) such as rheumatic, hypertensive, ischemic, cerebrovascular and inflammatory heart diseases (World Health Organization, 2007). As a proportion of total deaths from all-causes, CVD in the Asia-Pacific region ranges from less than 20% in countries such as Thailand, Philippines and Indonesia to 20–30% in urban China, Hong Kong, Japan, Korea and Malaysia (Khor, 2001).

Elevated cholesterol in the blood depends on several factors such as dietary intake, obesity, inherited diseases, socioeconomic status, lifestyle behavior, race, age as well as gender. The most common inherited form of hypercholesterolemia is called familial hypercholesterolemia (FH) which is defined as a genetic disorder that causes severe elevations in total cholesterol and low density lipoprotein in the blood and early CVD. An estimated FH prevalence worldwide is 1 in 500 (0.2%) in the general population and is associated with an LDL-C level between 270 to 300 mg/dL (15.0 - 16.6 mmol/L) (Livy, 2011). The FH clinical manifestation has been shown to be associated with increased CVD such as coronary heart disease (CHD) and death (Austin *et al.*, 2004). Furthermore, there is a link between hypercholesterolemia and obesity as high serum cholesterol is commonly found in obese person (Miettinen, 1971). Based on WHO survey, Malaysia ranked as the sixth country among Asia with the highest adult

obesity rate in 2010 (weightlossmalaysia.com). Women and family history of illness such as hypertension, diabetes and CVD are the risk factors for developing obesity among Malaysian adult.

Currently, there are many published papers on the relationship between high cholesterol level and CVD (Anum & Adera, 2004; Austin, *et al.*, 2004). High cholesterol in the blood is the root cause of obesity and plaque formation which in turn leads to CVDs. Nowadays, the management of this health problem is done in several stages and it is quite complicated. Patient with two or more cardiovascular risk factors and has established coronary heart disease in addition to hypercholesterolemia need to be referred to a specialist with expertise in that field. Furthermore, high costs for the medicines due to many health complications also need to be considered. In more severe cases, operation such as partial ileal bypass surgery is suggested to the hypercholesterolemia patient associated with CHD and this is an expensive and complicated procedure. Several side effects such as diarrhea and a higher incidence of kidney stones and gallstones were observed among the patients who had undergone partial ileal bypass.

The most widely prescribed drugs in the market for lowering serum LDL-C concentrations is HMG-CoA reductase inhibitors, also known as statins (Langsjoen, 2003). They include atorvastatin, fluvastatin, lovastatin, pitavastatin, pravastatin, rosuvastatin and simvastatin. However, there are several cases of intolerance to statin therapy which indicated the presence of clinically significant adverse effects such as symptoms of liver problems, unusual tiredness and weakness, allergies and muscle pain. Adverse effects on liver due to statins include elevations in liver function tests, dark urine, fatty change in the liver, and yellowing of the skin or eyes. This is supported

by an animal study conducted by Smith *et al.* (1991), which demonstrated that statins also induce hyperplasia of bile canaliculi, induce gallstone formation, impair bile flow, and elevate serum bile acids and conjugated bilirubin.

Hypercholesterolemia is also a known risk factor for fatty infiltration of the liver which can progress to primary biliary cirrhosis and other forms of cholestatic liver disease (Assy, 2000). Accumulation of fat in the liver cells is also known as steatosis or non-alcoholic fatty liver disease (NAFLD). NAFLD is a condition where excess fats are deposited in liver cells (more than 5 – 10% of the liver's weight) (Reddy, 2006). This health problem is caused by obesity, high blood cholesterol, high blood sugar or the result of other unmanaged disease states. NAFLD is generally discovered by the presence of elevated aminotransferases such as alanine transaminase (ALT) and aspartate transaminase (AST). There are no exact data available for the prevalence of NAFLD in Malaysia. However, in conjunction with the previous study on high cholesterol patient, the estimated prevalence of NAFLD in Malaysia is approximately 15 – 30% of the general adult population (thestar.com). Additionally, the previous epidemiology study showed that about 60% of Malaysian with slightly high in cholesterol levels has NAFLD (Magosso *et al.*, 2010). Therefore, it is clear that NAFLD is linked to hypercholesterolemia and also has been reported to be an independent risk factor for CVD as well.

There is an urgent need to find an alternative way to overcome the problem of complex management of hypercholesterolemia and other related risk factors, high cost of medicines and operation as well as adverse effects of prescribed drugs. Furthermore, as the incidence of hypercholesterolemia, related cardiovascular events, obesity and NAFLD kept increasing around the world including Malaysia, studies on natural

substances with hypocholesterolemic effects have been intensively carried out during the last few years. The key point here is to reduce high cholesterol level in order to prevent other related health complications such as CVDs and NAFLD.

Plant medicinal uses have increased in the world as an alternative solution to health problems. In the previous studies, *Pleurotus* spp. is well-known as their ability to reduce cholesterol (Alam *et al.*, 2011; Bobek *et al.*, 1994 and Bobek *et al.*, 1995). Therefore, in this preliminary study, we would like to accomplish the following objectives:

- To establish dietatical model for inducing hypercholesterolemia in rats.
- To observe any reduction in body weight of hypercholesterolemic rats after PSC and atorvastatin treatment.
- To establish the effective dose of PSC in reducing plasma liver enzymes activities in hypercholesterolemic rats.
- To determine and compare enzymatic activities between PSC and atorvastatin treatment in hypercholesterolemic rats.
- To compare histological changes in hypercholesterolemic rats after PSC and atorvastatin treatment.

CHAPTER 2

LITERATURE REVIEW

2.1 Oyster mushroom

Chang *et al.* (1992), defined mushroom as a 'microfungus' with a distinctive fruiting bodies, can be either epigeous or hypogeous, easily seen with naked eye and picked by hand. Mushrooms mostly belong to the family Agaricaceae in the class of Basidiomycetes. They are categorized in saprophytic, parasitic and mycorrhizal mode of living. There are 22 000 mushroom species have been identified and exist in nature, however, only a few are used as edible mushrooms. Most Asian countries use traditionally wild edible mushrooms as nutritional foods and medicines.

Pleurotus mushroom is one of the edible mushroom species which commonly known as oyster mushroom. They grow wildly in tropical and subtropical areas and can be easily artificially cultivated (Pornariya *et al.*, 2009). Mushrooms of *Pleurotus* genus include *P. ostreatus*, *P. sajor-caju, P. florida*, *P. flabellatus*, *P. highking* 51, *P. cystidiosus*, *P. sapidus*, *P. eryngii*, *P. tuberregium*, *P. ulmarius*, *P. pulmonarius*, *P. citrinopileatus*, *P. geesteranus*, and others (Chang *et al.*, 1989; Kaul, 2001; Khan, 2010).

Pleurotus mushrooms have high content of protein, carbohydrates, dietary fibers (Table 2.1), minerals (i.e. potassium, phosphorus, iron, zinc, calcium, magnesium, manganese, copper) (Table 2.2), and vitamins (Table 2.3) and low in fat content (Khan & Tania, 2012). The protein contains all nine essential amino acids required by humans. (Table 2.4).

Table 2.1: Approximate composition of macronutrients of some important *Pleurotus* mushrooms. (Source: Khan & Tania, 2012)

Mushroom spp.	Proteins*	Carbohydrate*	Lipids*	Fibers*	Minerals*	Moisture#
P. ostreatus	17-42	37-48	0.5-5	24-31	4-10	85-87
P. sajor-caju	16-38	37-40	1-5	22-31	5-9	85-87
P. florida	15-21	40-43	1-5	23-27	8-12	87-88
P. cystidiosus	17-18	43-45	5-6	25-26	7-8	86-87
P. highking 51	20-21	36-37	5-6	30-31	5-6	85-86
P. geestaranus	19-20	45-46	3-4	26-27	5-6	85-86
P. eryngii	11-12	39-40	7-8	28-29	4-5	85-90
P. tuber-regium	13-17	53-54	0.2.2	15-16	4-10	88-89
P. flabellatus	21-22	59-60	1-2	10-12	6-7	91-94

*g/100g dried mushroom; # % of fresh mushroom

Table 2.2: Approximate trace elements (minerals) content of some important *Pleurotus* mushrooms (mg/100g dried mushroom). (Source: Khan & Tania, 2012)

Mushroom spp.	K	Ca	P	Na	Mg	Zn	Fe	Mn	Cu	Se
P.ostreatus	1400	2-36	ND	3	9-17	3-27	55-65	0.5-3	0.65	0.011
P.sajor-caju	1600	3-23	ND	2	5-21	3-21	33-54	0.5-3	0.8	0.025
P.florida	1460	0.5-34	13.7	0.3	1-14	0.5-16	0.05-44	0.5-3	0.05	0.013
P.flabellatus	1537	120	1616	686	40	145	209	10	22	ND
P.tuber-regium	300	3-200	5-10	2-8	0.7-2	2-2.5	30	2	0.3-2	ND

Note: K=potassium; Ca=calcium; Na=sodium; Mg=magnesium; Zn=zinc; Fe=iron; Mn=manganese; Cu=copper; Se=selenium; ND=not detected

Table 2.3: Approximate vitamins content of *Pleurotus ostreatus* mushroom. (Source: Khan & Tania, 2012)

Vitamins in P.ostreatus	**Contents (mg/100g dried mushroom)**
Thiamin	1.9 – 2.0
Riboflavin	1.8 – 5.1
Niacin	30 – 65
Folate	0.3 – 0.7
Ascorbic acid	28 – 35

Table 2.4: Approximate essential amino acid composition of some important *Pleurotus* mushrooms (presented as mg/100g of dried mushroom). (Source: Khan and Tania, 2012)

Mushroom spp.	Leu	Val	Lys	Ile	Thr	Tyr	Met	Phe
P. ostreatus	16.4	10.5	11.3	9.9	9.4	6.9	2.7	11.1
P. sajor-caju	14.6	10.1	5.8	11.2	8.9	5.9	2.7	9.2
P. eryngii	10.8	7.4	7.3	7.2	6.8	4.9	1.7	7.1
P. tuber-regium	28.4	33.4	27.4	21.1	31.4	4.2	4.8	21.9

Leu = leucine; Val = valine; Lys = lysine; Ile = isoleucine; Thr = threonine; Tyr = tyrosine; Met = methionine; Phe = phenylalanine

Recently, numerous studies have focused on the therapeutic effect of oyster mushroom. Previous studies on extracts powder of fruit bodies or mycelium of *Pleurotus* mushrooms have been reported to have anticancer (Gu and Sivam, 2006; Martin and Brophy, 2010), antihypercholesterolemic (Alam *et al.*, 2011; Bobek *et al.*, 1994; Bobek *et al.*, 1995), antihypertensive (Miyazawa *et al.*, 2008; Hagiwara *et al.*, 2005), antidiabetic (Chorváthová *et al.*, 1993; Krishna & Usha, 2009; Hu *et al.*, 2006; Kim *et al.*, 2010), anti-inflammatory (Andrej *et al.*, 2011), hepatoprotective (Jayakumar *et al.*, 2006; Arunavadas & Umadevi, 2008; Sumi *et al.*, 2010), antioxidant (Jayakumar *et al.*, 2006; Khan *et al.*, 2011; Liu *et al.*, 2010) and antimicrobial activities (Wolff *et al.*, 2008; Karaman *et al.*, 2010). In addition, studies have shown that some mushrooms may have potential to reduce body weight (Alam *et al.*, 2008; Alam *et al.*, 2009).

In the present study, *Pleurotus sajor-caju* (PSC) was used. PSC is an edible cosmopolitan mushroom (Zadrazil & Kurtzman, 1982). It is originating from India and believed to be indigenous to South East Asia where it is then being commercially cultivated. Earlier report on PSC showed that it is one of the *Pleurotus* spp. which is low in calories and fat but rich in proteins and dietary fibers (Khan & Tania, 2012). The various therapeutic effects of PSC have been studied which include anticancer (Dalonso *et al.*, 2010; Ngai *et al.*, 2004), antidiabetic (Agrawal *et al.*, 2010), antioxidant (Khan *et al.*, 2011), antimicrobial (Ngai *et al.*, 2004; Kidukuli *et al.*, 2010), antihyperlipidemic and hepatoprotective activity (Alam *et al.*, 2009).

2.2 Statins

Statins which also known as 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors are one of the most widely prescribed drugs on the market (Stapleton *et al.*, 2010). There are two types of statins available; natural statin and

synthetic statin. Natural statin includes lovastatin which is produced by the *Penicillium* sp. (Endo *et al.*, 1976), *Monascus ruber* (Juzlova *et al.*, 1996) and most commonly by *Aspergillus terreus* (Alberts *et al.*, 1980; Novak *et al.*, 1997). Atorvastatin is categorized as fully synthetic statin. It is used for the treatment of hypercholesterolemia. Although there is a variety type of statin drugs, they are still producing a qualitatively similar effect on the lipid profile. Atorvastatin is taken orally at 10, 20, 40 and 80 mg/day for the hypercholesterolemia treatment (Valentovic, 2007). Additionally, atorvastatin was believed to prolong inhibition of CoA-reductase compared to other statins. This may be due to longer residence of the drug or its metabolites in the liver (Naoumova *et al.*, 1997). Animal study done by Movahedian *et al.* (2010) also demonstrated that 20 mg/kg atorvastatin was effective in lowering blood cholesterol level in rats. Thus, in this preliminary study we decided to use 20 mg/kg atorvastatin which categorized as positive control group.

HMG-CoA reductase is an important enzyme in cholesterol biosynthesis. Statin works in the body by inhibiting HMG CoA-reductase (Alberts *et al.*, 1980). As a result, HMG-CoA will not be converted to mevalonate, thus, inhibits the cholesterol biosynthesis (Tobert, 2003). However, the mechanism of statin to reduce plasma cholesterol level is not simply reduction in cholesterol biosynthesis. The most importantly is the lower density lipoprotein (LDL) receptor involvement. This is supported by work done by Brown and Goldstein (1986) and Kovanen *et al.* (1981) which showed that induction of LDR receptor is crucial to the effectiveness of the statin drug class. Here, statin lower plasma cholesterol level by increasing the uptake of LDL via the LDL receptor. In addition, these drugs also decrease the production of apolipoprotein-B-containing lipoproteins by the liver (Arad *et al.*, 1992) especially LDL

cholesterol and, to a lesser extent, plasma triglyceride (TG), and a small increase in high density lipoprotein (HDL).

Previous studies have reported that several oyster mushrooms strain contain statin (Gunde and Cimerman, 1995; Julio *et al.*, 2003), which has similar mechanism as statin drugs. Mostly, statins were detected in fruiting bodies of oyster mushroom (Gunde *et al.*, 1993; Gunde and Cimerman, 1995). The mechanism of oyster mushroom in lowering cholesterol level is quite similar to statin drug except this drug may cause some side effects such as symptoms of liver problems, unusual tiredness and weakness, allergies and muscle pain. Symptoms of liver adverse effects of statin drug include increases in hepatic transaminases, dark urine, fatty change in the liver (steatosis), and yellowing of the skin or eyes. Animal studies have demonstrated that statins also induce hyperplasia of bile canaliculi, induce gallstone formation, impair bile flow, and elevate serum bile acids and conjugated bilirubin (Smith *et al.*, 1991).

Moreover, another study done by Folkers *et al.* (1990), suggested that side effects of statins can be caused by the statin-induced deficiency of coenzyme Q_{10} (CoQ_{10}). CoQ_{10} which is also known as ubiquinone is a naturally occurring, fat-soluble nutrient with characteristics common to vitamins and like-vitamins and is important for the optimal functioning of an organism (Bliznakov, 2002). It is functions as cellular energy production in eukaryotes. Statin therapy was associated with a lowering of CoQ_{10} blood levels and resulted in a measureable decline of cardiac function (Karl Folkers *et al.*, 1990). Since then, many other studies (Caliskan *et al.*, 2000; Satoh *et al.*, 2000) confirmed the connection between statin treatment and CoQ biosynthesis. A reduction of CoQ_{10} is associated with impairment of myocardial function, liver dysfunction as well as myopathies. A statin-induced CoQ deficiency in the blood can be compensated by oral CoQ_{10} administration (Bargossi *et al.*, 1994). Thus, person with

statin therapy should be complemented with other supplement (oral CoQ_{10}) in order to support the deficient cellular functions as well as to minimize oxidative stress.

The biosynthesis of CoQ requires the availability of several vitamins or their coenzyme forms: vitamin B2, B6, B12, C, folic acid, niacinamide, pantothenic acid as well as many trace elements (Folkers K., 1969). Therefore, it is suggested that *Pleurotus* mushrooms can be one of the alternative treatments besides statin drugs as they are rich in vitamins and trace elements (minerals), safe to be consumed, non-toxic, no side effects and no need to take other supplements as compared to statin treatment.

2.3 Effect of oyster mushroom on liver enzyme activities

Liver is the largest internal organ which is made up of numerous lobules and packed with parenchymal cells called hepatocytes. Liver functions as carbohydrate metabolism, fat metabolism, bile production, detoxification, storage of vitamins, breakdown of haemoglobin, synthesis of plasma protein and play a major role in breakdown and synthesis of cholesterol. Cholesterol is needed by the body in certain amount as a component of cell membranes, to produce some hormones such testosterone and estrogen, to make vitamin D as well as bile acid production. In this study, enzyme activities in liver were assessed as liver is the major organ responsible for maintenance of homeostasis related to lipid metabolism.

Nowadays, there are many *in vivo* evidences indicating the effect of *Pleurotus* spp. on liver enzymes activities (Alam, 2009; Alam, 2011; Khan, 2011; Bobek, 1995). The functional integrity of liver was assessed by measuring total protein, albumin, globulin, albumin/globulin (A/G) ratio, serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and gamma-glutamyltransferase (GGT). This is known as liver function tests (LFTs) which are used

to determine the presence or absence of liver disease, to make specific diagnoses, to determine severity and to monitor the course of disease.

Total protein is a combined measure of the concentration of proteins in the blood and there are two major types of protein that will be measured specifically which are known as albumin and globulin. Albumin is the major plasma protein produced by the liver at a rate of 10 – 15g per day. It is important for maintenance of plasma oncotic pressure as well as to transports small molecules and drugs. Albumin levels depend on several factors such as nutritional status, catabolism, hormonal factors and urinary and gastrointestinal losses. Decreased serum albumin reflects not only liver disease but also due to protein loss (nephrosis, enteropathy and burns), catabolic states as well as malnutrition (Dufour *et al.*, 2000). Hypoalbuminaemia from liver-related causes is more common in chronic liver disease rather than acute disease due to albumin long half-life (21 days). Globulin is a protein that includes gamma globulin (antibody) and variety of enzymes and transport proteins and usually involves in immune system. A low globulin level is one of the signs of liver damage.

Bilirubin is the breakdown product of red blood cells (the haem component). Liver function test measures the total bilirubin which is a combination of conjugated and unconjugated bilirubin. Serum concentration of bilirubin is an important indicator of liver functional loss. It is a marker of liver's ability to take up bilirubin from plasma to the hepatocytes, conjugate it with glucuronic acid to become water soluble and excrete in the bile. Elevated serum bilirubin can indicates intrahepatic or extrahepatic obstruction to bile outflow, cholestasis, hemolysis or other problems with the liver.

Serum AST and ALT are intracellular enzymes which are released when hepatocytes are damaged. AST is also known as serum glutamate oxaloacetate

transaminase (SGOT) catalyzes the reversible transfer of the amino group from aspartic acid to α-ketoglutaric acid to produce oxalacetic acid and glutamic acid. On the other hands, ALT, also called serum glutamate pyruvate transaminase (SGPT) transfer of the amino group from alanine to α-ketoglutaric acid produces pyruvic acid and glutamic acid. Serum concentration of ALT is more specific than AST as it is confined to the liver, whereas AST can also be found in skeletal muscle, heart muscle, kidney and erythrocytes. Enzyme ALT was first thought to be confined in cytoplasm of the hepatocyte only, but previous research showed that it is also can be found in mitochondria (Swick *et al.*, 1965). Approximately 80% of AST can be found in mitochondria of the hepatocytes (Pincus, 1996).

ALP is found in biliary epithelium and bile canalicular region of hepatocytes, which catalyzes the hydrolysis of phosphate esters. ALP is also present in other several tissues such as bone, intestine and placenta. Its function to be involved in metabolite transport across cell membrane, but not well established (Kathryn *et al.*, 2008). Elevated ALP suggested intrahepatic or extrahepatic biliary obstruction. The last common enzyme in LFT is GGT. GGT transfers a gamma-glutamyl group between peptides and is involved in amino acid transfer. Its concentration is mainly high in the liver. These four enzymes are the commonly markers for liver injury. However, further test such as liver biopsy need to be done in order to assess the etiology of elevated LFTs and severity of liver disease.

In the previous study by Alam *et al.* (2011) showed that plasma ALT and AST were found to be higher in hypercholesterolemic rats compared to control rats. ALT and AST are released into serum when the cytoplasmic hepatocytes are damaged and result in mild hepatocellular injury or might progress to more severe hepatocellular injury when mitochondrial membrane is damaged (Pincus, 1996). Therefore, treatment with

Pleurotus sp. was believed to reduce plasma AST, ALT and ALP activities in hypercholesterolemic rats (Alam *et al.*, 2011).

2.4 Effect of oyster mushroom on body weight and histological changes in liver of hypercholesterolemic rat

A significant correlation exists between serum cholesterol and obesity (Tanner, 1951; Montoye *et al.*, 1966) and hypercholesterolemia is frequently found in patients with obesity. Obesity is also recognized as the risk factor contributing to development other diseases such as diabetic, hypertension and CVD. Moreover, the leading cause of steatosis is linked to obesity-associated fatty liver disease. Besides obesity, hypertriglyceridemia and altered glucose homeostasis have also been associated with hepatic steatosis (Marchesini *et al.*, 2003). Hepatic fatty liver disease is also known as hepatic steatosis, manifests as accumulation of large (macrovesicular) or small (microvesicular) intracytoplasmic fat droplets that predominantly affect liver parenchymal cells (Reddy & Rao, 2006) and mainly present in perivenular regions (acinar zone 3). The prevalence of steatosis in obese individuals is about 75% (Adam *et al.*, 2005).

The simple pathogenic mechanism of hepatic steatosis begins with the accumulation of fat in hepatocytes as a result from metabolic derangements related to central obesity and insulin resistance (Hübscher, 2006). Accumulation of triglyceride in the liver is due to increase delivery of free fatty acids to the liver combine with impairment of fatty acid metabolism in hepatocytes. This preliminary study aimed to observe microscopically any fatty infiltration on the liver surface which may be a sign of steatosis in hypercholesterolemic and PSC-treated rats. The study will be conducted

by using scanning electron microscope (SEM) in which no previous study has been done yet.

Previous studies were focused more on the histological changes by using light microscope and transmission electron microscope (TEM). There are fatty changes observed in hepatocytes of high-fat diet compared to treatment groups (Altunkaynak & Ozbek, 2009; Nepal *et al.*, 2011; Zou *et al.*, 2006). Moreover, study done by Alam *et al.* (2011), showed that liver tissues of 5% mushroom-fed hypercholesterolemic rats were almost similar to normocholesterolemic rats. Therefore, on the way to support previous findings, the present study aimed to demonstrate any signs of steatosis or fatty infiltration in the liver surface of hypercholesteromic and hypercholesterolemic-treatment groups by using SEM. We assumed that it must be a correlation between dose of oyster mushroom and fatty infiltration observed on the liver surface of hypercholesterolemic and hypercholesterolemic-treatment rat.

On the other hand, several studies have been conducted on the ability of oyster mushroom to reduce body weight in hypercholesterolemic rats (Alam *et al.*, 2009, Alam *et al.*, 2011). This finding is significance because hypercholesterolemia is commonly associated with obesity which may cause other health complications such as artheroslerosis, coronary heart disease and others. Furthermore, in one experimental approach, steatosis was the dominant finding in the liver of hypercholesterolemic rats (Bobek *et al.*, 1998) which associated with a significant increase in liver weight (Matos *et al.*, 2005). However, it was less manifest in groups with higher oyster mushroom treatment (Bobek *et al.*, 1998). It was concluded that oyster mushroom have beneficial effect in reducing body weight and fatty changes in liver.

CHAPTER 3

MATERIALS AND METHODS

3.1 *Pleurotus sajor-caju* (PSC) extraction

The extraction of oyster mushroom (PSC) was carried out by using a Soxhlet apparatus (Figure 3.1a), which consists of an extractor, a tubular condenser, a solvent storage flask and an electrical heater. Oyster mushroom (PSC) was supplied by the National Kenaf and Tobacco Board of Malaysia from Bachok Kelantan, Malaysia. PSC mushroom was already in the ground dry powder form. Then, 60 g of ground dry sample of PSC was weighed into the extraction thimble made of thick filter paper. The extraction thimble was then inserted into the main chamber of Soxhlet extractor. Approximately 600 mL of water were added into a round bottom flask, which functions as the solvent. The Soxhlet extractor was attached to the flask containing the solvent and was then equipped with a condenser tube on the top. Water solvent in the flask was heated by using an electric heater and its vapour condense in the condenser tube. The condenser tube ensures that any solvent vapour cools and drips back down into the thimble containing the PSC as warm solvent. The desired compound of PSC was extracted by making contact with warm solvent collected in the chamber. The chamber was automatically emptied by a siphon side arm when the Soxhlet chamber was full. The solvent was running back down to the distillation flask. This cycle was repeated for three to four days until a drop of solvent from the siphon tube does not leave residue when evaporated. After the extraction was completed, the solvent was allowed to cool. The solvent in the flask was then transferred to another glass bottle and sent to the UPMS for the freeze dried process. Product of the freeze dried was in a dry powder form (Figure 3.1b).

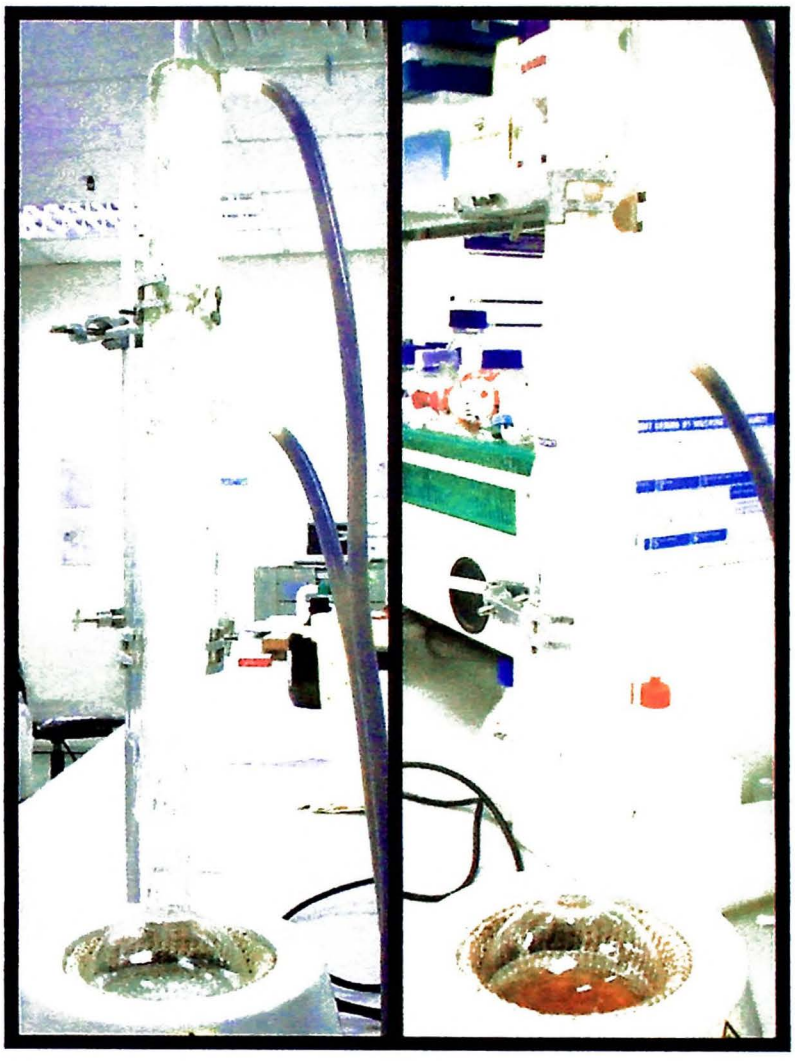

Figure 3.1a: Water extraction of PSC by using Soxhlet apparatus

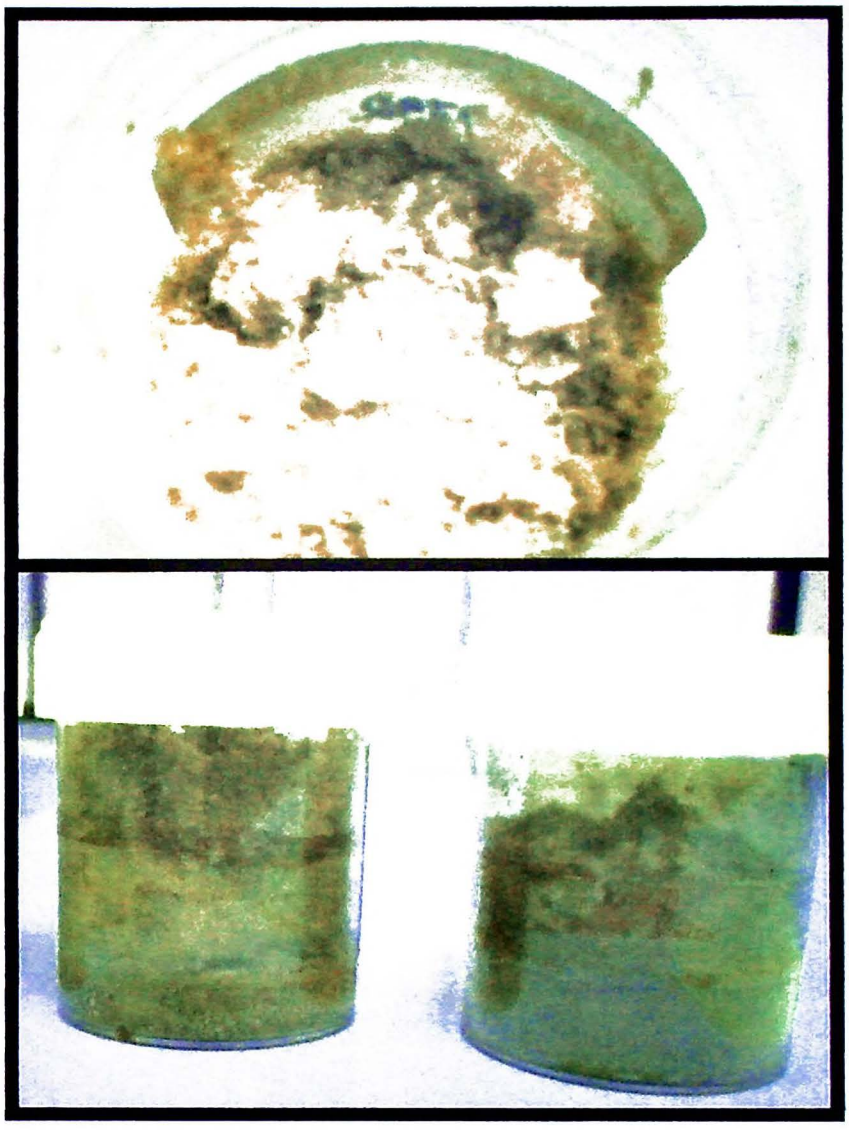

Figure 3.1b: PSC extract after freeze dried

3.2 Animal study

3.2.1 Preparation of high-cholesterol diet (Figure 3.2)

The hypercholesterolemic diet was prepared based on this ratio; 32 g of ghee per 68 g of rat pellet. For one day feeding, the diet was prepared using the following procedure; 340 g of commercial rat's pellet was grounded using an automatic grinder. The powder form of rat's pellet was mixed with 160 g of ghee to total up to 500 g of high-cholesterol diet per day. The mixture was then shaped into pellet again by making it into a ball form. After shaping, pellets were dried for about 15 minutes and used for feeding all rats except normal control group.

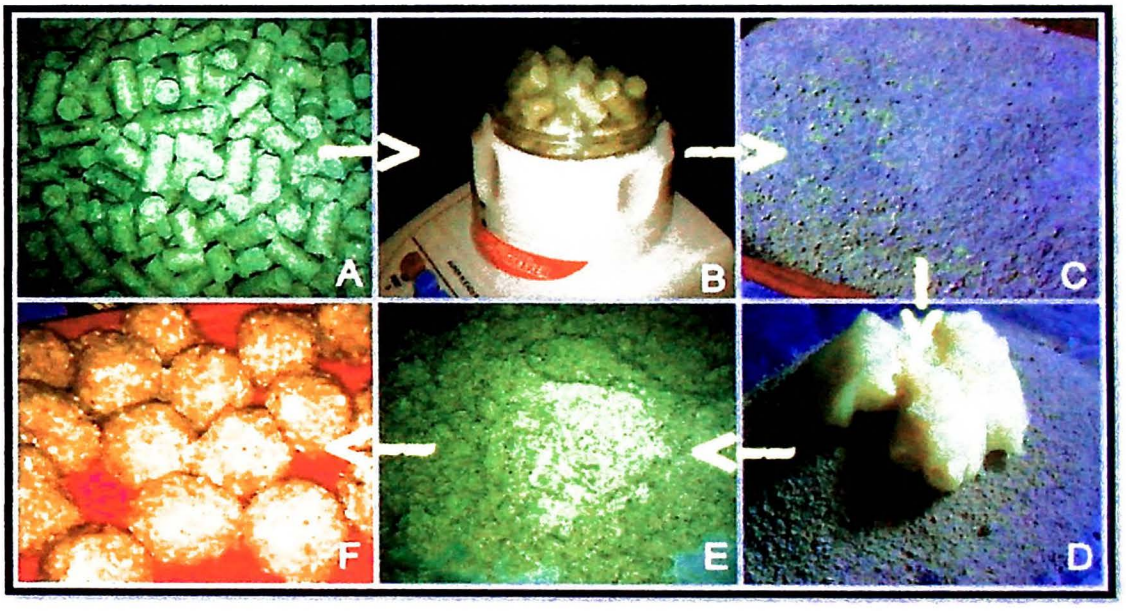

Figure 3.2a: Preparation of high-cholesterol diet. (A: normal rat pellet form; B: rat pellet was grounded; C: product of grounded rat pellet; D: ghee was added to grounded rat pellet; E: mixing product of rat pellet with ghee; F: mixing product was shaped into pellet again by making it into a ball form)

3.2.2 Preparation of diluted PSC extract (Figure 3.2b) and atorvastatin (Figure 3.2c)

Carboxymethylcellulose (CMC) was obtained from Sigma Chemical, USA in a powder form. 1 g of CMC powder was diluted with 200 mL of distilled water by using double boiling technique to produce 0.5% concentration. PSC extract was then diluted in CMC solution as follow; 10 mg of PSC in 0.3 mL of CMC. From here, it was diluted again to produce 0.5 mL final volume by adding 0.2 mL distilled water.

The atorvastatin was purchased as 10-mg tablets from a retail pharmacy. For atorvastatin preparation, the tablet was crunched finely with a mortar. For each 10 mg of atorvastatin, it was diluted with 0.2 mL distilled water.

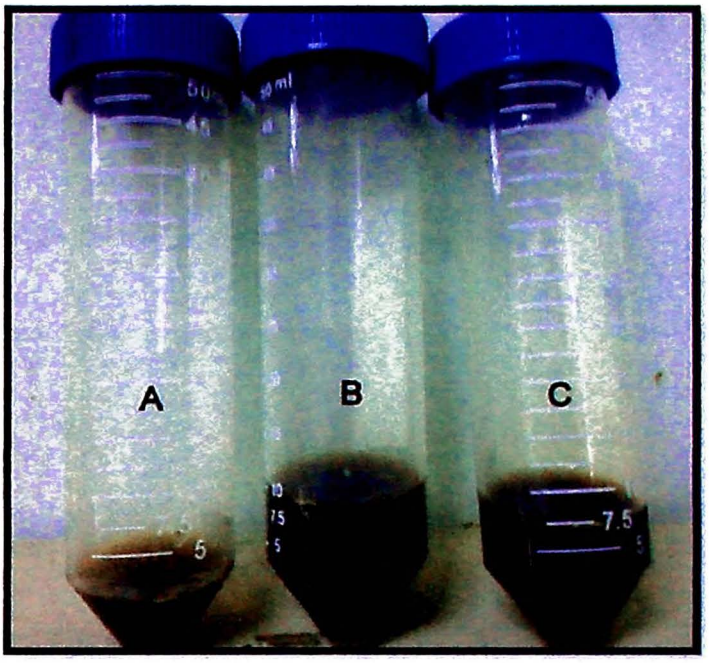

Figure 3.2b: Product of diluted PSC extract.
(A: 20 mg/kg-PSC; B: 100 mg/kg-PSC; C: 200 mg/kg-PSC)

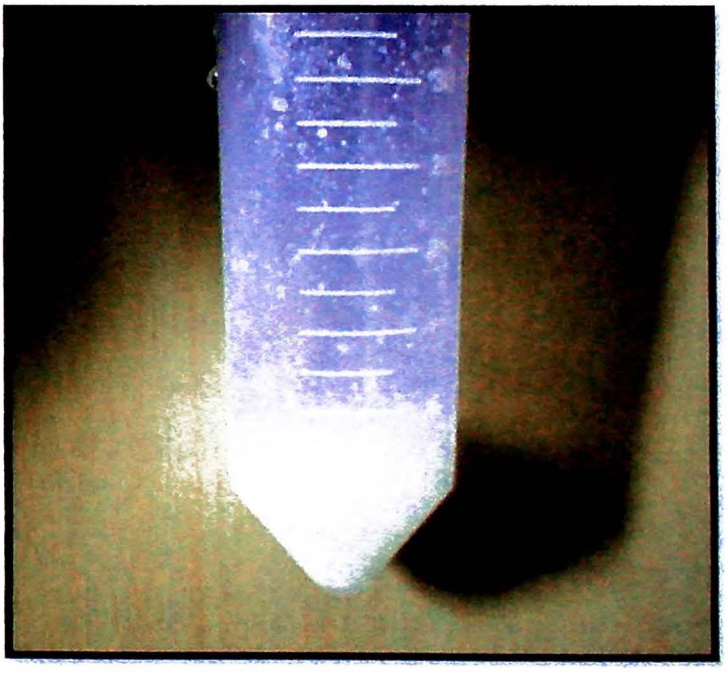

Figure 3.2c: Product of diluted atorvastatin (20mg/kg)

3.2.3 Preparation of 6% sodium pentobarbital

Given,

M1: 200 mg/mL = 20% of pentobarbital

M2: 60 mg/mL = 6% of pentobarbital

To prepare 10 mL of 6% pentobarbital, the calculation was as follow:

$$M1V1 = M2V2$$

$$(200mg)V1 = (60mg)(10mL)$$

$$V1 = 3\ mL$$

Thus, in order to produce 6% of pentobarbital, 3 mL volume of 20% pentobarbital was needed. Seven mL of distilled water was added to sum up the total volume to 10 mL.

3.2.4 Experimental study

The procedure and protocol described below were approved by Animal Ethics Committee USM (USM/Animal Ethics Approval/ 2013/ (83) (446)). Twenty male Wistar rats (Figure 8.6) weighing 250-370 g were obtained from Laboratory Animal Unit Universiti Sains Malaysia (LARUSM). All rats were maintained in an animal facility under standard laboratory conditions for 1 week prior to experiment. They were housed in plastic cages at room temperature of 22 ± 1°C under a 12-h light-dark cycle. For the beginning, rats were fed with a basal diet for one week as an adaptation period. Rats were divided randomly into six feed groups (3 rats/group): control group was fed a basal diet; while the remaining five groups were given 32 g ghee per 68 g pellet (high-cholesterol diet). Rats were fed for two weeks and were allowed free access to water.