EFFECTS OF ANTI-OXIDATIVE AND CHOLESTEROL LOWERING CAPACITIES OF SELECTED EDIBLE-MEDICINAL MUSHROOMS TOWARDS AMELIORATION OF ALZHEIMER'S DISEASE

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ABSTRACT

Alzheimer's disease (AD) is a neuro-degenerative disorder affecting mainly the elderly people. Though more than 40 million people are suffering from AD complications up to present date, there is hardly any treatment effective in withstanding the progression of AD. Thus, demand for natural, safe and cost-effective AD therapeutics has got momentum. Oxidative stress (OS) and increased plasma cholesterol levels have been implicated among the numerous pathomechanistic factors of AD. Despite some discrepencies, strategies aimed at lowering OS and hypercholesterolemia seems promising as AD therapeutics. Mushrooms have been highly hailed for providing numerous health benefits including anti-oxidative and hypocholesterolemic potentialities. However, combined anti-oxidative and hypocholesterolemic effect of edible-medicinal mushrooms have not been reported though these two aspects have been studied separately. Also, as an AD therapeutic agent, mushroom has not been interpreted through their anti-oxidative and hypocholesterolemic properties. Thus, the present study has been designed to elucidate the anti-oxidative, hypocholesterolemic and AD ameliorating properties of the selected edible-medicinal mushrooms: *H. erinaceus, L. edodes, F. velutipes* and *G. lucidum*. Five solvent-solvent partitioned fractions (methanol:dichloromethane, dichloromethane, hexane, ethylacetate, aqueous and hot water extract (HWE) of each of the four mushroom species were screened through *in vitro* anti-oxidant tests including 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging, Folin-Ciocalteu assay, lipid peroxidation (LPO) and low density lipo-protein (LDL) oxidation inhibition tests. Among all, the HWE of *G. lucidum* had been found possessing the best *in vitro* anti-oxidative capacity and highest content of polyphenols, tri-terpenoids and sterols. Thus, this mushroom extract had been chosen for the rest of the studies. *In vivo* anti-oxidative and hypocholesterolemic studies upon rats showed increasing effect towards plasma and liver anti-oxidant enzymes (catalase,

glutathione peroxidase and superoxide dismutase) while total cholesterol (TC), triacylglycerol (TG), LDL-cholesterol were decreased and HDL-cholesterol was increasd. This mushroom extract demonstrated absence of adverse effect upon the organ function tests. As derangement of memory and learning abilities is the most notable complication associated with AD, the effect of the HWE of *G. lucidum* had been tested upon the AD model rats. Feeding of HWE of *G. lucidum* was found to improve memory and learning abilities of the AD rats. This cognitive improvement has been supported by elevated levels of memory related neurotransmitters in the respective rats. Transmission electron microscopic (TEM) studies demonstrated pronged neuronal dendrites in the *G. lucidum* HWE treated rats than those of the non-treated. Finally, brain comparative proteomics have identified differentially expressed proteins involved in neurotransmission, metabolism, cellular stress response and misfolding repairment. Improved functional network had been observed among the proteome of the *G. lucidum* HWE treated rats through STRING analysis. Ingenuity pathway analysis (IPA) identified nervous system development, cell-cell signaling and interaction, molecular transport and cell death and survival among the top-most functional networks among the experimental subjects. Thus, AD ameliorating effect of *G. lucidum* through anti-oxidative and hypocholesterolemic performances might be implicated in AD therapeutics.

ABSTRAK

Penyakit Alzheimer (AD) adalah gangguan degeneratif neuro yang mempunyai kesan ke atas orang tua lazimnya. Walaupun lebih daripada 40 juta orang yang menderita komplikasi AD sehingga kini, tiada rawatan yang berkesan yang wujud untuk menahan perkembangan AD. Oleh itu, keperluan untuk sebatian terapeutik bercirikan semulajadi, selamat dan kos efektif telah mendapat perhatian. Tekanan oksidatif (OS) dan peningkatan paras kolesterol plasma adalah faktor penting di antara pelbagai faktor-faktor yang menyebabkan AD. Walaupun terdapat beberapa perbezaan, strategi untuk menurunkan OS dan hiperkolesterolemia berpotensi sebagai terapeutik AD. Cendawan dikenali dengan pelbagai manfaat kesihatan termasuk anti-oksida dan potensi hipokolesterolemik. Walau bagaimanapun gabungan ciri anti-pengoksidaan dan kesan hipokolesterolemik cendawan yang boleh dimakan atau ubatan tidak dilaporkan sedangkan kedua-dua aspek telah dikaji secara berasingan. Juga, cendawan masih tidak dianggap sebagai agen terapeutik AD, berdasarkan ciri-ciri antioksida dan hipokolesterolemik. Oleh itu, kajian ini telah dilakukan untuk menjelaskan sifat-sifat anti-pengoksidaan dan hypocholesterolemia cendawan terpilih (*H. erinaceus , L. edodes, F. velutipes* dan *G. lucidum*) dalam mencegah AD. Lima pecahan pelarut-pelarut (methanol : diklorometana, diklorometana, heksana, etil asetat, akueous dan ekstrak air panas (HWE) bagi setiap empat spesies cendawan telah disaring melalui ujian *in vitro* antioksida termasuk memerangkap radikal bebas 2,2-diphenyl-1-picrylhydrazyl (DPPH), esei Folin-Ciocalteu, pengoksidaan lipid (LPO) dan ujian perencatan pengoksidaan lipoprotein ketumpatan rendah. Antara semua, HWE *G. lucidum* telah didapati memiliki potensi terbaik kapasiti anti-oksida *in vitro* dan kandungan tertinggi polifenol, tri-terpenoid dan sterol. Oleh itu, ekstrak cendawan ini telah dipilih untuk kajian selanjutnya. Kajian *in vivo* anti-oksida dan hipokolesterolemik ke atas tikus menunjukkan kesan peningkatan paras enzim anti-oksida plasma dan hati

(katalase, glutation peroksidase, superoxide dismutase) manakala jumlah kolesterol (TC), triasigliserol (TG), LDL-kolesterol berkurangan dan HDL-kolesterol meningkatkan kesan dari ekstrak cendawan ini tanpa memberi kesan buruk kepada ujian fungsi organ. Oleh kerana gangguan daya ingatan dan kebolehanan belajar adalah komplikasi yang paling ketara yang dikaitkan dengan AD, kesan HWE *G. lucidum* telah diuji ke atas tikus model AD. Pemakanan HWE *G. lucidum* telah didapati meningkatkan daya ingatan dan kebolehan pembelajaran tikus AD. Peningkatan kognitif telah disokong oleh peningkatan tahap neurotransmitter memori yang berkaitan dalam tikus masing-masing. Bukti lanjut dari kajian histopatologi (DAPI) yang menunjukkan peningkatan sel-sel neuron dan pemanjangan ketara dendrit dalam tikus yang dirawat dengan *G. lucidum* HWE (kajian TEM). Akhirnya, perbandingan proteomik otak telah mengenal pasti protein terzahir yang terlibat dalam penghantaran maklumat, metabolisme, tindak balas tekanan selular dan pembaikan pelipatan. Pemuliharaan rangkaian berfungsi telah meningkat di kalangan proteome tikus dirawat oleh *G. lucidum* HWE berdasarkan analisis STRING. Analisis laluan 'ingenuity' (IPA) mengenal pasti pembangunan sistem saraf, isyarat sel-sel dan interaksi, pengangkutan molekul dan sel mati dan hidup di kalangan rangkaian berfungsi paling atas di kalangan subjek eksperimen. Oleh itu, *G. lucidum* membendung kesan AD melalui ciri-ciri anti-oksida dan hipokolesterolemik mungkin berpotensi sebagai calon untuk terapeutik AD.

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Last but not the least, let we make a new world free of AD through mushroom-based therapeutic approaches.

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LIST OF SYMBOLS AND ABBREVIATIONS

Abbreviations	Definitions
Aβ	Amyloid beta
AD	Alzheimer's disease
AI	Atherogenic index
APP	Horseradish peroxidase
BDNF	Brain derived neurotrophic factor
BBB	Blood brain barrier
BM	Barnes maze
CD	Conjuated diene
DPPH	2,2 –diphenyl 1 picrylhydrazil
ELISA	Enzyme linked immunosorbent assay
FC	Fold change
GC-MS/MS	Gas chromatography tandem mass spectrometry
HDL-C	High density lipo-protein cholesterol
IC_{50}	Concentration that produces 50% inhibition
IPA	Ingenuity pathway analysis
LC-MS/MS	Liquid-chromatographt tandem mass spectrometry
LDL-C	Low-density lipo-protein cholesterol
LPO	Lipid peroxidation
LTP	Long-term potentiation
MDA	Malondialdehyde
NOR	Novel object recognition
PSD 95	Post-synaptic density 95
RME	Reference memory error
Rt	Retention time
SNAP 25	Synaptosomal associated protein 25
TBARS	Thiobarbituric acid reactive substances
TC	Total cholesterol
TG	Triacylglycerol
TNFα	Tumor necrosis factor alpha
VAchT	Vesicular acetylcholine transporter
WME	Working memory error

CHAPTER 1: GENERAL INTRODUCTION

1.1 Current trend and future challenges of Alzheimer's disease

Alzhemier's disease (AD), a neurodegenerative disorder and the most common form of dementia, poses grave threat towards the ever increasing lifespan of the humanity. By 2050, the global figure of the centenarians (people aged 100 years) is expected to reach up to 2 million (Alzheimer's Association, 2016). During their lifetime, one in every eight male and one in every four female has been predicted to develop AD (Alzheimer's Association, 2016). There are more than 40 million people worldwide suffering from AD (Selkoe & Hardy, 2016). Although AD is an age-onset physical complication, manifested usually after the age of 60, its initiation and progression occurs during early stages of life (Swerdlow, 2007).

According to the amyloid cascade hypothesis, AD is caused by the accumulation of amyloid beta peptide (Aβ), derived from the plasma membrane bound amyloid precursor protein (APP), inside the neurons called as neurofibrillary tangles or outside the neurons called as plaques (Hardy & Selkoe, 2002). Aβ aggregates cause neuronal cell toxicity, cell function loss and/or cell death leading to AD complications (Hardy & Selkoe, 2002) .

Oxidative stress (OS) and hypercholesterolemia (elevated level of blood cholesterol) are the most common etiological factors coinciding AD (Stampfer, 2006). Elevated level of plasma cholesterol, through the formation of the "lipid rafts" in the plasma membrane bilayer, facilitates the deposition of the neurotoxic Aβ (Ehehalt *et al.*, 2003). Cholesterol lowering drugs, statins or 3-hydroxy-3-methylglutaryl coenzyme A (HMG-

CoA) reductase inhibitors have been reported to be protective against AD development (DeKosky, 2005; Sierra *et al.*, 2011).

1.2 Research gap and importance of the present work

Although AD is going to plague the aged humanity worldwide, till today, there is no specific drug for the treatment of AD; the available medico-strategies just delay the worsening of the symptoms. Yet, the synthetic drugs are highly expensive and cause numerous side effects. Therefore, to aid the ever increasing global aged and AD prone populace, finding out of safe, less expensive and easy to achieve therapeutic agents has emerged as an urgent need.

As modern life-style and dietary pattern evoke OS and hypercholesterolemia highly, lifestyle modification involving functional food based therapeutic approaches seem imperative in withstanding the progression of AD. In this context, mushrooms spur high due to their anti-oxidative and hypocholesterolemic effects. In AD model animals, mushrooms have been found boosting up neurogenesis and improving pathophysiological complications (Mori *et al*., 2011; Zhou *et al*., 2010) . Still today, there is scarcity of therapeutic approach against AD through combined management of OS and hypercholesterolemia. In this regard, evaluation of mushrooms as the AD ameliorating agent beacons promising and the present study is aimed at elucidating the anti-oxidative potentiality of edible *(H. ericaneus, F. velutipes* and *L. edodes)* and medicinal mushrooms *(G. lucidum),* followed by the cholesterol lowering and AD ameliorating effects of *G. lucidum.*

1.3 Research questions

Whether oxidative stress attenuating and cholesterol lowering capacities of selected mushroom species are effective in enhancing memory and learning abilities of the AD subjects?

1.4 Research objectives

Objectives of the present research are

1. to determine the *in vitro* anti-oxidative effect of the solvent – solvent partitioned fractions and of hot water extract (HWE) of *Ganoderma lucidum* (GL), *Hericium ericaneus* (Lion's mane, LM), *Lentinula edodes* (Shiitake, SK) and *Flammulina velutipes* (Enoki, EN) mushrooms.

2. to identify the bio-active component present in the selected mushroom fractions and extracts.

3. to evaluate the *in vivo* anti-oxidative and hypocholesterolemic effect of the HWE of *G. lucidum* on rat models.

4. to investigate memory and learning related behavioral improvements by *G. lucidum* HWE towards AD model rats.

5. to perform proteomics analysis and protein-protein interaction of the AD model rats' brain tissue.

1.5 Outline of the thesis

Comparing the *in vitro* anti-oxidative performance of different solvent – solvent partitioned fractions and HWE of *G. lucidum, H. erinaceus, L. edodes* and *F. velutipes,* this study selects HWE of *G. lucidum* as the potent extract **(Chapter 3)** to further with identification of bio-active components **(Chapter 4)** and *in vivo* studies. **Chapter 5** describes the *in vivo* anti-oxidative and hypocholesterolemic performance of the HWE of *G. lucidum.* AD ameliorating behavioral, antibody and ELISA and transmission electron microscopic outcomes of the HWE of *G. lucidum* have been described in **Chapter 6. Chapter 7** outlines the proteomics study of the selected animal models.

Chapter 8 deals with the general discussion and **Chapter 9** contains the concluding remarks and future aspect of the present study.

CHAPTER 2: LITERATURE REVIEW

2.1 Oxidative stress (OS) and its physiological complications

In normal physiological condition, reactive oxygen species (ROS) and reactive nitrogen species (RNS) are generated as a part of cellular metabolic, signaling and defensive actions (Finkel & Holbrook, 2000). As they are highly reactive, they undergo reactions with other reactive anions and/or cations and turn into neutral molecules or the bodily systems neutralize, removes or attenuates them through different mechanisms (Valko *et al.*, 2007). Thus, these reactive species have very short half-lives (Valko *et al.*, 2007). But, when the level of their generation outnumbers their dismissal through enzymatic or non-enzymatic processes, the state of oxidative stress (OS) emanates. Persistent oxidative stress (OS) mediates structural and functional alteration of biomolecules (carbohydrates, proteins, lipids, nucleic acids), poses threat towards normal functioning of the tissues and organs that result in numerous pathophysiological consequences such as cardiovascular diseases (CVD), neurodegenerative diseases including AD, diabetes and aging (Dalle-Donne *et al.*, 2006; Valko *et al.*, 2007).

2.2 Anti-oxidative defense and demand for natural anti-oxidant

Traditionally an "anti-oxidant" is a substance that prevents oxidation. In biological sense, an "anti-oxidant" is a natural or synthetic substance capable of withstanding or prolonging the deteriorating effect of oxygen. Halliwell, (1990) defined biological antioxidant as a substance that at small concentration can oppose the oxidative modification of the biomolecules. Food science considers anti-oxidants as the substances that slow down or thwart the detrimental upshot of the reactive species upon foods (Huang *et al*., 2005). From biochemical and medicinal viewpoint, antioxidants are

the enzymatic or non-enzymatic substances having the potential of reverting oxidation (Kedare & Singh, 2011). Most of the natural anti-oxidants possess polyphenolic structures.

Evolutionarily, cells have adapted themselves to cope with the constant exposure of ROS with multiple defense strategies (Kedare & Singh, 2011). Cells thwart OS through physical defense involving stabilization of plasma membrane and steric hindrance. Anti-oxidative enzymatic defense system includes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) counterbalance the production of ROS (Valko *et al.*, 2007). SOD first converts superoxide into relatively less harmful hydrogen peroxide and later on, CAT and GPx turns hydrogen peroxide into water (Lobo *et al.*, 2010; Valko *et al.*, 2007). Also, non-enzymatic intracellular anti-oxidants act directly by scavenging free radicals or through breaking of the free radical generating chain reactions and this type of anti-oxidant include glutathione, flavonoids, pyruvate, vitamin C (ascorbic acid), vitamin E (α tocopherol) and carotenoids (Lobo *et al.*, 2010). Cells synthesize a trivial amount of the direct-acting anti-oxidants and most of them must be supplied through diet for maintenance of the normal cellular functioning against OS (Lobo *et al.*, 2010). Thus, the supply of the exogenous anti-oxidants (natural or synthetic) seemed promising as a preventive therapeutic stratagem against ROS-mediated pathophysiology. Though, both natural and synthetic anti-oxidants fall under choice in this regard the second form of anti-oxidant suffers from numerous side effects. To cite some examples, synthetic anti-oxidant butylated hydroxyl toluene (BHT) has been associated with lung carcinogenesis, butylated hydroxyl anisole negatively regulate mitogen-activated protein kinase (MAPK) and cause stomach cancer (Botterweck *et al.*, 2000; Kahl, 1984; Umemura *et al.*, 2001). In short, adverse effects generated from the usage of the synthetic anti-oxidants outweigh their benefits and their usage has faced severe criticism both in terms of dosage and regulatory perspectives

(Shahidi & Zhong, 2005). Thus, demand for natural, dietary anti-oxidants has got momentum (Lobo *et al.*, 2010).

2.3 Unperturbed cellular signaling with anti-oxidant usage

ROS and RNS participate as messengers in the physiological redox signaling processes (Brigelius-Flohé & Flohé, 2011; Forman *et al.*, 2014; Holmstrom & Finkel, 2014; Murphy *et al.*, 2011; Sawa *et al.*, 2013). ROS and RNS mediate post-translational modification to the thiol moieties of the proteins involved in cell signaling through formation of sulfoxides, disulphides, sulfenic acid and also by S-glutathionylation and S-nitrosylation (Naviaux, 2012; Sies, 2015). Thus, the redox signaling based concept of OS incorporates it as a signal that provokes oxidative type of reactions and/or impairs the redox harmony that results in two consequences: heightened cellular defense, which can be termed as eustress and/or cellular damage that can be termed as distress (Niki, 2009, 2016). At physiological level, low level of OS had been deemed as an adaptive strategy i.e. eustress that associates augmented accumulation of defensive components including antioxidants, proteins and enzymes (Niki, 2009; Noguchi, 2008; Surh *et al.*, 2009). Role of ROS as the metabolic and longevity signaling molecules had also been reckoned (Chen *et al.,* 2005; Ristow & Schmeisser, 2011).

As ROS and RNS include a diverse array of components differing in structure and function, their removal by anti-oxidative strategy do not directly affect the beneficial roles of ROS and RNS in signaling (Niki, 2016).

2.4 Hypercholesterolemia

Cholesterol is an integral component of the animal cell membrane and a precursor of vitamin D3 and steroid hormones. Cholesterol and triacylglycerols are carried in the blood with the aid of the lipo-proteins. Based on the density, lipo-proteins are of five

types: very low density lipo-protein (VLDL), intermediate density lipo-protein (IDL), low-density lipo-protein (LDL) and high density lipo-protein (HDL). Hypercholesterolemia refers to the elevated level of cholesterol in the blood. It is a form of hyperlipidemia (elevated lipid level in blood) and also ascribed as dyslipidemia (Durrington, 2003). Cholesterol is generated both from dietary sources and endogenously. Cholesterol metabolism occurs in the liver. Under normal physiological system, there occurs a balance between its biosynthesis and removal in the form of bile acids and excretion through intestine. Hypercholesterolemia stems from the increased synthesis and/or decreased removal of endogenous cholesterol (due to defective clearance activity of VLDL as well as increased catabolism of VLDL into IDL and LDL) and also from the excessive supply of dietary cholesterol or cholesterol precursors. The rate-limiting step of cholesterol biosynthesis is mediated by the 3-hydroxy 3-methyl glutaryl co-enzyme A (HMG Co-A) reductase (HMGR). Cellular level of cholesterol regulates its activity: higher cholesterol level reduces whereas lower cholesterol level stimulates its functioning.

2.4.1 Management of hypercholesterolemia

The Fifth Joint Task Force of the European Society of Cardiology and Other Societies on Cardiovascular Disease Prevention in Clinical Practice had suggested life-style and dietary modification as the first line and pharmacological intervention through utilization of lipid lowering drugs as the second line of treatment strategy against hypercholesterolemia (Perk *et al.*, 2012). Life style modification involves loss of weight, consumption of diet containing less cholesterol and higher saturated fatty acid, giving up of cigarette smoking and alcohol drinking, intake of unsaturated fatty acids such as DHA and regular physical exercise (Brown *et al.*, 2009; Galani & Schneider, 2007; Klein *et al.*, 2004). However, combination of both life style modification and pharmacological treatment sound better in real life (Last *et al.*, 2011). Currently, seven

types of drugs are available for treating hypercholesterolemia that include statins (inhibitors of 3-hydroxy-3-methyl glutaryl co-enzyme A reductase), fibrates, bile acid sequestrants, derivatives of nicotinic acid, estrogen replacement therapy and monoclonal antibodies to proprotein convertase subtilisin/ kexin type 9 inhibitor (Bergeron *et al.*, 2015). Following are the important features of these hypocholesterolemic agents

2.4.1.1 Statins

Statins inhibit intracellular cholesterol biosynthesis by inhibiting the activity of the HMG Co-A reductase and also have HDL-C increasing, LDL receptor increasing and triacylglycerol lowering effects (Liao & Laufs, 2005). Its side effects include myalgia, release of muscle and liver enzymes in the blood, contraindication with other drugs, nausea, headache, dyspepsia, constipation, insomnia, diarrhea and abdominal pain (Sinzinger *et al.*, 2002).

2.4.1.2 Fibrates

Fibrates stimulate the expression of the peroxisome proliferator-activated receptor (PPAR)-α, which later regulates the inhibition of hepatic synthesis and secretion of triglycerides. They also stimulate the degradation and clearance of triglyceride-rich lipoproteins through enhanced expression of lipoprotein lipase (Staels *et al.*, 1998). This increased clearance of triglycerides results from a stimulation of the expression of lipoprotein lipase (Zhang *et al.*, 2001).

Its side effects include increased level of liver enzymes in the blood, gastrointestinal disturbance, myalgia, myositis, dermatitis, insomnia and impotency (Elisaf, 2002).

2.4.1.3 Bile acid sequestrants

Bile acid sequestrants are the anion-exchange resins. They bind the bile acids in the gut and inhibit their entero-hepatic recycling process. As a consequence, synthesis of hepatic LDL receptors are upregulated for obtaining cholesterol to compensate the production of bile acids that ultimately results in reduction of LDL-C level (Einarsson *et al.*, 1991).

They are very often unpalatable and are not absorbed. Their side effects include constipation, abdominal bloating, diarrhea, headache and dizziness (Insull, 2006).

2.4.1.4 Nicotinic acid derivatives

Vitamin B_3 (nicotinic acid) derivatives had been suggested having suppressing effect up on the hormone sensitive lipase of adipocyte and thus causes reduced synthesis of non-esterified fatty acid, TG, VLDL and LDL as well as increased synthesis of HDL (Kamanna & Kashyap, 2000). Its inhibitory effect up on diacylglycerol acyltransferase-2, the key enzyme of hepatic TG synthesis, also aids in its anti-hypercholesterolemic effect (Kamanna & Kashyap, 2008).

Its side effects include hypotension, hyperuricemia, abdominal discomfort, flushing, rhinitis, nausea, rash, pruritus and headache (Gille *et al.*, 2008).

2.4.1.5 Estrogen replacement therapy (ERT)

Decreased level of estrogen especially in the post-menopausal women had been associated with increased rate of atherosclerosis (Mikkola & Clarkson, 2002). ERT had been found beneficial in the younger post-menopausal women who are at the earlier stage of hyperlipidemia and atherosclerosis (Mikkola & Clarkson, 2002). But, older (more than 65 years old) post-menopausal women having pre-existing atherosclerosis seemed unresponsive towards ERT (Mikkola & Clarkson, 2002).

ERT has contraindication with liver diseases, thromboembolic diseases and breast cancer. It also causes headache, bloating and breast tenderness (Ross *et al.*, 2000).

2.4.1.6 Inhibitors of proprotein convertase subtilisin/kexin type 9 (PCSK9)

Binding with the heapatic LDL receptors, PCSK9 lowers LDL uptake and increases blood LDL levels. Recently developed inhibitors (monoclonal antibodies) to PCSK9, lowers the production of PCSK9 and reduces their binding with the LDL receptors and thus lowers production of LDL and CVD risk (Bergeron *et al.*, 2015).

However, due to the multiple adverse effects of the synthetic anti-hypercholesterolemic medications, food and dietary supplement based approaches have gained the alternative therapeutic attention. In this regard dietary fiber and complex polysaccharide rich foods such as mushrooms have highly been adored.

2.5 Alzheimer's disease (AD)

2.5.1 General features

Alzheimer's disease (AD) is the most common form of dementia, the deteriorated behavioral and mental state that disrupts the affected person's everyday life. AD had been named after German neuro-pathologist and psychiatrist Alois Alzheimer who first described this neurodegenerative disease in 1906 (Hippius & Neundorfer, 2003). AD symptoms start with gradual decrement of the capability learning and memory. This occurs due to the neuronal degeneration in the brain region associated with memory and learning (Binetti *et al.*, 1996). As a consequence the affected person becomes unable to perform daily normal activities, becomes confused about time and space, faces problem in planning and executing even errands (Waldemar *et al.*, 2007). At advanced stage, the AD patients suffer from difficulties in speaking and writing, sleeping and awakening and even cannot recall their own names (Waldemar *et al.*, 2007). They also face problem in remembering their personal history, recognizing relatives and family

members (Bäckman *et al.*, 2004). They need help in dressing and toileting properly and cannot control their bladder and bowel activities. They become irritated and suspicious even about their caregivers though they become dependent on their caregivers and family members. At the final stage, they become unresponsive towards conversation and their language becomes limited to little words and finally they fully lose tendency of speaking (Taler & Phillips, 2008). Their muscle becomes rigid and cannot swallow well or move by themselves. They also become vulnerable to infectious diseases (Förstl & Kurz, 1999).

2.5.2 Epidemiology and Prevalence of AD

According to the Alzheimer's statistics 2016, more than 44 million people are suffering from AD worldwide. Prevalence of AD is highest in the Western Europe, followed very closely by the USA while lowest in the sub-Saharan Africa. AD is the prime cause of disability in later age of life. In 2016, global cost of AD is estimated to be 605 billion US dollar (http://www.alzheimers.net/resources/alzheimers-statistics/). Prevalence of AD increases up to 15 times during the age range of 65 – 80 years. AD prevalence is higher in the developed countries than those of the developing and least developed (Mayeux & Stern, 2012). This might be due to the increased life span of the people living in the developed countries. As the developing countries are also harboring increased number of aged people, AD prevalence trend is also upward in those countries (Prince *et al.*, 2015). AD is posing threat to the global economic policy as it impacts world economy negatively.

2.5.3 Types of AD

Generally, AD occurs in the aged people. Sometimes, the younger and mid – aged people also suffer from it. Genetic and environmental factors also influence its causation and combined types of AD can be described based on the criteria

2.5.3.1 Early onset and late onset

Early onset AD (EOAD) develops before age 65 and is less common (about 5% of all the AD cases) while the late-onset AD (LOAD) is the most common form of AD and affects people over 65 years of age (Zetterberg & Mattsson, 2014).

Both the age-onset types can fall into either familial or sporadic forms.

2.5.3.2 Familial and sporadic

Early onset familial form is most rare and found in about 1 percent of total AD cases (Rogaev *et al.*, 1995). These patients bear fault in either chromosome 1 (*PS2*gene) or in chromosome 14 (*PS1* gene) or in chromosome 21 (*APP* gene) (Rogaev *et al.*, 1995). Age range of these patients is 40-50 years and sometimes may arise even at 30 years of age. People with Down's syndrome (bearing extra copy of chromosome 21) are at increased risk of developing early onset familial AD (Munter *et al.*, 2010). The apolipoprotein E (APOE) gene, especially its APOE-ε 4 allelle is considered as the genetic risk factor of late onset familial AD (Sando *et al.*, 2008).

Sporadic form of EOAD is also uncommon (about 10% of total AD cases) and the people with Down's syndrome are at increased risk of it. Sporadic form of LOAD, dubbed as the sporadic AD, is the most common (about 90% of total AD cases) form that affects people over 65 years old (Zetterberg & Mattsson, 2014). In every five year, there increases two-fold risk of sporadic AD development after 65 years age (Bermejo-Pareja *et al.*, 2008).

2.5.4 Impaired memory – the most prominent feature of AD

2.5.4.1 Memory and learning

Memory is the recalling process of any information learned previously (Squire & Kandel, 2000). Learning is related with the acquirement process of something while

memory denotes the acquired expression of something. Thus, learning is a process involved in memory. Memory process involves multiple phases: encoding (information acquisition), storage (recording of the encoded information into short - or long – term form) and retrieval (recalling of the stored memory) (Izquierdo & Medina, 1997). Hippocampus, cerebral cortex, basal ganglia, amygdala and cerebellum of the brain participate in memory and learning abilities.

From neurochemical point of view, memory and learning abilities are the outcomes of the synaptic plasticity, changing (either strengthened or weakened) ability of the synapses in response to the stimuli (Abbott & Nelson, 2000). It may be of short-term (milliseconds) or long-term (minutes to hours) duration. The short – term synaptic plasticity alters the strength of neurotransmission while the long – term one changes the number and structure of the synapses. If synaptic plasticity strengthens the synapses, the state is termed as synaptic potentiation and if weakens, synaptic depression (Teyler & DiScenna, 1987). Based on the duration, both potentiation and depression may of short – and long–term. The short short–term potentiation (STP) is reversible while the long-term potentiation (LTP) is irreversible. The former (STP) is the neuro-molecular analog of short-term memory (STM) and the latter of long–term memory (LTM).

2.5.4.2 Types of memory

Following the modified model of Atkinson and Shiffrin (1968), different types of memory has been illustrated in Figure 2.1.

A. Short – term memory (STM): It is the memory of the most recent events and lasts for only a few seconds to minutes. As a bio-electrical type of memory, it incorporates changes in synaptic neurotransmission, enzyme activation and protein phosphorylation but no *de novo* protein synthesis. It may be re-classified into sensory (ability to retain sensory information immediately after

the original stimulus has elapsed) and working (organized or manipulated form of the STM) memory types. The executive form of working memory is an important aspect of behavior as it maintains guidance, decision-making and reasoning while the visual memory deals with the memories formed from visual experience.

B. Long – term Memory (LTM): Recalling of the permanent information ranging from days, months to years even throughout one's lifetime are of long – term memory (LTM) type. It is a biochemical type of memory that can be sub-divided into procedural and declarative forms. Procedural memory sub-type of LTM is the memory of motor skills such as learning of procedures or skills and the declarative sub-type refers to the memory of facts and figures. Declarative memory is also known as the explicit memory which may be either semantic (related to general knowledge such as word meaning, concept, ideas) or of spatial (related to spatial orientation and environment) and/or episodic (related with specific experience).

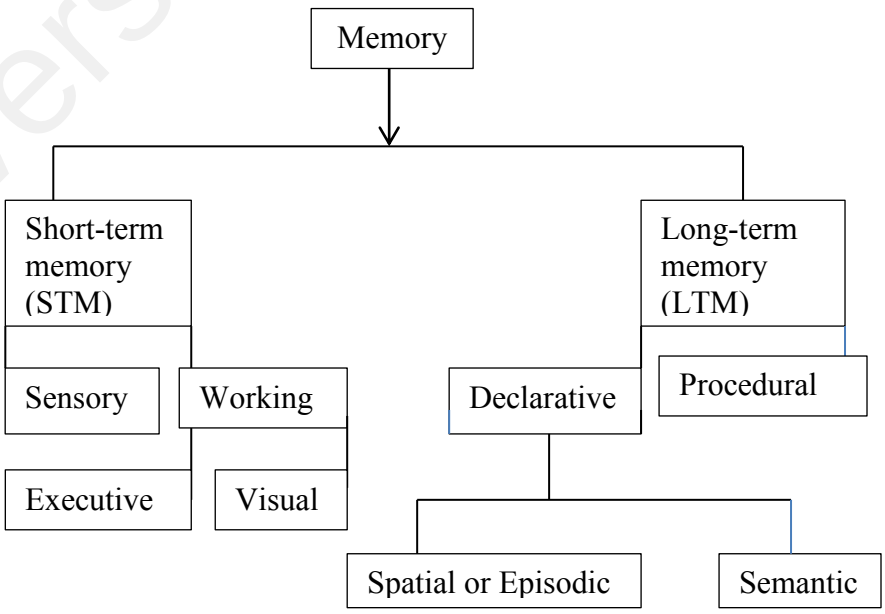


Figure 2.1: Types of memory

2.5.4.3 Synapses at the ground of memory formation

Synapses, the interconnections of two nerve endings, are the ground of neurochemical and neuro-electrical signal transmission (Kleim *et al.,* 2002). Neurotransmitters released from the pre-synaptic membranes bind with the post-synaptic membrane receptor and causes signal transduction mediated polarization and/or depolarization (Ferreira & Klein, 2011; Yan *et al.,* 2016). Heightened synaptic connection and transmission induce enhanced LTP and improved memory. On the other hand, weakened synaptic connections and lowered transmission cause LTD and poor memory (Ferreira & Klein, 2011; Yan *et al.,* 2016).

2.5.4.4 Pre – and post – synaptic protein links in memory formation

A number of pre – and post – synaptic proteins participate in memory consolidation (Figure 2.2). Without the functioning of these proteins, the proper consolidation and recall of memory processes become impaired. Pre – synaptic proteins involved in memory consolidation include synaptosomal – associated protein 25 KD (SNAP 25), syntaxin 1B, synaptogamin 1, synaptojanin I, synaptophysin, synapsin I and II, amphiphysin, Munc 13-1, Munc 18, dynamin 1, Rab 3A, SV 2A, CASK, VDCC α2δ1, VDCC (N type)α1B, ELKS, Piccolo, Bassoon, neurexin – 1 and – 3 and Tomosyn b (Rosenberg *et al.,* 2014). The post – synaptic memory related proteins are post – synaptic density protein 95 KD (PSD 95), PSD 93, TrkB, Glu A2 and A3, Glu N1 and N2B, $GABA_BR$ – 1, ProSAP1/Shank2, SAP 97, SAP 102, Homer 1, CaMKIIα, CaMKIIβ, Neuroligin 3 and 3, Cortactin B, α Actinin 1, Drebrin, N-CAM and Agrin (Rosenberg *et al.,* 2014).

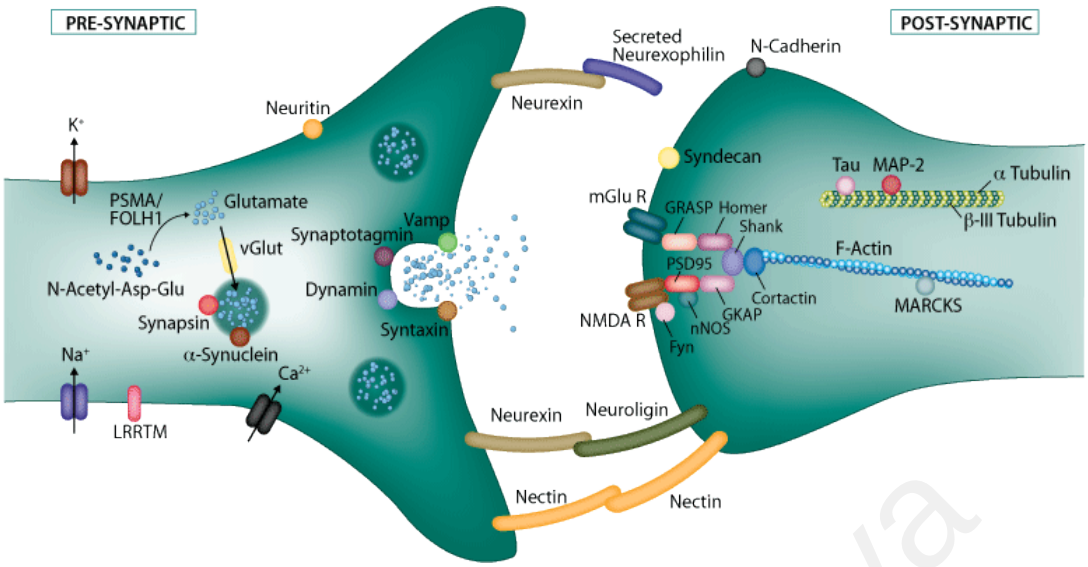


Figure 2.2: Pre – and post – synaptic proteins involved in memory processing (Adapted from https://resources.rndsystems.com)

2.5.4.5 Memory and learning ability disruption in AD

Disruption of memory is the first among the six cognitive domains most commonly altered in AD. Other five domains perturbed in AD are associated with learning abilities that include executive functioning, language, visuo-spatial functioning, attention and upset (Ross, 2009). At the onset, memory disruption might be mistakenly treated as the forgetfulness associated with the normal aging processes. As the diseases progresses, worsen memory and personality change make the everyday life challenging to them. As memory loss is the most expressive hallmark of AD, improvement of disrupted memory has been the focus of the AD treatment strategies.

2.5.5 Causes of AD

Like other chronic diseases, AD had been believed to be the result of multiple causative factors. Various hypotheses had been put forward to explain the cause and mechanism of AD. The most common ones are described below.

2.5.5.1 Genetic predisposition

About 0.1% of the early-onset familial AD occurs due to autosomal dominant inheritance. Mutation in any of the gens *APP, PS1 and PS2* or their combined effect might be the cause of AD.

2.5.5.2 Amyloid plaque formation

Glenner and Wong (1988) first proposed that amyloidogenesis of a particular protein might be a causative agent of AD . According to the amyloid cascade hypothesis of Aβ formation, Aβ is formed from APP by the sequential cleavage of β- and γ-secretase (Hardy & Higgins, 1992) (Figure 2.3). Normally, APP is cleaved by the α-secretase and does not produce the harmful Aβ; but in AD, cleavage by β-secretase is favored (Hardy & Higgins, 1992). Increased production and decreased clearance of Aβ leads to the deposition of Aβ plaques that cause neurodegeneration and AD consequences (Figure 2.4) (Selkoe & Hardy, 2016).

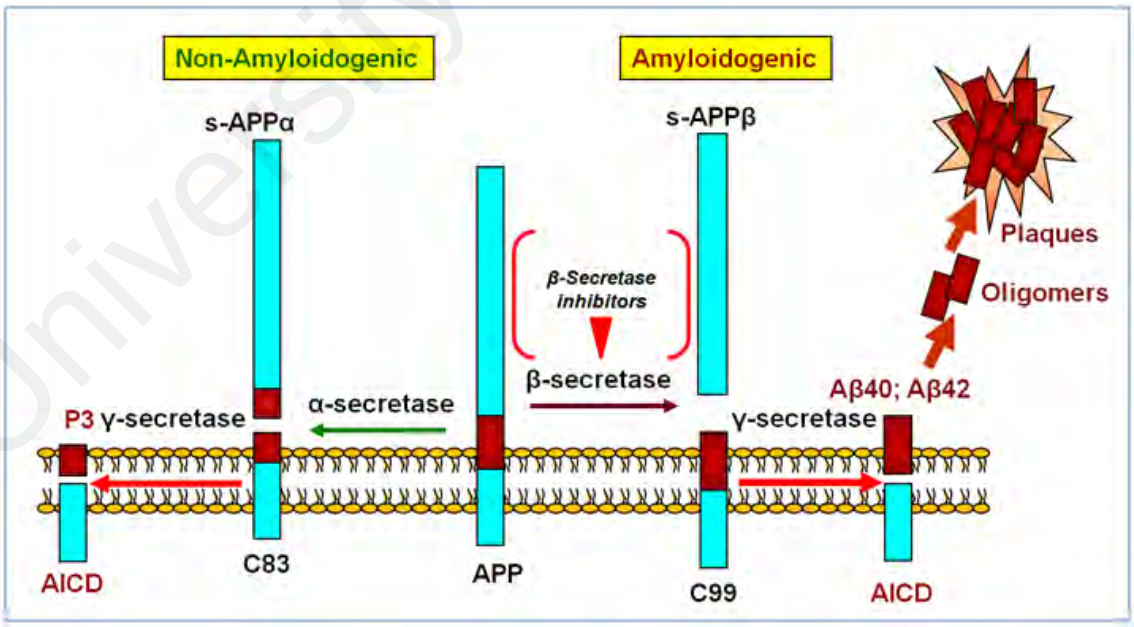


Figure 2.3: Amyloid β production (Zhang, 2012).

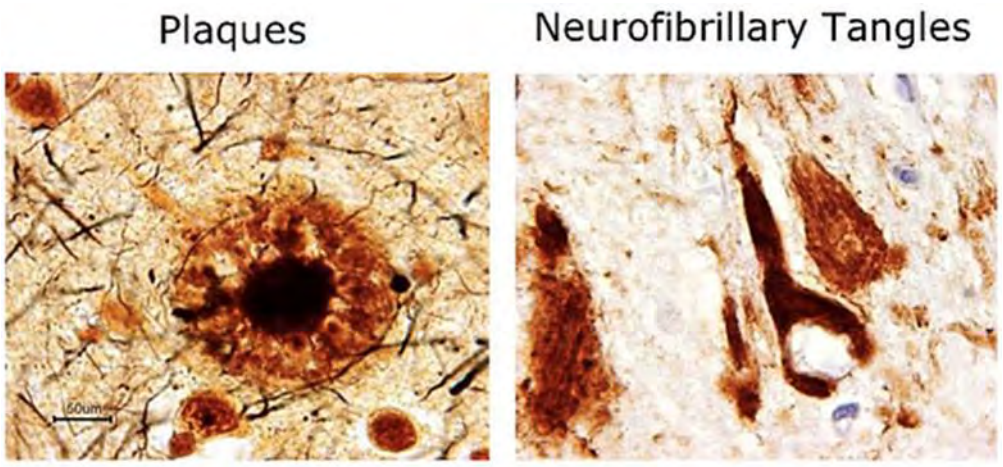


Figure 2.4: Photomicrograph of AD brain (Adapted from Robbins and Cotran Pathologic Basis of Disease, 7th edition).

The last twenty-five years have observed the dominating prevalence of the amyloid cascade hypothesis amongst all the others (Hardy & Selkoe, 2002; Selkoe & Hardy, 2016b). Some discrepancy of this hypothesis is that amyloid plaques are not always linked with cognitive impairments and cognitive-normal subjects with increased amyloid deposition in the brain had been reported (Benilova *et al.,* 2012; Giannakopoulos *et al.,* 2003; Ingelsson *et al.,* 2004; Perez-Nievas *et al.,* 2013). Thus, a reappraisal of the amyloid cascade hypothesis had emerged stating that oligomeric Aβ are neurotoxic and sets the ground for synaptic dysfunction involving tau hyperphosphorylation and neurofibrillary tangles (NFT) formation ultimately resulting in neuronal loss (Hardy, 2006; Selkoe & Hardy, 2016b; Spires-Jones & Hyman, 2014).

Another approach towards interpreting the role of Aβ in AD pathogenesis is the "modern amyloid cascade hypothesis" which states that at low (picomolar) level, Aβ is not detrimental, rather memory enhancer and only at higher level, it impairs cognition; its soluble forms rather than the plaques are neurotoxic (Morley & Farr, 2014).

However, studies relating mutations in the genes *APP, PS-1, PS-2* and *Apo E4* support the amyloid cascade hypothesis of AD (Tanzi, 2012). Thus, dyshomeostasis of Aβ remains as the prime reason behind AD pathogenesis (Selkoe and Hardy 2016b).

Although, some mid - and late – stage clinical trials have failed to prove the amyloid cascade hypothesis fully, it would not be legitimate to abandon this hypothesis as the discrepancies of the clinical trials might occur due to flawed experimental design, choice of the experimental subjects, toxic biochemical reactions such as liver toxicity evoked by BACE1 inhibitors and non-selective inhibition of the γ – secretase and immunospecificity (De Strooper, 2014; Saxena, 2010; Toyn & Ahlijanian, 2014; Yan *et al.,* 2016). Considering the ever-increasing number of AD subjects in the next two decades, much research emphasizing the amyloid cascade hypothesis in parallel with those of others seem promising.

2.5.5.3 Neurofibrillary tangle formation

Neurofibrillary tangles (NFT) are formed mainly of tau, the microtubule associated protein. Tau regulates neuronal polarity, connects axonal microtubule with neuro-membrane and provides stability. It has about thirty sites potential for phosphorylation and in AD, tau is highly phosphorylated that disrupts its connectivity and stability providing function (Geschwind, 2003). Hyperphosphorylated tau becomes aggregated and tangled (Figure 2.4). It loses association with microtubules and cannot process axonal transportation, causes neurotoxicity and impairs mitochondrial respiratory chain that leads towards neuronal death (Figure 2.4) (Götz *et al.,* 2006; Santacruz *et al.,* 2005). Tau also aids in Aβ-mediated neurotoxicity and neuronal death (Rapoport *et al.,* 2002). Also, kinases involved in tau phosphorylation such as cyclin dependent kinase 5 (CDK 5), glycogen synthase kinase 3 beta (GSK 3β) and microtubule affinity regulatory

kinase (MARK) link AD pathogenesis with OS, inflammation, depleted energy generation and disrupted cellular communication (Ballatore *et al.,* 2007).

A drawback of this hypothesis is that though NFT impairs cognitive functioning, mutation in tau showed only fronto - parietal dementia (FTD) and not AD (Giannakopoulos *et al.,* 2003; Goedert & Jakes, 2005; Ingelsson *et al.,* 2004).

2.5.5.4 Oxidative stress

Oxidative stress hypothesis underscores "oxidative stress" as the upstream of AD pathogenesis (Nunomura *et al.,* 2006; Nunomura *et al.,* 2001). As ageing continues, the brain encounters oxidative and metabolic stress of multiple sorts. Besides, the brain itself consumes the highest oxygen, contains the highest amount of oxidation-prone unsaturated fatty acids as well as relatively low amount of anti-oxidative enzyme especially catalase. Early-onset, familial AD pathogenesis has been exacerbated through oxidative stress as revealed by the germline mutation studies. In addition, late-onset, familial and sporadic AD has also been implicated to be associated with oxidative stress through apolipoprotein E (Apo E) ε4 allele(s) (Tamaoka *et al.,* 2000). AD risk factors involving hypertension, diabetes mellitus, stroke, smoking and intoxication as well as life-style-oriented mediators have been directly or indirectly linked with increased oxidative stress and decreased anti-oxidative defenses (Tamaoka *et al.,* 2000). Oxidation of lipid and nucleic acids in biological fluids along with decreased plasma level of antioxidants and total anti-oxidative capacity have been found reported in AD patients (Nunomura *et al.,* 2006). Studies involving cell lines also tally this (Misonou *et al.,* 2000; Paola *et al.,* 2000). This result was further established by the finding that anti-oxidant vitamin E (α - tocopherol) decreased both Aβ and Tau lesions in animal models (Nakashima *et al.,* 2004). Compared to the normal subjects, Aβ plaques in AD brains contain higher amount of redox active metal ions (Cu^{2+}, Zn^{2+}, Fe^{2+}) that can induce localized OS (Lovell *et al.,* 1998; Roberts *et al.,* 2012).

In addition, OS stimulates several kinases such as glycogen synthase kinase -3 and mitogen activated protein kinases (MAPK) that are active participants of AD pathogenesis. They phosphorylate Tau protein, and once phosphorylated, Tau is more vulnerable for oxidation that leads to neurofibrillary tangle (NFT) formation (Liu *et al.,* 2005). Thus, NFTs are also resultant products of neuronal oxidative stress.

Increased biomolecular oxidation is an important hallmark of AD. Increased level of carbonyls (protein oxidation product); MDA, TBARS, 4-hydroxy-2-trans nonenal (HNE) and isoprostanes (lipid peroxidation products); 8-hydroxy guanosine (8 OHG) and 8-hydroxy-2 deoxy-guanosine (nucleic acid oxidation products); glycation and glycoxidation (sugar oxidation products) in AD brains bear testimony towards OS induced AD pathogenesis (Butterfield *et al.,* 2001). OS driven mitochondrial dysfunction is another aspect of AD pathogenesis. Mitochondrial DNA contribute to the production of 13 proteins needed for respiratory chain and ROS-mediated mutation in mitochondrial DNA leads towards stable neuronal dysfunction as the neurons do not divide (Santos *et al.,* 2013). More importantly, AD neuronal mitochondria had been detected as the potential site of Aβ accumulation, where it induces OS and disrupts the redox activities of some enzymes (Caspersen *et al.,* 2005; Lustbader *et al.,* 2004; Manczak *et al.,* 2006; Petersen *et al.,* 2008). Thus, OS induced mitochondrial dysfunction linked with impaired metabolic activity affect neuronal function and memory.

2.5.5.5 Protein oxidation in AD

Proteins are highly vulnerable to oxidative modification due to presence of functional groups in their constituent amino acids. Oxidative modification of proteins often leads towards their functional impairment. Normally, proteasome degrades and removes the oxidized proteins from the cell. Some enzymes such as glutathione reductase, thioredoxin and methionine sulfoxide reductase also participate in the repairing and

removing activity towards the mildly oxidized proteins (Friguet, 2006). Free radicals either oxidatively modify the amino acid side chains of the proteins and hence alter the protein backbone or cause protein fragmentation. Mechanistically, carbonyl groups (C=O) are either introduced into the protein's structure or are produced as in case of the reaction between proteins with those of the lipid peroxidation products such as MDA (Berlett & Stadtman, 1997). Peroxynitrite ($ONOO^-$) mediated nitration of the protein tyrosine side chain is another aspect of post - translational protein modification (PTM). The resultant 3-nitrotyrosine hampers phosphorylation of the tyrosine residue by tyrosine kinase (TK) that affects TK mediated signal transduction process including those of nerve growth factor (NGF) and brain derived neurotrophic factor (BDNF) resulting in apoptosis (Mangialasche *et al.,* 2009). Peroxynitrite can also oxidize proteins.

2.5.5.6 Mitochondrial cascade hypothesis

According to this hypothesis, aging causes accumulation of toxic components through oxidative damage of the mitochondria and these toxic accumulants lead to "the 3-R response": reset (production of beta amyloids as an adaptive response towards the oxidative damage of the neurons), remove (reduced synaptic communication and apoptotic removal of the neurons) and replace (production of neurofibrillary tangles) (Swerdlow & Khan, 2004).

2.5.5.7 Inflammation

Inflammatory hypothesis suggests that AD pathologies emanate from the inflammatory response towards extracellular amyloid-β deposits. At the outset, inflammatory response is neuroprotective as it is aimed at clearing Aβ (Rogers *et al.,* 2002; Wyss-Coray & Mucke, 2002). However, continuous overload of Aβ surpasses the beneficial effects and turns into deleterious (Wyss-Coray & Mucke, 2002). Inflammatory response is governed by the microglia and increases over time through

Tau. The inflammatory markers (TNF α, IL-2, IL-6, CRP) have been implicated to be directly or indirectly linked with cognitive decline and AD (Bettcher & Kramer, 2014). Aβ initiates inflammation by binding with complement C1, microglial toll-line receptor (TLR) and receptor for advance glycation end products (RAGE) (Tacnet-Delorme *et al.,* 2001; Tahara *et al.,* 2006). Inflammatory and transcription factors (AP_{-1}, NF-κB) become activated that generate ROS and activate the cholinergic neurons and involve astrocytes in the inflammatory cascade (Parvathenani *et al.,* 2003; Suzuki *et al.,* 2004). Repeated induction by Aβ and supply of ATP to the microglia add fuel to this vicious cycle.

2.5.5.8 Synaptic dysfunction

Aβ released from the presynaptic membrane can bind to the post-synaptic membrane and modulate post-synaptic functions (Kamenetz *et al.,* 2003). Low level of Aβ can induce synaptic plasticity and maintain LTP (Puzzo *et al.,* 2011). On the contrary, increased Aβ level; dimeric, trimeric and oligomeric toxic forms of Aβ facilitates towards impaired LTP and evokes LTD, neuronal and synaptic loss of activity (Palop & Mucke, 2010; Sheng *et al.,* 2012; Spires-Jones & Hyman, 2014; Tu *et al.,* 2014; Yan *et al.,* 2016). Also, abnormality in the expression and function of the pre – and post – synaptic proteins also facilitate impaired memory and AD pathogenesis (Ardiles *et al.,* 2012; Ferreira & Klein, 2011; Hernandez & Abel, 2008; Sutton & Schuman, 2006) . Thus, Aβ mediated synaptic dysfunction disrupts the memory forming events and results in AD consequences.

2.5.5.9 Cholinergic hypothesis

Acetylcholine is an excitatory neurotransmitter of the CNS, neuromuscular junction and is essential for neuronal functioning and for memory and learning abilities. According to the cholinergic hypothesis of AD, decreased availability of the cholinergic

neurotransmitter acetylcholine (Ach) leads towards AD consequences (Terry & Buccafusco, 2003). Produced by acetylcholine transferase from acetyl-CoA and choline, it is released into the synaptic cleft and upon binding to the post – synaptic neuron, exerts signal transduction. Acetyl choline esterase (AchE) breaks down Ach and does not allow prolonged action of Ach into the post–synaptic neuron that affects memory and learning abilities. Thus, treatment strategies have been developed based on this that agents having anti-acetyl choline esterase (AchEI) activity would have ameliorating effects on AD. Since 1970s, different AchEIs have been developed namely donepezil, galantamine, rivastigmine and tacrine (Craig *et al.,* 2011). AchEIs have been reported to improve cognitive and behavioral performance of the AD subjects.

2.5.5.10 Dopaminergic hypothesis

Dopamine can act both as an excitatory and inhibitory neurotransmitter as well as the precursor of other neuro-transmitters, anti-oxidant and anti-amyloidogenic substance (Himeno *et al.,* 2011). Dopaminergic hypothesis of AD describes Aβ – induced impaired level of dopamine in the hippocampus leads towards disrupted cognitive functions (Guzmán-Ramos *et al.,* 2012; Martorana & Koch, 2013). However, detailed clinical studies are called for establishing this hypothesis.

2.5.5.11 Excitotoxicity and Ca^{2+}dyshomeostasis

Hyperactivation of the NMDA (N-methyl D-aspartate) receptor by Aβ or by L-glutamate destroys the post-synaptic Ca^{2+} homeostasis, increases intra-neuronal Ca^{2+} concentration, leading towards neuronal death (Hynd *et al.,* 2004; Koh *et al.,* 1990). NMDA receptor excitotoxicity also stimulates tau expression and hyper phosphorylation (Amadoro *et al.,* 2006). Increased level of intracellular Ca^{2+} and Ca^{2+}–dependent protease calpin associated with tau and decreased level of Ca^{2+} buffering protein calbindin correspond towards Ca^{2+} dyshomeostasis in AD brains (LaFerla,

2002). Endoplasmic reticulum stress mediated Ca^{2+} dyshomeostasis may also cause neuronal death in AD (Katayama *et al.,* 2004).

2.5.5.12 Decreased level of neurotrophic factors

Human brain harbor mainly four types of neurotrophic factors such as brain derived neurotrophic factor (BDNF), nerve growth factor (NGF), neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4). Among them, the role of BDNF had been vastly studied. In AD subjects, decreased level of BDNF had been observed in the hippocampus and cortex implying its role in memory and learning and in neuro-protection (Lee *et al.,* 2005).

2.5.5.13 Defective endocytosis

Neurotransmitters left at the synaptic cleft are endocytosed and re-used for further synaptic transmission. Endocytosis of the neurotransmitters is essential for maintenance of proper signal transmission. Defective endocytosis impairs constant signaling between the neurons that affects memory formation and recalling (Nixon, 2007). Defective endocytosis also affects neuronal survival as they fail to receive extracellular regulatory signals (Nixon, 2005, 2007). Endosomal alteration is an earlier event in AD pathogenesis (Cataldo *et al.,* 2004; Nixon, 2005, 2007).

2.5.5.14 Defected energy metabolism

Altered enzymatic activity of glucose, glycogen metabolism and impaired function of the mitochondrial respiratory chain result in reduced energy (ATP) generation that deplete the neurons of vital support. Insulin insufficiency and resistance in diabetes also pose threat to the neuronal survivality and parallels AD progression (Suzanne & Wands, 2008).

2.5.5.15 Vascular hypothesis

Vascular hypothesis has been described as a two-hit process: vascular risk factors (such as injury, APOE4, hypercholesterolemia, hypertension, hypoglycemia) disrupts the blood brain barrier, decreases blood flow causing reduced amyloid-β clearance and increased accumulation of amyloid-β. Though highly activated, the microglia fail to remove excessive amyloid-β burden that results in inflammation and oxidative stress that intensifies synaptic loss, neuronal dysfunction, neurodegeneration and accelerated dementia (Stanimirovic & Friedman, 2012). Thus, amyloid-β leads towards self-propagation of AD.

2.5.5.16 ABSENT hypothesis

The ABSENT (Amyloid Beta Synergistic Endothelial and Neuronal Toxicity) is hypothesis is based on the research findings that not any one hypothesis by itself can fully explain the mechanism underpinning AD and the consequent histopathological alteration. It explains the toxicity of Aβ to both vascular and neuronal tissues along with their synergistic effect on the onset and progression of AD (Roy & Rauk, 2005). ABSENT hypothesis indicates Aβ as the sole generator of the free radicals that later leads to AD pathologies (Roy & Rauk, 2005).

2.5.5.17 Sink hypothesis

According to the sink hypothesis of AD, there occurs a concentration dependent shift of Aβ from the brain into the blood. Decreased level of blood Aβ level is an indicator of its lowered clearance and increased deposition and increased risk of AD (Yamamoto *et al.,* 2014; Zhang, Yan & Lee, 2011). Capacity of the blood albumin for binding of Aβ determines their level in the blood and increased level of blood albumin-bound Aβ is an indicator of increased clearance of Aβ from the brain as well as decreased brain Aβ deposition (DeMattos *et al.,* 2002; Yamamoto *et al.,* 2014).

2.6 Influence of cholesterol upon Aβ production

2.6.1 Lipid raft based Aβ production

In normal physiological system, Aβ is produced in neuronal and non-neuronal cells (Busciglio *et al.,* 1993). It is a 4 KDa peptide, hydrophobic in nature, generated from the amyloidogenic processing of amyloid precursor protein (APP). APP is a type I transmembrane glycoprotein expressed in every cell and its gene is in chromosome 21. APP undergoes two sequential enzymatic cleavages: first with the membrane bound aspartyl protease, β site APP cleaving enzyme (BACE, β secretase) that generates N-terminal extracellular soluble β-secretase-cleaved APP (sAPP or sAPPβ) and transmembraner carboxyl terminal fragment (CTFβ or C99 fragment) (Figure 2.3) (Golde *et al.,* 2000). In the second step, CTFβ is cleaved by γ-secretase that produces Aβ peptides of whose two most common are 40 amino acid long, Aβ40 and 42 amino acid long, Aβ42 (Figure 2.3) (Golde *et al.,* 2000). Between Aβ1-40 and Aβ1-42, the second one along with its insoluble oligomers are much neurotoxic and potent in forming senile plaques in AD pathogenesis (Walsh & Selkoe, 2007). Their neurodegenerative mode of action involves *inter alia* oxidative stress, inflammatory and synaptic dysregulation (Mattson, 2004; Oddo *et al.,* 2003; Reddy & Beal, 2008). Also, a cognate CTFγ is produced (Figure 2.3). In the amyloidogenic pathway, about 90% of the produced Aβ are of Aβ40 type. In the non-amyloidogenic (secretory) pathway, α-secretase cleaves APP to generate the secreted derivative, sAPPα and CTFα (Golde *et al.,* 2000). Later, γ-secretase cleaves CTFα and produces CTFγ (Figure 2.3) (Golde *et al.,* 2000). Indeed, γ-secretase can act only after the activities of α and β secretases and α and β secretases very often compete for APP (Skovronsky *et al.,* 2000). Thus, stimulation of one pathway coincides with inhibition of the other. However, in normo-cellular condition, the non-amyloidogenic (secretory) pathway prevails.

Experimental evidence suggests that the amyloidogenic processes occur in the lipid rafts or in the membrane of trans Golgi network and endosomes while the non-amyloidogenics occur beyond the lipid rafts of the plasma membrane (Ehehalt *et al.,* 2003; Kojro *et al.,* 2001; Lee *et al.,* 1998; Vetrivel *et al.,* 2004). Lipid rafts are membrane microdomains that are cholesterol rich, gangliosides and glycosyl phosphatidyl inositol anchored proteins supposed to be participants of cell signaling (Simons & Ikonen, 1997).

Normally, membranes of endoplasmic reticulum and Golgi apparatus contain low level of cholesterol. Increased cholesterol level or altered cholesterol distribution in these membranes mediate increased lipid raft production which subsequently lead towards increased Aβ generation (Ehehalt *et al.,* 2003; Schroeder *et al.,* 2001). Increased intracellular cholesterol alter membrane lipid composition, augment β- and γ-secretase activities and stimulate APP cleavage resulting in increased Aβ production (Burns & Duff, 2003; Burns *et al.,* 2003; Puglielli *et al.,* 2003; Shobab *et al.,* 2005; Wahrle *et al.,* 2002). Intracellular cholesterol, at elevated level, attaches with the transmembrane domain of APP that leads to APP's insertion into the lipid raft and Aβ generation (Beel *et al.,* 2010). Not only production but also aggregation and fibrillogenesis of Aβ in AD brain is facilitated by the membrane cholesterol level through pooling of Aβ with lipid raft component GM1 ganglioside (Kakio *et al.,* 2002). On the contrary, lowered level of cholesterol decreases Aβ fibrillogenesis through reduced binding of Aβ with lipid raft component GM1 (Mizuno *et al.,* 1999) . Even, altered sub-cellular distribution of cholesterol affects Aβ production (Jin *et al.,* 2004). However, the amyloidogenic pathway is highly favored with the esterified cholesterol rather than the non-esterified one (Bhattacharyya & Kovacs, 2010; Puglielli *et al.,* 2001).

2.6.2 Apo E4 driven Aβ production

Apolipoprotein E is the main carrier of cholesterol in the central nervous system (CNS) and also an important constituent of very low density lipoproteins (VLDL). Among its three allelles (ε2, ε3 and ε4), individuals carrying the ε4 allele are at heightened risk of developing AD as the ApoE ε4/ε4 genotype increases fibrinogenesis in the brains of Alzheimer's disease patients (Hultman *et al.,* 2013). However, ε3 is less potent in causing AD while ε2 allele has been associated with decreasing AD risk (Hooijmans & Kiliaan, 2008).

ApoE ε4 shows gender specific effect (women more vulnerable than men) and also gene dosage effect (homozygous carriers are more at risk than their heterozygous counterparts) (Hooijmans & Kiliaan, 2008). People carrying APOE ε4 usually experience elevated blood cholesterol levels that later on aids in Aβ accumulation and AD pathology (Eichner *et al.,* 2002; Hooijmans & Kiliaan, 2008; Kivipelto *et al.,* 2002). Elevated cholesterol in the brain also triggers Aβ deposition.

However, some schools of thought have put forward contradiction to this mechanism (Dietschy & Turley, 2001; Ledesma & Dotti, 2006). Thus, more explanatory mechanism might be that the APOE ε4 allele upsurges serum cholesterol levels through atherosclerosis followed by hypoperfusion of specific brain areas *en route* to Aβ deposition and neuronal degeneration, the hallmarks of AD. Besides, ApoE4 has been associated with vascular diseases including coronary artery disease, atherosclerosis, diabetes, hypertension and obesity (Davignon *et al.,* 1988; Wellington, 2004). The underlying mechanism might be its diminished clearance role towards LDL and chylomicrons. ApoE4 has also been reported influencing cerebral hemodynamics such as leakage of the blood-brain barrier (Zipser *et al.,* 2007) and cerebral amyloid angiopathy (Kinnecom *et al.,* 2007).

2.7 Association among OS, hypercholesterolemia and AD

The association between hypercholesterolemia and increased risk of AD pathogenesis had been emanated from multiple studies. Hypercholesterolemia linked increased deposition of Aβ in the rabbit hippocampus was first demonstrated by Sparks *et al*. (1994). Later, Refolo *et al.* (2000) showed that transgenic mice fed hypercholesterolemic diet become much prone to development of AD (Refolo *et al.,* 2000). In their following studies, Refolo *et al*. (2001) observed that feeding of cholesterol lowering drugs to the transgenic AD mice reduces the risk of AD development by about 50% (Refolo *et al.,* 2001). Increased brain level of cholesterol has been found during AD progression (Wood *et al.,* 2002). The role of cholesterol in AD pathogenesis becomes clearer from the opposite relationship between plasma HDL level and risk of developing AD (Wolf *et al.,* 2004). HDL can slow down the aggregation of Aβ and thus prevents Aβ toxicity (Olesen & Dagø, 2000). Epidemiological studies suggest 2-3 times greater risk of late-age dementia and AD in people having mid-life hypercholesterolemia than the normo-cholesterolemics (Anstey *et al.,* 2008). Heightened AD neuropathologies have been observed in experimentally-induced hypercholesterolemic animals (Shie *et al.,* 2002). Alike to these findings, decreased neuronal cholesterol levels has been found to be inhibitory towards Aβ formation (Buxbaum *et al.,* 2001; Wolozin, 2001). Correlation between neuritic plaques and hypercholesterolemia had further been evidenced from autopsy studies of the young patients (Pappolla *et al.,* 2003). Major cholesterol metabolite 24S-hydroxycholesterol, had been found in higher concentrations in the plasma and cerebrospinal fluid of the AD patients (Schönknecht *et al.,* 2002). Thus, hypercholesterolemia plays pivotal role in the progression of AD (Mathew *et al.,* 2011).

Brain is much prone to OS due especially to its high lipid and low anti-oxidative defense arsenal content and its enormous (one fourth of total respired) oxygen

utilization (Ansari & Scheff, 2010; Mazzetti *et al.,* 2015; Reed, 2011; Sottero *et al.,* 2009). Inside brain, neurons are more vulnerable to OS and direct association between OS and Aβ generation had been observed in both animal models in human subjects (McLellan *et al.,* 2003). Transgenic AD model mice (Tg19959) having partially defective anti-oxidant enzyme MnSOD, demonstrated accelerated OS as well as Aβ level in brain. Two to three-fold overexpression of the same enzyme in the same model animals showed lowered OS (representing 50% increased level of catalase along with 50% reduced level of protein oxidation), about 33% decreased level of Aβ deposition and restoration of memory deficit (Dumont *et al.,* 2009). In line with these, deficiency of Cu/ZnSOD1 in Tg2576 AD model mice enhanced OS-driven Aβ oligomerization and memory loss (Murakami *et al.,* 2011).

Increased lipid peroxidation and reduced anti-oxidant status had been linked *apriori* to the onset of AD symptoms (Chen *et al.,* 2008; Resende *et al.,* 2008; Su *et al.,* 2010). Indeed, Aβ itself acts as a pro-oxidant (Combs *et al.,* 2001; Ding *et al.,* 2007). Methionine35 within the Aβ sequence make it much vulnerable to OS and its replacement with cysteine lowers its tendency towards oxidation (Butterfield & Boyd-Kimball, 2005; Butterfield *et al.,* 2013). Thus, it is plausible that the Aβ oligomers interpolate in the lipid bilayers and generate lipid peroxidation whose sequel continues to other biomolecules (Butterfield *et al.,* 2001; Naylor *et al.,* 2008; Tamagno *et al.,* 2006). Mitochondrial dysfunction often generates OS that is detrimental towards cellular survival and aids in AD pathogenesis (Castellani *et al.,* 2002; Melov *et al.,* 2007; Reddy, 2011; Yao, Jia *et al.,* 2009). Amyloid precursor protein (APP) and Aβ act as the mitochondrial deregulators that further the state of OS (Anandatheerthavarada *et al.,* 2003; Caspersen *et al.,* 2005; Manczak *et al.,* 2006).

Cholesterol cannot cross the blood brain barrier (BBB) *per se* and to get rid of excessive cholesterol, the brain converts it into relatively polar oxysterol 24 – OH and oxysterol 27 – OH, aided by the 24-hydroxylase and 27-hydroxylase, respectively. Oxysterols are capable of crossing the BBB and is exposed to the systemic circulation and then eliminated (Björkhem *et al.,* 2009; Björkhem & Meaney, 2004; Meaney *et al.,* 2007). Oxysterol 27 - OH can re-enter into the brain, exert much hypercholesterolemic condition there and propel Aβ production (Björkhem, 2006; Heverin *et al.,* 2005; Sharma *et al.,* 2008). Thus, OS along with hypercholesterolemia might be implicated in the production, deposition and oligomerization of Aβ and thus an important antecedent in AD pathogenesis (Oda *et al.,* 2010).

Thus, strategies effective in lowering plasma cholesterol or inhibiting the biosynthesis of cholesterol sound imperative for withstanding OS, hypercholesterolemia and AD pathogenesis.

2.8 Mushroom – the functional food and traditional medicine

A functional food possesses bio-active components capable of providing nutritional as well as disease preventive and therapeutic support to its consumer (Augustyniak *et al.,* 2010). Mushrooms have long been associated with multiple aspects of human civilization such as their utilization in human diet and medicine due to their delicacy, nutritional and therapeutic values (Valverde *et al.,* 2015; Wasser 2014). Usage of mushrooms for medicinal purposes dates back to the Paleolithic age (more than 2000 years ago) and their psychedelic functionality had placed them among the divine foods and in mythology (Sanodiya *et al.,* 2009). Though, traditionally being used for a long time for various culinary and ailing purposes, mushrooms seem befitting as the functional foods against OS, hypercholesterolemia and AD (Guillamón *et al.,* 2010;

Sirtori *et al.,* 2009; Wasser, 2014). From the nutritional point of view, mushrooms possess considerably higher amount of carbohydrate, protein, vitamins and minerals and lower amount of fats (Barros *et al.,* 2007; Cheung, 2010). Their content of unsaturated fatty acids predominate over the saturated ones (Barros *et al.,* 2007; Cheung, 2010) . Their carbohydrate content varies from 35% to 70% of their total dry weight and they are rich sources of vitamins and minerals (Cheung, 2010; Furlani & Godoy, 2008; Mattila *et al.,* 2001). They are also rich sources of dietary fibers that aid in their CVD preventive actions (Cheung, 2013; Nile & Park, 2014). Traditional Chinese medicine had used mushrooms in various ailments such as *Cordyceps* against hepatic diseases, *Pleurotus* for gastro-intestinal disorders and *Ganoderma* for neuro-psychological problems and as an energizing agent (Aida *et al.,* 2009; Paterson, 2006). Their therapeutic usage still remains in practice and some mushrooms had been included in the Chinese Pharmacopoeia (National Pharmacopoeia Committee, 2010).

2.9 Current trends in AD treatment

2.9.1.1 Enhancement of neurotransmitter level and neurotransmission

As different neurotransmitters and their signaling processes are affected in AD, treatment strategies have focused on improving both the levels of neurotransmitters and neurotransmission. Food and drug administration (FDA, USA) had approved four drugs (galantamine, donepezil, rivastigmine and memantine) as neurotransmitter modulating in AD. Galantamine, donepezil and rivastigmine inhibit AchE activity and modulate cholinergic neurotransmitter signaling processes (Davis & Couch, 2014). Meta analysis of the epidemiological studies indicates protection of the drugs against cognitive, behavioral and personality aberration associated with AD (Lanctôt *et al.,* 2009). Unfortunately, these drugs can not alter AD pathogenesis rather they improve only the AD symptoms temporarily, their side effects are ample and can affect cholinergic system beyond the brain and they are suitable for the preliminary AD patients only

(Hansen *et al.,* 2008; Winblad & Jelic, 2004). AD pathogenesis involves glutamate – mediated enhanced excitotoxicity of the NMDA receptor and memantine blocks this excitotoxicity through non-competitive antagonism (Danysz & Parsons, 2012). Neuro-protective role of memantine from glutamate mediated excitotoxicity and behavioral improvement of the mild to moderate AD patients had been reported (Areosa *et al.,* 2005). It also has cholinergic system improving effect and the combined donepezil and memantine therapy showed donepezil like cognitive and behavioral improvement in the mild to moderate AD subjects (Tariot *et al.,* 2004). Lecozotan, the antagonist of 5-hydroxytryptamin (5-HT) or serotonin receptor, aimed at improving the level of 5 – HT in AD subjects, also suffers from excessive side effects (Booth *et al.,* 2012).

Thus, the current treatment strategies aimed at improving neurotransmitter signaling could improve AD pathogenesis very little and the improved behavioral attainments were of very short duration. Thus, treatment strategy capable of sustained AD modulating effects involving much protection against neurodegeneration and cognitive improvement is quintessential.

2.9.1.2 Modulation of the secretases

As the β- and γ – secretases are involved in generation of Aβ, their controlled activity would aid in maintaining AD pathogenesis. Transgenic mice deficient of the β – secretase (BACE1) had shown decreased accumulation of brain Aβ level (Ghosh *et al.,* 2012). Inhibition of the γ – secretase also showed promising outcomes (Siemers *et al.,* 2005). Also, reduced Aβ deposition has been observed in case of the α – secretase stimulator bryostatin that induced the non-amyloidogenic pathway of APP processing (Etcheberrigaray *et al.,* 2004).

2.9.1.3 Immunotherapy

Both active and passive immunization of the transgenic animals with the anti - Aβ antibodies showed microglia – mediated clearance of Aβ plaques from the AD model animals (Bard *et al.,* 2000; Schenk *et al.,* 2004). However, adverse side effects including T – cell mediated encephalopathy led towards Aβ truncation at the C-terminal end and development of an immune-conjugate with the N – terminal of Aβ peptide attached to a carrier protein and having reduced risk (Schenk *et al.,* 2004).

2.9.1.4 Inhibition of Aβ fibrillation

Inhibitors designed towards the promoters of Aβ-Apo E and Aβ – Aβ interaction halt the conversion of Aβ in the β sheets and thus hinder the fibrillation process. Alzhemed (NC - 531) mimics glycosaminoglycan, the promoter of Aβ fibrillation and thus under trial as an anti – AD drug (Geerts, 2004). Similarly, the metal chelators (such as clioquinol, PBT-1 against Cu^{2+} and Zn^{2+}) can lower Aβ level and fibrillation by chelating metal ions (Cherny *et al.,* 2001). Longer Aβ peptides (such as Aβ 42, 43) are more self-aggregating and much AD-causing than their shorter counterparts such as Aβ 40. Even, Aβ 40 may be amyloidogenesis preventing (Dickson *et al.,* 2007)

2.9.1.5 Inhibition of tau kinases

Phosphorylation of tau by kinases like CDK5, GSK - 3β leads towards NFT formation and inhibition of the kinases are target of AD therapeutics. However, tau phosphorylation is interplay of multiple kinases and phosphatases and thus development of anti – AD drug using this approach seems cumbersome.

2.9.1.6 Anti-inflammatory drugs

As AD is associated of neuro-inflammation, usage of anti-inflammatory drugs seemed pertinent in AD therapeutics. Epidemiologically, non-steroidal anti-inflammatory drugs (NSAIDs) could reduce the risk of AD and lower Aβ deposition in

transgenic mice brain (Aisen, 2002; Aisen *et al.,* 2003). NSAIDs have been supposed to be effective if used prior to neuro-degeneration occurs and have been suggested to be use in the mid-age, much earlier than the AD symptoms appear (Aisen, 2002; Aisen *et al.,* 2003).

2.9.1.7 Cholesterol lowering drugs

Retrospective case control analyses showed that the cholesterol lowering drugs, statins, lead towards reduced production and deposition of Aβ (Wolozin *et al.,* 2000). About 74% reduced risk of AD had been implicated with the usage of the statins (Wolozin *et al.,* 2000). Statins can act as the facilitator of Aβ clearance from the brains (Fassbender *et al.,* 2001). They have also been praised to be neuro-protective (Sierra *et al.,* 2010). However, reports relating very little cognitive improvement and no amelioration of the plasma and CSF Aβ levels of the statin – utilized AD subjects have also been produced (Höglund *et al.,* 2005; Simons *et al.,* 2002; Sparks *et al.,* 2005) . Thus, large scale clinical trials are imperative for elucidating the effect of statin therapy on AD (DeKosky, 2005; Refolo *et al.,* 2001). AD ameliorating effect of the statins is also subject to their types, dosages, time of treatment regimen and also the stage of AD (Sierra *et al.,* 2010).

2.9.1.8 Anti-oxidant supplementation

Usage of the anti-oxidants seemed pertinent in withstanding OS mediated AD pathogenesis (Engelhart *et al.,* 2002). Large observational studies have implicated the AD lowering effect of vitamin E as its anti-oxidative performance (Sano *et al.,* 1997). Also, mild to no-effect of vitamin E on AD amelioration have also been found in randomized controlled trials (Petersen *et al.,* 2005; Sano *et al.,* 2005).

2.9.1.9 Estrogen replacement therapy

Estrogen has been reported to be neuro-protective and has been added in AD therapeutics (Simpkins *et al.,* 1997). Although epidemiological studies had linked reduced risk of AD in the post-menopausal estrogen supplemented women, large scale randomized controlled studies are yet to confirm its AD ameliorating effect (Mulnard *et al.,* 2000; Tang *et al.,* 1996).

2.9.1.10 Caregiving

Caregiving to the AD patients is highly recommended even at the early stage of the disease as they become fully dependent on the family members and relatives at the severe stage. Supporting the AD patients with their daily activities such as aiding in feeding, brushing, toilet using, road crossing add to the safety of the patients and save financial burden of the family (https://www.caregiver.org/alzheimers-disease-caregiving). Lifestyle modification of the family members including maintenance of blood pressure, sugar and lipid levels in the normal range, intake of balanced diet, avoidance of stress, regular physical exercise and free discussion among the family members are among the mostly suggested caregiving techniques (https://www.caregiver.org/alzheimers-disease-caregiving).

2.10 Therapeutic suitability of edible – medicinal mushrooms against AD

2.10.1 Synaptic degeneration prevention

Synaptic degeneration (loss of synaptic connection)-driven neurodegeneration is an important step in AD pathogenesis. Loss of synaptic density proteins such as synaptophysin, synaptotagmin and PSD - 95 parallel Aβ-induced synaptotoxicity during AD progression (Reddy, 2011). Thus, neurotransmission becomes severely weakened. Aqueous extract of *G. lucidum* at 500 µg/ml concentrations *in vitro,* contributed significantly to the restoration of synaptic density protein, synaptophysin and thus

attenuated Aβ-induced synaptotoxicity in rat cortical neurons (Lai *et al.,* 2008). Mechanisms involved attenuation of phosphorylation of c-Jun N-terminal kinase (JNK), c-Jun, and p38 MAP kinase (Lai *et al.,* 2008).

Aβ causes decreased synaptophysin immunoreactivity along with its increased accumulation and aggregation in AD neuritis (Stagi *et al.,* 2005). Thus, axonal transport of synaptic vesicles becomes blocked. Stress signaling pathway JNK participates in this mechanism where NO exacerbates the situation. Inhibition of the phosphorylation of the JNK can overcome the blockage of synaptophysin transport (Stagi *et al.,* 2005). Aqueous extract of *G. lucidum* (500 µg/ml, 14 days treatment *in vitro*) significantly inhibited the phosphorylation of JNK in Aβ-stressed rat cortical neurons that potentiated it to overcome the Aβ-induced blockage of synaptophysin transport (Lai *et al.,* 2008).

2.10.2 Reversion of neuronal apoptosis

Neuronal apoptosis is another phenomenon in AD pathogenesis where Aβ stimulates the activity of caspase-3 (Harada & Sugimoto, 1999). Apoptotic signaling pathway involves the protein kinase pathways including the JNK c-Jun and p38 MAP kinase (Harada *et al.,* 1999). Aqueous extract of *G. lucidum* reverted back Aβ-induced neuronal apoptosis by inhibiting these pathways (Lai *et al.,* 2008). Specifically, *G. lucidum* inhibited phosphorylation at serine 67 and serine 73 of c-Jun, the substrate for JNK (Lai *et al.,* 2008). Orally administered *G. lucidum* polysaccharide (GLPS) at 100, 200 and 400mg/kg body weight significantly lowered neuronal apoptosis in rats (Zhou *et al.,* 2010b). In cultured rat cortical neurons, (GLPS) at 0.1, 1.0 and 10 µg/ml, reduced neuronal apoptosis in a dose dependent manner. GLPS induced neuro-protective mechanism involves downregulation of the expression of caspases-3, -8 and -9 and Bax, and inhibition of the reduction of Bcl-2 expression resulting in altered Bcl-2/Bax ratio (Zhou *et al.,* 2010b). Lysophosphatidylethanolamine (LPE), derived from *G. frondosa*

stimulated the activation of MAP kinase of rat pheochromocytoma PC12 cells and showed anti-apoptotic effects such as suppressed cell condensation and DNA ladder generation (Nishina *et al.,* 2006). LPE also upregulated the expression of neurofilament M and thus promoted neuronal differentiation of PC 12 cells (Nishina *et al.,* 2006).

2.10.3 Aβ deposition lowering

Mouse diet supplemented with 0.3%, 0.6% and 1.8% of *G. lucidum* powder, had been found to significantly lower Aβ deposition in their brain along with increased anti-oxidative enzymatic levels and improved memory-related learning abilities (Wang *et al.,* 2004). Studies involving SH-SY5Y neuroblastoma cell lines identified enhanced non-amyloidogenic protein secretion (sAPPα) activity of *G. lucidum*. In this case, *G. lucidum* mycelial extract mimicked the nerve growth factor (NGF) activity and it stimulated the phosphorylation of ERK 1/2 and PKC and involved the signaling cascades of PI3K and ERK (Pinweha *et al.,* 2008). *Hericium erinaceus* has also been reported to lower Aβ plaque burden in the APPswe/PS1dE9 transgenic mouse model of AD. Also, the number of plaque-activated microglia and astrocytes in cerebral cortex and hippocampus were diminished (Tsai-Teng *et al.,* 2016).

2.10.4 Neurite outgrowth and neurogeneration stimulation

Normal maintenance and differentiation of the neurons require supportive assistance from the neurotrophic factors (neurotrophins) such as brain derived neurotrophic factor (BDNF), glia derived neurotrophic factor (GDNF), nerve growth factor (NGF) and neurotrophin 3 (NT-3). The NGFs are polypeptides and too large to cross the BBB. Positive role of *H. erinaceus* upon neurite outgrowth and NGF generation have been reported by several studies (Lai *et al.,* 2013; Mori *et al.,* 2008, 2011 and Phan *et al.,* 2014). Mori *et al.* (2008) found that among four mushroom species, *Hericium erinaceus*, *Pleurotus eryngii*, *Grifola frondosa* and *Agaricus blazei*, only *H. erinaceus*

extract promoted NGF mRNA expression in a concentration-dependent manner in 1321N1 human astrocytoma cells. However, hericenones C, D and E, constituents of *H. erinaceus,* failed to promote NGF gene expression in 1321N1 cells. Also, ddY mice fed 5% *H. erinaceus* dry powder for 7 days showed an increase in the level of NGF mRNA expression in the hippocampus. Another study by Mori *et al.* (2011) showed that *H. erinaceus* prevented intracerebroventricularly injected Aβ (25-35) peptide-induced learning and memory deficit in mice. Short-term memory relating to space and visual recognition was ameliorated (Mori *et al.* 2011). The proposed mechanism was through stimulating NGF activity (Mori *et al.* 2011). The bio-active component involved was identified to be hericinones C, D and E (Mori *et al.,* 2008 and 2011). Increased ratio of nerve growth factor (NGF) to NGF precursor (proNGF) and hippocampal neurogenesis have also been reported in case of the *H. erinaceus* ethanolic extract fed APPswe/PS1dE9 AD model mice (Tsai-teng *et al.,* 2016). In an attempt to identify the neuro-supportive component of *H. erinaceus,* Park *et al.* (2002) demonstrated that an exopolysaccharide from its culture broth had neurite outgrowth promotive effect in PC12 cells. Lipophilic fraction of *G. lucidum* at 125 and 500 mg/L was found to promote neurtie outgrowth, stimulate NGF activity and potentiate PC 12 neuronal differentiation (Zhang *et al.,* 2005). Seow *et al.* (2013) reported the comparatively enhanced neuritogenic effect of the aqueous extract of *G. neo-japonicum* than those of the *G. lucidum* and *G. frondosa* in PC 12 cells. At 50 μg/ml concentration, the *G. neo-japonicum* stimulated 14.22% neuritogenesis, whereas at 75 μg/ml concentration, the effect was 12.61% for the *G. lucidum* and 12.07% for the *G. frondosa* extract (Seow *et al.,* 2013). Bio-components present in these mushroom extracts activated the MEK/ERK1/2 and PI3K/Akt signaling pathways to promote neuritogenesis (Seow *et al.,* 2013). Similar pattern of neurite-outgrowth stimulatory effect along with restoration of impaired memory in rats have been observed for *Tremella fuciformis* (Kim *et al.,*

2007; Park *et al.,* 2012). At 20 µg/ml concentration, the aqueous sclerotium of *Lignosus rhinocerotis* induced PC 12 cells' neurite outgrowth of 24.4% (Eik *et al.,* 2011). In scopolamine- induced learning and memory deficient rats, *Cordyceps militaris* reverted back memory loss of the rats and promoted dose dependent (5-20 µg/ml) neuritogenesis in Neuro2A mouse neuroblastoma cells (Lee *et al.,* 2011).

2.10.5 Acetylcholine esterase inhibitory effects

Decreased availability of the cholinergic neurotransmitter acetylcholine leads towards AD consequences (Inestrosa *et al.,* 1996; Recanatini *et al.,* 2004). Acetylcholine esterase inhibitors (AChEIs) inhibit the enzyme acetylcholinesterase, which in turn inhibits the breakdown of acetylcholine into acetate and choline and prolongs its duration of action (Inestrosa *et al.,* 1996; Recanatini *et al.,* 2004). *Ganoderma lucidum* at 2 mg/ml concentration showed 57% inhibition of the acetylcholine esterase activity *in vitro* (Hasnat *et al.,* 2013). Lanostenoid tri-terpens of *G. lucidum* have been supposed to act as the AchEIs and thus protect the cholinergic neurons (Lee *et al.,* 2011; Zhang *et al.,* 2011). Restoration of impaired memory in rats by *T. fuciformis* had been reported to be through activation of the cholinergic system (Park *et al.,* 2012).

2.10.6 Dopaminergic activity

Shielding effect against dopaminergic neuronal loss through reduced production of microglial proinflammatory factors have been associated with *G. lucidum* (Zhang *et al.,* 2011). Methanolic extract of G. *lucidum* at 400 µg/ml, significantly inhibited the dopaminergic neurodegeneration of MES 23.5 cell membranes (Zhang *et al.,* 2011).

2.10.7 BACE1 inhibitory effect

Beta-site APP-cleaving enzyme 1 (BACE1) catalyzes the regulatory step for the production of toxic Aβ whose inhibition seems promising in controlling AD

pathogenesis (Yan *et al.,* 2016). Methanolic extract of *L. edodes* was found to inhibit by 40.1% of the BACE1 activity followed by *P. eryngii* (33.7%), *F. velutipes* (22.3%) and *A. bisporus* (18.3%) (Seo *et al.,* 2008). Through characterization of the cell-free extracts of different bacteria, fungi and yeast, Lee *et al.,* (2007) identified the BACE1 inhibitory effects (percentage scale) of mushrooms second only to *Saccharomyces cerevisiae*. Mushroom species having anti-BACE1 effects were *F. velutipes, P. ostreatus, G. frondosa, Dictyophora echinovolvata, Pholiota adipose, Fomitella fraxinea* and *Inonotus obliquus* (Lee *et al.,* 2007). BACE1 inhibitory components identified in *A. polytricha* has been detected as heat stable (Bennett *et al.,* 2013). Polyphenolic component hispidin, isolated from *Phellinus linteus,* had been found to non-competitively inhibit the BACE1 (Park *et al.,* 2004). BACE1 inhibitory effect of *A. polytricha* has also been indicated to be hispidine mediated (Bennett *et al.,* 2013).

AD hallmarks of neurodegenerative and memory related behavioral impairment is intricately linked with different types of proteins – their expression, suppression, deactivation, hyperactivation – it seems plausible to explore the proteomics of the experimental subjects. Thus, the following section has been designed to review the literature featured with AD proteomics.

1.1 Proteomics

"Proteomics", as a term had first been used in 1997 and this approach has facilitated highly in prognosis, diagnosis and in validating therapeutic approaches towards multiple diseases (James, 1997) . "Proteome" stands for the entire set of proteins of an organism and "proteomics" denotes towards the study of the proteome (Anderson & Anderson, 1998). Proteomics approaches seem promising both for deciphering the patho-mechanism and for identifying the bio-markers associated with AD. Biomarkers detection beacons towards the presence or absence, severity, trend and predictive

information of a pathological condition. Thus, biomarkers predicting the risk and providing support towards amelioration of AD pathogenesis, is of utmost importance for mitigating the ever increasing onslaught of AD.

2.10.8 Merits of proteomics studies

A. Proteomics enable simultaneous study, identification and characterization of the specific protein itself, its isoforms and post-translational modified variants (Moya-Alvarado *et al.,* 2015).

B. Proteomics provides *de novo* hypothesis based future direction of the research through less biased experimental design (Moya-Alvarado *et al.,* 2015).

Demerits of proteomics studies

a. Highly abundant proteins may obscure the identification and quantitation of the less abundant ones.

b. MS suffer from accuracy of providing reproducible results for the peptides (Gillette & Carr, 2013).

2.10.9 Importance of proteomics approach in AD therapeutics

Proteomics approach would aid much in high-throughput screening of the etiology and therapeutic approaches for AD. Cellular proteomics studies might shed light on AD biomarker development and elucidate the AD pathomechanism. Proteomics reveal that oxidative stress driven alteration of metabolic enzymes and mitochondrial functioning stand among the causative factors of AD pathogenesis (Moya-Alvarado *et al.,* 2015).

Neuropathological hallmarks of AD involves amyloid plaques and neurofibrillary tangles (NFT) (Serrano-Pozo *et al.,* 2011). Amyloid plaques consist mainly of extracellular deposition of Aβ peptides (Serrano-Pozo *et al.,* 2011). Proteomics of the postmortem amyloid plaques including liquid chromatography combined with tandem mass spectrometry (LC-MS/MS) had shown that in addition to Aβ, amyloid plaques

harbor about 488 types of proteins (Liao *et al.,* 2004) . Of them, twenty six had been marked as plaque specific (Liao *et al.,* 2004) .

Contrary to the amyloid plaques, NFTs are intra-neuronal aggregates of misfolded and/or hyperphosphorylated tau protein. Upon neuronal death, the NFTs appear as extracellular (Serrano-Pozo *et al.,* 2011). Proteomics approaches involving LC-MS/MS had identified sixty three NFT associated proteins in addition to tau (Wang *et al.,* 2005) . The most prominent were apo-lipoprotein E, α-synuclein and glyceraldehyde 3 phosphate dehydrogenase (GAPDH) (Wang *et al.,* 2005) .

Besides amyloid plaques and NFT, neuropil threads and dystrophic neurites containing hyperphosphorylated and misfolded tau proteins, loss of neuronal neuropils and synapses, astrogliosis and microgliosis are other hallmarks of AD. Very often, synaptic and axonal degeneration result in cognitive impairment as well as dendritic atrophy, the retrograde degeneration of axons, and the eventual atrophy of dendrites and perikarya (Serrano-Pozo *et al.,* 2011). Loss of memory remains as the top-notch symptom of AD.

Proteomics endeavor towards AD pathogenesis and amelioration goes beyond amyloid plaque and NFT pathology and search for AD biomarkers and biochemical analyses that would be helpful in early diagnosis and treatment of AD. AD diagnosis through biomarkers detection is at upper hand than those of the cognitive function tests as subtle change in cognitive tests may impair the accuracy of the neuropsychological performances (Jack *et al.,* 2011). AD standard biomarkers involve positron emission tomography (PET) that detects brain functioning through measurement of glucose metabolism and magnetic resonance imaging (MRI) that exposes brain atrophy and CSF analysis for tau and phosphorylated tau proteins (Moya-Alvarado *et al.,* 2015).

As mitigation of OS and hypercholesterolemia have been found to be linked with decreased Aβ generation and AD pathogenesis, therapeutic approaches targeted towards combined anti-oxidative and hypocholesterolemic outcomes seem pertinent in withstanding AD pathogenesis. Still today, there is hardly any report dealing with the combined anti-oxidative and cholesterol lowering approach for AD amelioration. Likewise, though numerous health benefits of *G. lucidum* have been reported since hoary past, its AD ameliorating effect through anti-oxidative and cholesterol lowering strategy has remained unexplored. Thus, in the present study, the anti-oxidative and cholesterol lowering performance of *G. lucidum* has been incorporated for evaluating AD ameliorating potentiality of this mushroom. Following chapters vividly describe the processes applied, results obtained and their justification that answer to the research questions outlined in the previous chapter.

CHAPTER 3: *IN VITRO* ANTI-OXIDATIVE INVESTIGATION OF SELECTED CULINARY-MEDICINAL MUSHROOMS

3.1 Introduction

Over the last few decades, natural substances possessing anti-oxidative effects have received immense therapeutic importance against OS and associated physiological disorders sucha as AD (Mancuso *et al.,* 2012). Mushrooms abound with numerous anti-oxidants (Valverde *et al.,* 2015). The most notable among them are the polyphenolics, polysaccharides such as β-D glucan, tri-terpenoids and sterols (Agarwal *et al.,* 2012; Guillamón *et al.,* 2010; Kao *et al.,* 2011). Mushrooms' possession of nutraceuticals, vitamins, minerals, other bio-components and comestibility has enabled to classify them into edible and medicinal types. Thus, comparative studies among mushrooms of both types seem apt in determining the most potent one followed by furthering its biomedical importance of any sort. In the present study, *H. erinaceus, F. velutipes* and *L. edodes* have been chosen as the edible mushrooms while *G. lucidum* as the medicinal to compare their *in vitro* anti-oxidative potentiality *en route* to associating the best one's content of bio-active components, *in vivo* anti-oxidative and cholesterol lowering capacities with those of AD ameliorating performances.

Conventionally, *in vitro* anti-oxidative tests are preferred *apriori* to *in vivo.* This may be due to the ease and to preliminarily screen the anti-oxidative potentiality of any compound. When a compound fulfills the criteria of *in vitro* anti-oxidative pursuit, then comes the context of *in vivo* experimentation. Thus, the first attempt of the present study was to test the *in vitro* anti-oxidative potentiality of the selected edible-medicinal mushrooms that would seek answer of the first objective stated in chapter 1 (Section 1.5).

In vitro, non-enzymatic antioxidant assays can broadly be classified into two major groups based on their mode of action:

a. Electron Transfer (ET) based and

b.. Hydrogen Atom Transfer (HAT) based.

ET measures the reducing capacity of an antioxidant through donation of electron to the oxidant. The oxidant becomes reduced that is manifested by the changed color. The intensity of the color developed corresponds to the antioxidant capacity and is measured. Examples include 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging, Folin-Ciocalteu (FC) assay, ferric reducing ability of plasma (FRAP), trolox equivalent anti-oxidant capacity (TEAC) assay, total antioxidant assay using Cu^{2+} as the oxidant.

HAT measures the hydrogen atom donating capacity of an antioxidant (reductant). The process is based on the principle that both the antioxidant and the substrate competitively compete for the free radical (usually a peroxyl radical generated upon heating) while they decompose an azo compound. Examples include inhibition of lipid peroxidation, oxygen radical absorbance capacity (ORAC) assay, total radical trapping anti-oxidant parameter (TRAP) assay and crocin bleaching assay (CBA). As the transfer of hydrogen atom is the key step in chain breaking reactions, the HAT type assays are suitable for measuring radical chain breaking anti-oxidant capacities.

Beyond these two broad groups, there are other tests such as total oxidant scavenging capacity (TOSC) assay, the chemiluminescence and the electrochemiluminescence assays (Prior *et al.,* 2005). Properties of anti-oxidant activity is highly governed by the structure of the antioxidant its solubility in the given reaction system and partition co-efficient, ionization potentiality and bond dissociation (Prior *et al.,* 2005). As part of the

in vitro anti-oxidative assessment of the present study, DPPH free radical scavenging, Folin-Ciocalteu assay and inhibitory effects of the mushroom extracts and fractions towards lipid peroxidation and LDL oxidation were performed.

3.2 Materials and Methods

3.2.1 Mushroom samples

All the mushroom samples *(G. lucidum, H. erinaceus, F. velutipes and L. edodes)* were purchased from the local market (Figure 3.1). Purchased as the dried and large slices, the *G. lucidum* was cut into fine pieces and ground with the grinder (Polymix, PX-MFC 90D, Kinematica, Switzerland) into powder. The other three species were fresh and delicate. These were then cut into small pieces, dried in the oven until became moisture-free (1-3 days) and ground into powder.

Mushroom species	Common name	Photo
Ganoderma lucidum	Ling Zhi/Reishi	
Hericium erinaceus	Lion's mane	
Flammulina velutipes	Enoki	
Lentinula edodes	Shiitake	

Figure 3.1: Selected edible-medicinal mushrooms of the present study

3.2.2 Preparation of Solvent-Solvent Partitioned Fractions of Mushrooms

Bio-functionality of the bio-components is highly affected by the extraction procedure and extracting solvents. We used five extracting solvents such as methanol, dichloromethane, ethyl acetate, hexane and water. Based on their density gradient and polarity, we performed gradual fractionation of the four mushroom species following an established method in our lab (Rahman *et al.,* 2014) (Figure 3.2). In brief, four litres of methanol:dichloromethane (2:1) mixed with each 200 gm powder of every mushroom species were stirred with the benchtop shaker (SK-300, Lab Companion, USA) at 150 rpm for 3 days at room temperature. Extraction and filtration using Whatman no. 1 filter paper were repeated thrice and at every time, the collected organic solution was evaporated using a rotary evaporator (Büchi Rotavapor R-114, Switzerland) that produced the M:DCM fraction. Dissolving the M:DCM fraction in 90% aqueous methanol, we partitioned it with hexane (3×100 mL). From the two partitioned layers (hexane fraction forming the top layer), hexane fraction was separated using a separatory funnel and then rotary evaporated till dryness to form the aqueous methanolic layer (M). It is a semisolid fraction whose successive dissolution in distilled water (100 mL) and three times partitioning with dichloromethane (DCM, 3×100 mL) turned out the DCM fraction and the aqueous fraction. Through rotary evaporation, we apportioned the DCM fraction and further partitioning of the aqueous part with ethyl acetate (EA, 3×100 mLs) accrued the EA fraction afloating the aqueous residue. We obtained the aqueous fraction through freeze-drying (Labconco) of the aqueous fraction (Figure 3.2).

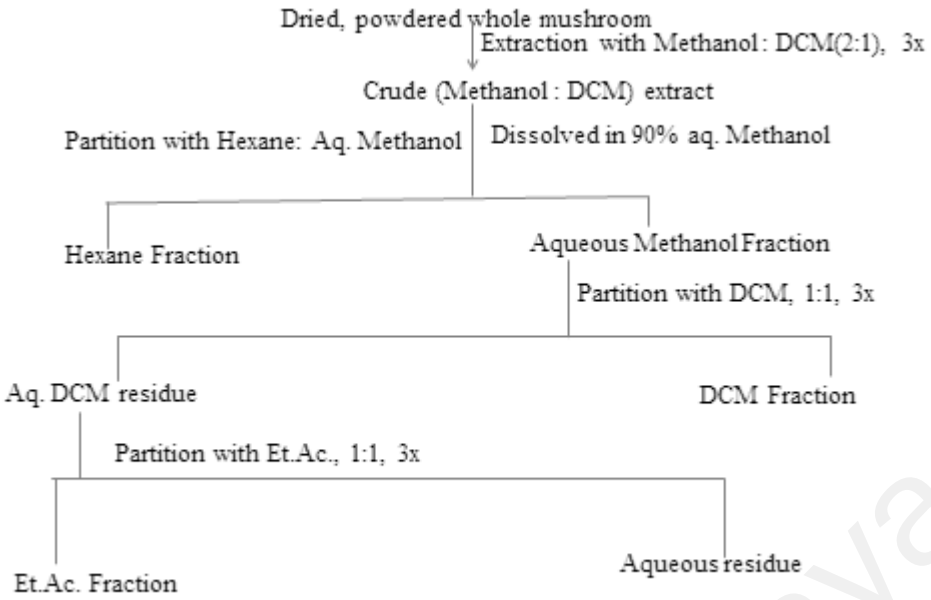


Figure 3.2: Preparation of the solvent-solvent partitioned fractions

3.2.3 Preparation of Hot Water Extracts

For reparing the hot water extracts (HWE) of the selected edible-medicinal mushrooms, the fruiting bodies were powdered and boiled in distilled water at the ratio of 1: 20 (w/v) at 100 °C for 45 minutes. After cooling, boiled mushrooms were removed by using Whatman No. 1 filter paper and the remaining aqueous part underwent freeze-drying (Labconco) that yielded the hot water extract of each mushroom species. We preserved the extracts in desiccator at room temperature for biochemical studies.

3.2.4 DPPH Free Radical Scavenging Assay

The principle of this assay is that the purple colored free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$) becomes reduced by the reducing agent or anti-oxidant present in the reaction mixture and is converted to the pale yellow colored hydrazine that corresponds to the decrease in absorbance at around 515-517 nm.

For determining the DPPH free radical scavenging effects of the mushroom fractions, we followed the modified method of Abdullah *et al*. (2011) . Each of the solvent fractions (0.1 mL volume of 1.0 mg/mL concentration) was mixed with each 3.9 mL of 0.06 mM DPPH dissolved in methanol. The mixture was shaken in darkness for 30 minutes at room temperature. The absorbance of the mixture was recorded at 515 nm. Using methanol as the blank and quercetin as the positive control (100 µg/mL), DPPH free radical scavenging percentage was calculated using the following equation:

$$\text{Radical scavenging activity (\%)} = (A_0\text{-}A_s) \times 100/A_0$$

where A_0 stands for the absorbance of 0.06 mM DPPH in methanol, and A_s denotes the reaction mixture's absorbance. The IC_{50} value (fraction concentration required for 50% scavenging) of the most potent solvent fraction was determined from the graph of the scavenging activity against respective mushroom fraction concentration.

3.2.5 Folin-Ciocalteu assay

Folin and Ciocalteu first developed a method to measure protein based on Folin-Looney reagent's ability to determine tyrosine and tryptophan residues present in the protein structure (Folin & Ciocalteu, 1927). Later, Singleton *et al.* (1999) unfurled its utility towards determining content of phenol in wine and thenceforth this method had been used for measuring total phenol (phenolics) content in different biological samples.

In the present study, the modified methodology of Slinkard and Singleton (1977) was used to detect the reducing capacity of the mushroom fractions. In succinct, 250 µL of 10% Folin-Ciocalteu reagent was added with equal volume of each of the fraction (100 µg/mL). Shaking of the mixture continued for 3 minutes in darkness. Addition of 10% sodium carbonate (500 µL) to the mixture followed 60 minutes incubation in the darkness. Then, absorbance was measured at 750 nm. Pursuance of the Folin-Ciocalteu

assay has been expressed as gallic acid equivalents (GAE) per gram of fraction utilizing the calibration curve of gallic acid (2-10 µg/mL).

3.2.6 Lipid Peroxidaton Inhbition Test

Oxidative modification of lipid structures especially in the carbon-carbon double bonds is referred to as lipid peroxidation. It generates various by products such as malondialdehyde (MDA) (Dianzani *et al.,* 2008). MDA and other aldehydes emanating from lipid peroxidation react with thiobarbituric acid (TBA) to form the adduct "TBA-MDA" that later on yield pink colored thiobarbituric acid reactive substances (TBARS) (Moselhy *et al.,* 2013). Spectroscopic measurement of TBARS at its absorption maximum of 532-535 nm indicates the extent of lipid peroxidation (Moselhy *et al.,* 2013).

The modified method of Daker *et al.* (2008) was used to determine the inhibitory effect of each of the mushroom fraction upon buffered egg yolk lipid peroxidation. Succinctly, emulsification of fowl egg yolk was performed with 0.1M phosphate buffer (pH 7.4) to achieve the final volume of 25 g/L. As an inducer of lipid peroxidation 100 µL of 1M ferrous sulphate (1M, 100 µL) was added to the mixture. Mushroom fractions (100 µL, 100 µg/mL) were introduced to the mixture and shaken vigorously. Following 60 minutes incubation at room temperature, freshly prepared 15% trichloroacetic acid (TCA, 0.5 mL) and 1% thiobarbituric acid (TBA, 1 mL) were added. The tubes containing the reaction mixtures were kept on the boiling water bath for 10 minutes, followed by cooling at room temperature. Precipitating the proteins through centrifugation at 3,500 g for 10 minutes, the supernatant (100 µL) was taken and TBARS formation was analyzed through measuring absorbance at 532 nm. As control, we used the buffered egg yolk with Fe^{2+} only. Percentage inhibition of lipid

peroxidation i.e. percentage inhibition of TBARS formation was calculated using the following equation:

Inhibition (%) = (A0-As) × 100/A0

Where, A0 is the absorbance of the control, and As is the absorbance of the reaction mixture containing the fraction. The IC_{50} value (fraction concentration required for 50% inhibition) of the most potent solvent fraction was determined from the graph of the scavenging activity against respective mushroom fraction concentration.

3.2.7 LDL Oxidation Inhibition Test

We studied two aspects of LDL oxidation for evaluating the individual mushroom fractions inhibitory effects towards LDL oxidation: lag time extension during conjugated diene (CD) formation and inhibition of malondialdehyde (MDA) production.

3.2.7.1 Conjugated diene (CD) measurement

Conjugated dienes (CD) are formed during the initial stage of lipid peroxidation due to abstraction of hydrogen atom from the methylene group (CH_2) of PUFA followed by the molecular rearrangement. Thus, anti-oxidative studies through measurement of CDs indicate the initial lipid peroxidation effect of any anti-oxidant. Exerting lipid peroxidation through Fenton reagent, quantification of CDs are done usually by measuring the lag phase and/or calculating the gradual increased rate of absorbance and/or percentage of inhibition of CD production (Antolovich *et al.,* 2002).

Experimentally, direct oxidation of LDL molecules is performed through low level of transition metal ions in acidic conditions. This approach of LDL oxidation at cell-free system by redox-active metal ions (Fe^{2+}, Cu^{2+}) has biochemical similitude with that of cellular systems (Yoshida & Kisugi, 2010). Mediated by transition metal ions, PUFAs present in the LDL molecule undergo peroxidative modification and molecular

rearrangement producing CDs. Endogenous antioxidants (α tocopherol) present in LDL molecule withstands the initial oxidizing process and this suppressed oxidative stage is known as the lag time of oxidation. The potency of anti-oxidant activity is measured through measuring the extended lag time of CD formation at 234 nm.

Method of Rahman *et al.* (2014) was followed to measure the mushroom fractions' potency in extending the lag time of CD formation. In short, human LDL underwent treatment with freshly prepared 0.1 mM FeSO4 solution at room temperature so that the final concentration of LDL becomes 150 µg protein/mL at pH 7.4 and final volume adjusted to 200 µL. Mushroom fractions' and HWE's anti-oxidative performance at 1 µg/mL concentration were studied at 20 minutes intervals for a period of 180 minutes at 234 nm. Distilled water, 0.1mM FeSO4 in ultrapure water (pH 7.4) and α-tocopherol LDL (10 µg/mL, Calbiochem) were used as the blank, negative -and positive control, respectively.

3.2.7.2 Inhibitory effect upon the formation of Malondialdehyde (MDA)

Propagation phase of rapid LDL oxidation quickly follows the lag phase where lipid peroxides are produced. Lastly, decomposition phase ensues wherein double bonds are broken and MDAs are formed. For evaluating MDA formation inhibitory effect of the mushroom fractions, we used the method of Rahman *et al.* (2014) . Freshly prepared 1M FeSO4 at room temperature was used to generate oxidative stress to human LDL (final concentration 150 µg protein/mL, final volume 200 µL, pH 7.4). Mushroom fractions were used at 1 mg/mL concentration and Distilled water, 0.1mM FeSO4 in ultrapure water (pH 7.4) and α-tocopherol LDL (100 µg/mL, Calbiochem) were used as the blank, negative -and positive control, respectively. All the reaction mixtures were incubated at 37 ºC for 180 minutes. Then, tricholoroacetic acid (TCA, 15%, 500 µL) and thiobarbituric acid (TBA, 1%, 1 mL), both freshly prepared, were added and

incubated at 100 ºC for 10 minutes followed by cooling at room temperature. An aliquot of the mixture taken to the ELISA plate was read at 532 nm. Using malondialdehyde dimethyl acetal as the standard at concentration range of 0 – 100 µM, a calibration curve was prepared (TBARS assay kit, item no. 10009055, Cayman Chemicals, USA). Exploiting the calibration curve, we determined the level of MDA produced and expressed as µmol MDA/mg protein LDL.

3.3 Results

3.3.1 DPPH Free Radical Scavenging Performance

As shown in Figure 3.3, all the mushroom extracts (1 mg/mL) and the positive control, quercetin (100 µg/mL) scavenged DPPH free radical and their rate of scavenging differed from each other (Raw data: Appendix A, Tables A - 1.1, 1.2, 1.3, 1.4, 1.5 and 1.6; Figures A - 1.1 and 1.2; Statistical data: Appendix C, Tables C- 1.1 and 1.2). Even, within the same mushroom species, different extract and solvent fractions had different scavenging activity.

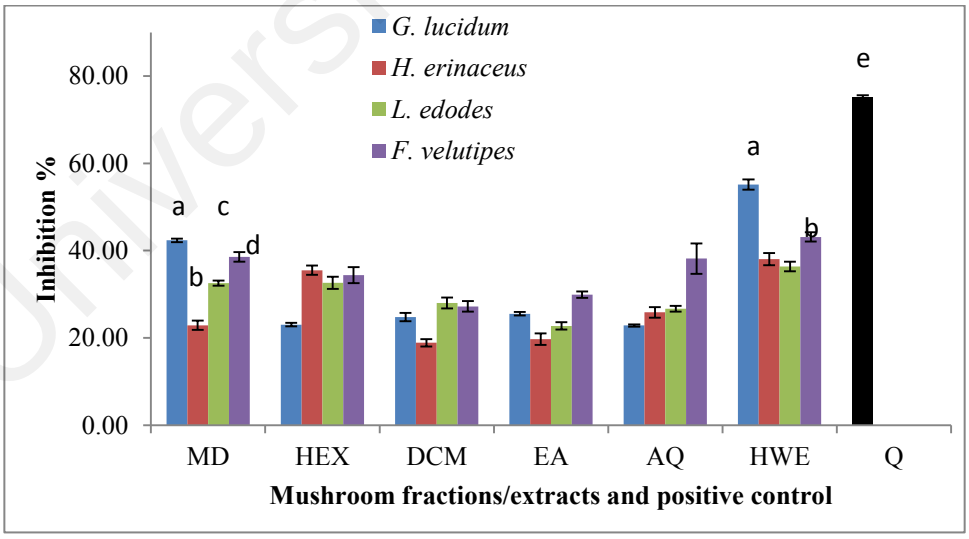


Figure 3.3: DPPH free radical scavenging effect

Data are expressed as mean±SE (Bar charts with different lower case superscripts are indicators of statistically significant difference at P≤0.05 level with one-way ANOVA and post-hoc Tukey's HSD test (n=3). Here, MD stands for methanol:dichloromethane; HEX for hexane; DCM for dichloromethane, EA for ethylacetate, AQ for aqueous and HWE for hot water extract. Mushroom fractions and HWE were used at 1 mg/mL while the positive control querecetin (Q) at 100 µg/mL concentrations.

3.3.2 Folin-Ciocalteu assay

The HWE of all the mushroom species showed the best reducing capacity as shown in Figure 3.4 (Raw data: Appendix A, Tables A - 2.1, 2.2, 2.3, 2.4, 2.5 and Figure A 2.1; Statistical data: Appendix C, Tables C - 2.1 and 2.2). Reducing capacity of each gram of the HWE of *G. lucidum, H. erinaceus, L. edodes and F. velutipes,* was 81.06, 73.63, 63.03 and 56.97 mg gallic acid equivalent (GAE), respectively. The values were significantly different among each other at 5% level of confidence. EA fraction of *G. lucidum* was second in terms of reducing capacity (75.3 mg GAE/g), following very closely by those of DCM (52.4 mg GAE/g) and MD (51.3 mg GAE/g). Compared to each of the fraction of other three species, significantly higher ($P \leq 0.05$) reducing capacity in the MD, DCM and EA fraction of *G. lucidum* was also observed.

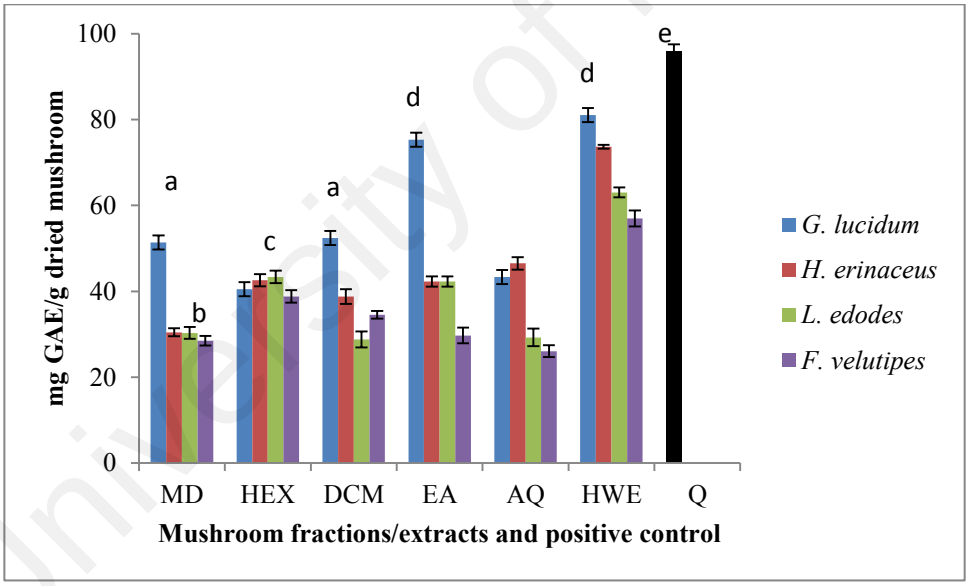


Figure 3.4: Folin-Ciocalteu assay

Data are expressed as mean±SE. Bar charts with different lower case superscripts are indicators of statistically significant difference at P≤0.05 level with one-way ANOVA and post-hoc Tukey's HSD test (n=3). Here, MD stands for methanol:dichloromethane; HEX for hexane; DCM for dichloromethane, EA for ethylacetate, AQ for aqueous and HWE for hot water extract. Mushroom fractions and HWE were used at 1 mg/mL while the positive control querecetin (Q) at 100 µg/mL concentrations.

3.3.3 Inhibition of Lipid Peroxidation

As depicted in Figure 3.5, the hexane fraction (at 1 mg/mL) of each mushroom species had the highest inhibitory effect towards lipid peroxidation (Raw data: Appendix A, Tables A - 3.1, 3.2, 3.3 and 3.4; Statistical data: Appendix C, Tables C - 3.1 and 3.2). Among the four mushroom species, the hexane fraction of *H. erinaceus* mostly inhibited (72.59%), followed sequentially by that of *L. edodes* (67.07%), *G. lucidum* (58.56%) and *F. velutipes* (55.39%). DCM fractions of all the mushrooms stood second to those of hexane in this inhibitory concert: *H. erinaceus* (66.59) > *L. edodes* (64.23) > *G. lucidum* (55.47) > *F. velutipes* (45.58). At 100 µg / mL, the positive control quercetin showed 80.54% inhibition of LPO and its IC_{50} value was 0.056 mg/mL. IC_{50} value of the hexane fraction of *H. erinaceus* was found to be 0.76 mg/mL.

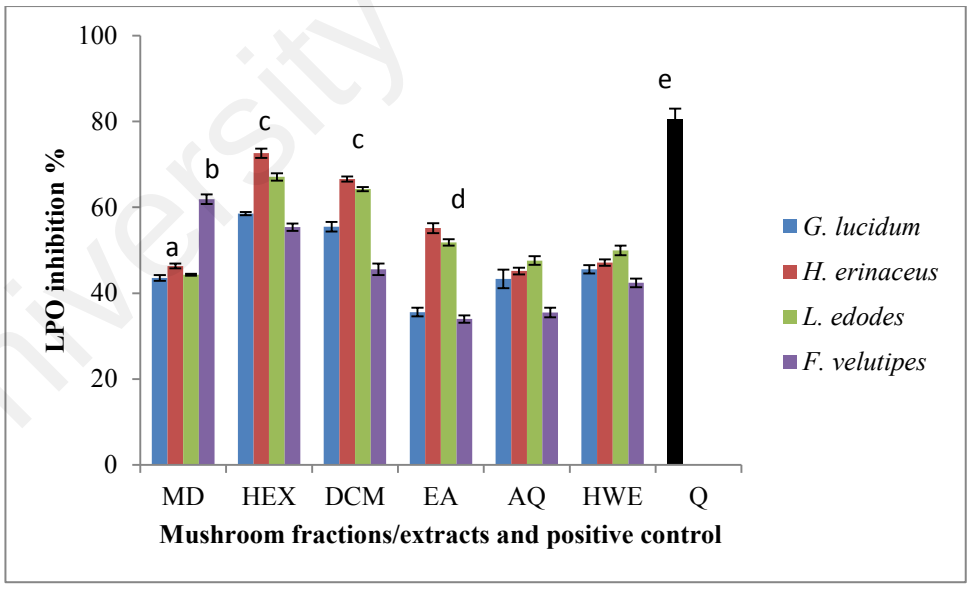


Figure 3.5: Lipid peroxidation inhibitory effect

Data are expressed as mean±SE. Bar charts with different lower case superscripts are indicators of statistically significant difference at P≤0.05 level with one-way ANOVA and post-hoc Tukey's HSD test (n=3). Here, MD stands for methanol:dichloromethane; HEX for hexane; DCM for dichloromethane, EA for ethylacetate, AQ for aqueous and HWE for hot water extract. Mushroom fractions and HWE were used at 1 mg/mL while the positive control querecetin (Q) at 100 µg/mL concentrations.

3.3.4 Inhibition of LDL Oxidation

3.3.4.1 Lengthening of the Lag time of CD Formation

At our experimental milieu, all the extracts of all the mushroom species showed lag time lengthening effect (Table 3.1) (Raw data: Appendix A, Tables A - 4.1, 4.2, 4.3 and 4.4; Statistical data: Appendix C, Tables C - 4.1 and 4.2) . However, their performance differed from each other. In case of every mushroom, the hexane fraction exerted the most lengthening effect (*G. lucidum* 127.67 minutes, *H. erinaceus* 120.43 minutes, *L. edodes* 115.78 minutes and *F. velutipes* 110.34 minutes) compared with the other fractions. Like that of lipid peroxidation inhibition (Figure 3.5), the DCM fractions of all the mushroom species stood second in lag time lengthening of CD formation (Table 3.1). Lengthening time of HWE of all the mushrooms was in the range of 80-86 minutes and third in the category. Effect of MD and EA was close to each other and their timing ranged from 40 to 50 minutes.

Table 3.1: LDL oxidation inhibitory effect of the mushroom fractions and extracts through lengthening the lag time of CD formation (minute)

Extract/ Fraction/ Standard	*G. lucidum*	*H. erinaceus*	*L. edodes*	*F. velutipes*
MD	43.33^{a}	37.67^{b}	33.33^{c}	26.00^{d}
HEX	127.67^{a}	120.43^{b}	115.78^{c}	110.34^{d}
DCM	80.67^{a}	67.00^{b}	113.33^{c}	101.67^{d}
EA	53.33^{a}	62.33^{b}	42.33^{c}	62.33^{d}
AQ	39.33^{a}	36.00^{b}	24.00^{c}	60.67^{d}
HWE	43.33^{abd}	41.67^{abd}	36.67	44.00^{abd}
α-tocopherol	138.33			

Data are expressed as mean±SE. Mean values with different lower case superscripts are indicators of statistically significant difference at $P \leq 0.05$ level with one-way ANOVA and post-hoc Tukey's HSD test (n=3). Here, MD stands for methanol:dichloromethane; HEX for hexane; DCM for dichloromethane, EA for ethylacetate, AQ for aqueous and HWE for hot water extract. Mushroom fractions and HWE were used at 1 mg/mL while the positive control, α-tocopherol at 100 µg/mL concentrations.

3.3.4.2 Malondialdehyde (MDA) assay

As shown in the figure 3.6, compared to the positive control, α-tocopherol, the hexane fractions of all the mushroom species were most potent among the six fractions. Level of MDA generation (µM/mg protein-LDL) was comparable *(L. edodes* 13.39*, G. lucidum* 14.64*, H. erinaceus* 15.90 and *F. velutipes* 15.48 µM/mg protein-LDL) with that of α-tocopherol (10.46 µM/mg protein-LDL) since the values were not statistically significant (Raw data: Appendix A, Tables A - 4.5, 4.6, 4.7, 4.8 and 4.9, Figure A 4.1; Statistical data: Appendix C, Tables C 4.3 and 4.4) .

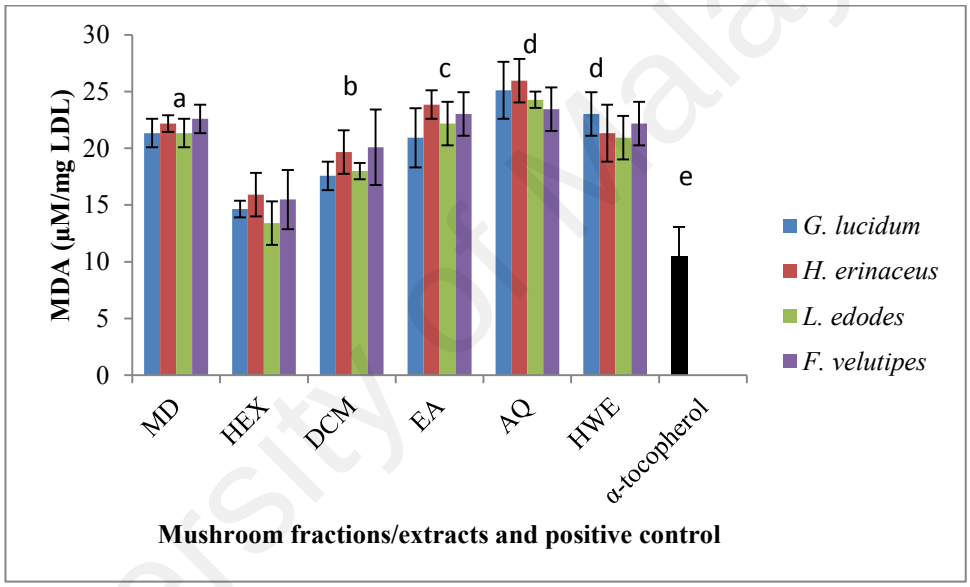


Figure 3.6: LDL oxidation inhibitory effect of the mushroom fractions and extracts through reduced production of MDA

Data are expressed as mean±SE. Mean values with different lower case superscripts are indicators of statistically significant difference at P≤0.05 level with one-way ANOVA and post-hoc Tukey's HSD test (n=3). Here, MD stands for methanol:dichloromethane; HEX for hexane; DCM for dichloromethane, EA for ethylacetate, AQ for aqueous, HWE for hot water extract and MDA for malondialdehyde. Mushroom fractions and HWE were used at 1 mg/mL while the positive control, α-tocopherol at 100 µg/mL concentrations.

3.4 Discussion

3.4.1 DPPH free radical scavenging effect

Anti-oxidants scavenge DPPH free radicals by donating hydrogen atom with changing of the purple color to yellow with concomitant decrease in absorbance at 515 nm. Among six fractions, the hot water extract (HWE) of *G. lucidum* mostly (55.13%, significant at $P \leq 0.05$ level) scavenged the DPPH free radical (Figure 3.3). The MD fraction of this mushroom stood second with 42.36% (significant at $P \leq 0.05$ level) scavenging effect. DPPH free radical scavenging effect of the MD fraction of *G. lucidum, H. erinaceus, L. edodes* and *F. velutipes* were significantly different from each other at 5% confidence level. Moderate scavenging effect (18.88-28%) was shown by the DCM, EA and AQ fractions of each of the mushroom species. IC_{50} of the most potent fraction, the HWE of *G. lucidum,* was 1.47 mg/mL compared to that of quercetin with 0.069 mg/.

DPPH free radical scavenging effect observed in the present study was in line with those reported by other researchers (Abdullah *et al.,* 2011; Mau *et al.,* 2002b; Mau *et al.,* 2005; Yildiz *et al.,* 2015). The methanolic extracts of *G. lucidum, G. lucidum* antler and *G. tsugae* have been reported to scavenge DPPH-free radical more than 70% (at 0.64 mg/mL concentration) and hydroxyl radical more than 50% (at 16 mg/mL concentration) (Mau, Lin & Chen, 2002). At 2.4 mg/mL concentration, ferrous ion chelating activity was 44.8% (*G. tsugae)*, 55.5% *(G. lucidum)* and 67.7% *(G. lucidum* antler*)* (Mau *et al.,* 2002a). Similar anti-oxidative prowess has been reported for the ethanolic extract of *G. lucidum* (Rajasekaran & Kalaimagal, 2011). The best *in vitro* anti-oxidative effect of the HWE of *G. lucidum* among different solvent (hot water, hydro-alcoholic, chloroform and petroleum ether) extracts of have also been observed by Agarwal *et al.* (2012). Abdullah *et al.* (2011) also ranked the HWE of *G. lucidum* first on the basis of anti-oxidant index (30.1%) among fourteen culinary-medicinal mushrooms.

3.4.2 Folin-Ciocalteu assay

Among all, the HWE of *G. lucidum* had been found possessing the best reducing capacity as evident by the Folin-Ciocalteu assay (Figure 3.4) (Raw data: Appendix A, Tables A - 2.1, 2.2, 2.3, 2.4, 2.5 and Figure A 2.1; Statistical data: Appendix C, Tables C - 2.1 and 2.2). Observed Folin-Ciocalteu assay results are compatiable with those of Yildiz *et al*. (2015). However, compared to some other reports, the current findings are higher and indicate better reducing capacities of the respective mushroom species (Abdullah *et al.,* 2011; Heleno *et al.,* 2015; Kim *et al.,* 2008; Mau *et al.,* 2002; Rawat *et al.,* 2013; Reis *et al.,* 2012; Wong *et al.,* 2009). Mau *et al.* (2002) also observed about four times higher reducing capacities of the *Ganoderma* species than those of the *Coriolus versicolor.*

3.4.3 Inhibition of lipid peroxidation

In the present study, inhibitory performance of the mushroom extracts upon the lipid peroxidation (LPO) of the buffered egg yolk was assessed through measurement of TBARS. As TBARS generation is linked with the extent of lipid peroxidation, the lower the level of TBARS indicates the better anti-oxidative capacity of the mushroom extract. In this sense, the hexane fractions of all the selected mushrooms stood first in inhibiting LPO (Figure 3.5). The other fractions and extracts varied among each other in the context of TBARS formation inhibition. Present findings are compatible with those of Mau *et al.* (2002) who regarded the better LPO inhibitory effect of *G. lucidum* with its higher content of polyphenolics. In addition, the hexane fractions of the present study might have possessed lipo-philic bio-components that provided with the enhanced anti-oxidative support in lowering LPO.

3.4.4 Inhibition of LDL oxidation

Based on established methods, *in vitro* LDL oxidation of human LDL was performed using transition metal ion Fe^{2+} (Lynch & Frei, 1993; Morgan & Leake, 1995; Visioli *et al.,* 2000). The proposition was such that redox-active metal ion (Fe^{2+}, Cu^{2+}) – mediated *in vitro* LDL oxidation is mechanistically alike to that of *in vivo* systems (Lapeyre *et al.,* 2005; Miranda *et al.,* 2000; Steinberg *et al.,* 1989; Turchi *et al.,* 2009). These ions participate in the peroxidative modification and molecular rearrangement of the poly unsaturated fatty acids (PUFAs) present in the LDL molecules and produce CD (Lynch & Frei, 1993; Morgan & Leake, 1995). At the initiation, LDL contained endogenous antioxidants guard against oxidative stress and thus there occurs a little OS, which is known as the lag phase of LDL oxidation. Upon addition of different mushroom extracts, the lag time had been extended (Table 3.1). Noteworthy, mushroom fraction-treated samples showed a gradual decrease in absorbance that is indicative of the decreased LDL oxidation as well as anti-oxidative potencies of the mushroom fractions.

As LDL oxidation is a step by step process, initial lag phase ends up quickly and rapid LDL oxidation phase (propagation phase) ensues with production of the lipid peroxides). At the third (decomposition) step, the double bonds break up with the generation of aldehydes such as malondialdehydes (MDA). In the present experimental arrangement, nucleophilic addition reaction of MDA with 2-thiobarbituric acid (TBA) produced thiobarbituric acid reactive substances (TBARS) (Lefevre *et al.,* 1997). Current investigation was to find out whether different solvent fractions of different mushrooms could inhibit or lower the production of MDA (in case of LDL oxidation) and TBARS (incase of lipid peroxidation). Obtained data suggest that the hexane fractions of all the mushrooms were most potent in inhibiting the formation of both MDA and TBARS (Figures 3.5 and 3.6). Lipophilic bio-components present in the non-polar hexane might impart in inhibiting lipid peroxidation and LDL oxidation in the

present experiment. Anti-oxidative mushroom bio-components might themselves become oxidized through quenching the free radicals and prevented lipid peroxidation and LDL oxidation (Turchi *et al.,* 2009). Probably, they had acted as electron donors towards Fe^{2+} and lowered the oxidative stress towards lipid and LDL molecules.

Conjugated dienes (CD) are formed during the initial phase of LDL oxidation. Anti-oxidant activity is usually determined based on the lengthening capacity of a substance upon the lag time of CD formation. Principally, the enhanced the lag time of any CD forming reaction, the lower the extent of LDL oxidation and the higher the anti-oxidative prowess of the anti-oxidant. In this context, the hexane fractions of all the mushrooms surpassed the rest of the fractions and the extracts. This trend was similar to those of LPO inhibition.

Malondialdehyde (MDA) is an intermediary product of LDL oxidation and we measured its level to test the potency of the mushroom extracts in withstanding the *in vitro* oxidation of human LDL. Among the four mushroom species, the *L. edodes* showed the best inhibition of LDL oxidation as indicated by the least production of MDA (Figure 3.6). DCM fractions of all the mushrooms stood next to those of hexane (Figure 3.6). The pattern of LDL oxidation inhibition tallied with those of lipid peroxidation inhibition (Figure 3.5). Aqueous fraction of all the mushrooms showed the least inhibitory potential while those of MD, EA and HWE were moderate. This may be due to the presence and outperformance of the lipo-philic anti-oxidants in the hexane fractions of the mushroom species studied.

3.5 Conclusion

The above mentioned *in vitro* anti-oxidative test results reveal that the HWE of *G. lucidum* excels most in scavenging DPPH free radicals and possesses the highest reducing capacity as assessed by the Folin-Ciocalteu outcomes. However, the hexane

fractions of all the mushrooms surpassed the respective HWEs in inhibiting lipid peroxidation and LDL oxidation. Though the hexane fraction of *H. erinaceus* and *L. edodes* mostly inhibited the formation of TBARS and MDA, respectively, the hexane fraction of *G. lucidum* lengthened the lag time of CD formation most. Thus, among the selected edible-medicinal mushrooms, the *in vitro* anti-oxidative performance of *G. lucidum* was the best. Among the five solvent-solvent partitioned fractions and HWEs of each of the four selected species, the HWE of *G. lucidum* possessed the highest free radical scavenging and reducing capacities. Thus, the HWE of *G. lucidum* was selected for assessing its *in vivo* anti-oxidative (chapter 5), cholesterol lowering (chapter 6) and AD ameliorating potentiality (chapter 6). Prior to those studies, bio-active components present in the HWE of *G. lucidum* and other potential extracts of the selected mushrooms have been determined whose description have been appended in the next chapter (chapter 4).

CHAPTER 4: DETERMINATION OF BIO-ACTIVE COMPONENTS IN THE SELECTED MUSHROOM FRACTIONS AND EXTRACTS

4.1 Introduction

Through *in vitro* tests described in the previous chapter, differential anti-oxidative performance of different fractions and extracts of different mushroom species have been observed. Stated objectives of *in vivo* anti-oxidative, cholesterol lowering and AD ameliorating effect of the selected mushrooms depend on the presence of the responsible bio-active components. Thus, it is imperative to identify the presence of the bio-active components in the potential mushroom fractions and extracts. This would fulfill the second objective of the present study.

Due to solvent-solvent partitioning, the selected mushroom fractions and extracts had been fractionated either into lipophilic or into hydrophilic types. As a consequence, determination of the bio-active components in the respective fractions and extracts are largely dependent on the solubility-based identification processes. Based on this principle, gas chromatography - tandem mass spectrometry (GC-MS/MS) for the lipophilic and liquid chromatography – tandem mass spectrometry (LC-MS/MS) for the hydrophilic fractions and extracts, respectively, were used to identify the bio-active components. The hexane fractions of *G. lucidum, H. erinaceus, L. edodes* were selected for GC-MS while the MD fraction of *F. velutipes* and the HWE of *G. lucidum* were selected for LC-MS analyses. Content of β-D glucan in the HWE of *G. lucidum* was also determined using commercially available kits.

4.2 Materials and methods

4.2.1 GC-MS/MS Analysis

For identifying bio-active components in the hexane fractions of *G. lucidum, H. erinaceus* and *L. edodes,* GC-MS/MS analysis was performed utilizing gas chromatography directly coupled to a mass spectrometer system (Agilent 7000 C triple quadruple GC/MS system, USA). The system was equipped with a HP-5ms silica capillary column (30 m x 250 μm, 0.25 μm film) with injector temperature 250°C, split less injection technique, injection volume of 1.5 μL, flow rate 1 mL/min, oven temperature 70°C – 300°C, helium gas as the carrier, electronic ionization mode with 70 eV ionization energy, ion source temperature 200 °C and interface temperature 300°C and masses scanned in the range of 50–1200 m/z. The peaks were identified comparing with the mass spectra library of the National Institute of Standards and Technology (NIST 14)/Environmental Protection Agency (EPA)/National Institutes of Health (NIH), USA.

4.2.2 LC-MS/MS Analysis

The LC/MS/MS analysis of the MD fraction of *F. velutipes* and the HWE of *G. lucidum* were performed using the Applied Bioscience's Sciex 6500 QTRAP tandem mass spectrometer (model 5017226-H, serial BL21931304) equipped with the UHPLC FX15 system of Perkinn Elmer. A Turbo V spray source connected the chromatography system with the ionization chamber. Mobile phase A consisted of 5mM ammonium formate and water with 0.1% formic acid v/v while mobile phase B consisted of 5mM ammonium formate and acetonitrile with 0.1% formic acid v/v. The process was accomplished with injection volume of 10 μL, flow rate of 400 μL / min using the Phenomenex aqua column (particle size 3 μM, 50 mm x 1.2 mm). A diode array detector recorded the LC spectra in the range of 50-1200 m/z.

4.2.3 Determination of β-D Glucan level

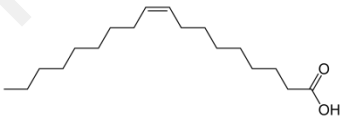
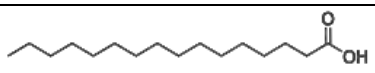
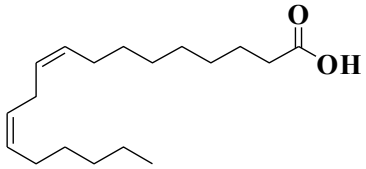
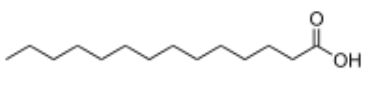
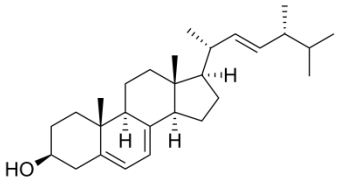
The level of β-D glucan in the HWE of *G. lucidum* was measured using the megazyme β glucan assay kit (yeast and mushroom, K YBGL 01/2016) (Megazyme, USA). Manufacturer's instructions supplied with the kit package were followed during experimentation and the calculation was performed using the calculating software provided by the manufacturer (K-YBGL_CALC, Megazyme, USA).

4.3 Results

4.3.1 GC-MS/MS Analysis of the Hexane Fraction of *G. lucidum*

The GC-MS/MS chromatogram of the hexane fraction of *G. lucidum* has been demonstrated at Figure A 5.1 of appendix A and the information relating the most abundant gano-components in Table 4.1 of this chapter.

Table 4.1: Identified bio-components in the hexane fraction of *G. lucidum*

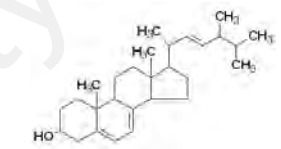
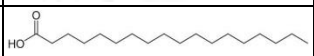
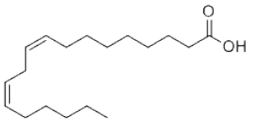
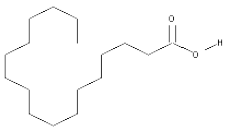
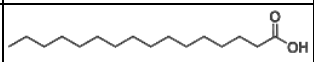
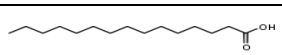
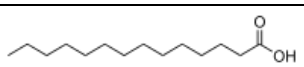
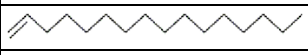
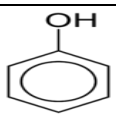
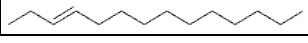
No.	Name	Structure	Retention time	Peak area (%)	M. formula	M. t (g/mol)
1	11-Octadecenoic acid, methyl ester (Vaccenic acid)		16.591	16.81	$C_{18}H_{34}O_2$	282.46
2	9-Octadecenoic acid (Oleic acid)		17.054	12.57	$C_{18}H_{34}O_2$	282.46
3	n-Hexadecanoic acid (Palmitic acid)		15.355	6.90	$C_{16}H_{32}O_2$	256.42
4	cis-9, cis-12 Octadecadienoic acid (Linoleic acid)		14.886	5.57	$C_{18}H_{32}O_2$	280.45
5	Tetradecanoic acid/ Myristic acid		13.226	1.18	$C_{14}H_{28}O_2$	228.37
5	5,6-Dihydro ergosterol		25.837	0.68	$C_{28}H_{46}O$	398.66

As found from the GC-MS analyses, the hexane fraction of *G. lucidum* abounds with 11-Octadecenoic acid, methyl ester (vaccenic acid), 9-Octadecenoic acid (oleicacid), n-Hexadecanoic acid (palmitic acid), cis-9, cis-12 Octadecadienoic acid (linoleic acid), tetradecanoic acid/myristic acid and 5,6-dihydro ergosterol. Some prior studies also identified the palmitic, oleic and linoleic fatty acids as the principal fatty acids present in the *G. lucidum* (Lv *et al.,* 2012; Stojković *et al.,* 2014).

4.3.2 GC-MS/MS Analysis of the Hexane Fraction of *H. erinaceus*

The GC-MS/MS chromatogram of the hexane fraction of *H. erinaceus* has been provided in Figure A 5.2 of appendix A and the identified bio-components are listed in Table 4.2 of this chapter. Based on the peak area percentage, the relative retention time (Rt) and respective quality, ten components were identified.

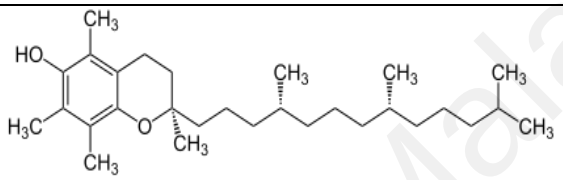
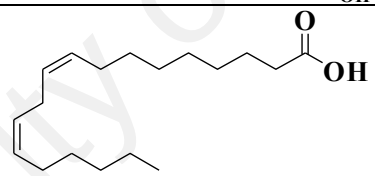
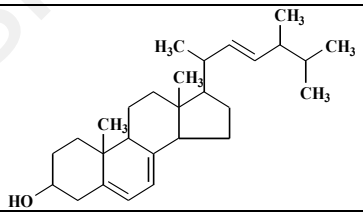
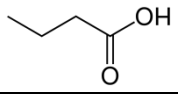
Table 4.2: Identified bio-components in the hexane fraction of *H. erinaceus*

No.	Name	Structure	Rt	Peak Area (%)	M. formula	M. wt. (g/ mol)
1	Ergosterol		25.72	5.20	$C_{28}H_{44}O$	396.65
2	Octadecanoic acid/Stearic acid		17.541	6.28	$C_{18}H_{36}O_2$	284.48
3	9,12-Octadecadienoic acid (Z,Z/*cis*-Linoleic acid)		17.35	27.31	$C_{18}H_{32}O_2$	280.45
4	n-Heptadecanoic acid/Margaric acid		16.83	2.15	$C_{17}H_{34}O_2$	270.45
5	n-Hexadecanoic acid /Palmitic acid		14.91	2.64	$C_{16}H_{32}O_2$	254.41
6	Pentadecanoic acid		14.37	1.57	$C_{15}H_{30}O_2$	242.39
7	Tetradecanoic acid/Myristic acid, methyl ester		13.24	0.58	$C_{14}H_{28}O_2$	228.37
8	Cetene (1-Hexadecene)		11.31	2.04	$C_{16}H_{32}$	224.43
9	Phenol		10.42	2.33	C_6H_6O	94.11
10	3-Tetradecene		8.86	1.23	$C_{14}H_{28}$	196.37

4.3.3 GC-MS/MS Analysis of the Hexane Fraction of *L. edodes*

As shown in Table 4.3 in this chapter and in Figure A 5.3 of appendix A, the most abundant bio-active components present in the hexane fraction of *L. edodes* were α-tocopherol (vitamin E), oleic acid, linoleic acid, ergosterol and butyric acid.

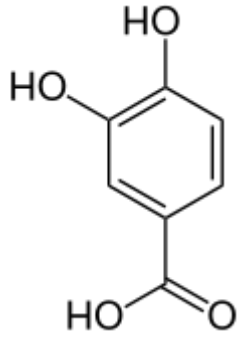
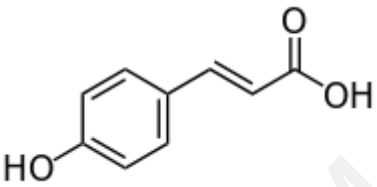
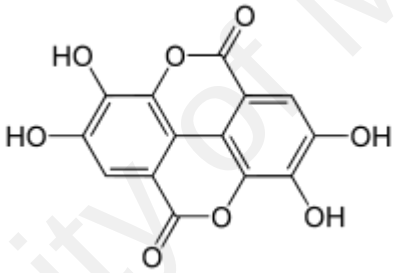
Table 4.3: Identified bio-components in the hexane fraction of *L. edodes*

No.	Bio-component	Structure	M. formula	M.wt (g/mol)
1	α-tocopherol		$C_{29}H_{50}O_2$	430.71
2	Oleic acid		$C_{18}H_{34}O_2$	282.46
3	Linoleic acid		$C_{18}H_{32}O_2$	280.45
4	Ergosterol		$C_{28}H_{44}O$	396.65
5	Butyric acid		$C_4H_8O_2$	88.11

4.3.4 LC-MS/MS Analysis of the MD Fraction of *F. velutipes*

Polyphenolic substances namely protocatechuic acid, p-coumaric acid and ellagic acid in the MD fraction of *F. velutipes* (Table 4.4; Appendix A, Figures A-5.4, 5.5, 5.6).

Table 4.4: Polyphenolic compounds identified in the MD fraction of *F. velutipes*

No.	Rt (m)	Mode (+/-)	Compound name and structure	Molecular formula	M. wt. (g/mol)
1	3.4	-	 Protocatechuic acid	$C_7H_6O_4$	154.12
2	5.2	-	 p-coumaric acid	$C_9H_8O_3$	164.16
3	7.68	-	 Ellagic acid	$C_{14}H_6O_8$	302.197

4.3.5 LC-MS/MS Analysis of the HWE of *G. lucidum*

As shown in the (Table 4.5, Appendix A, Figure A 5.7) LC-MS/MS analysis of the HWE of *G. lucidum* revealed the presence of phenolics (protocatechuic acid, cinnamic acid, p-hydorxy cinnamic acid, gallic acid, caffeic acid), monogalloyl glucose, caffeoyl glucose, Lucidenic acid A and F, Elfvingic acid and tri-terpenoids (ganoderic acids A, B, C, D, F, AM1, LM2 and hydroxy ganoderic acid). However, some of the peaks could not be related (834, 835, 838.5, 839, 1034.6 m/z, Da) with the known bio-components and those remained unidentified (Table 4.5).

Table 4.5: Gano - components identified in the HWE of *G. lucidum*

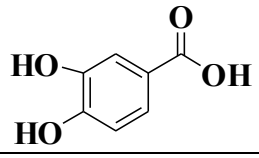
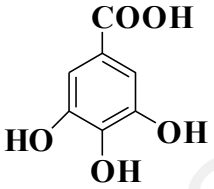
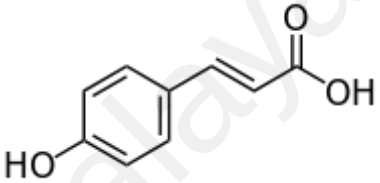
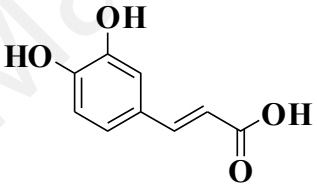
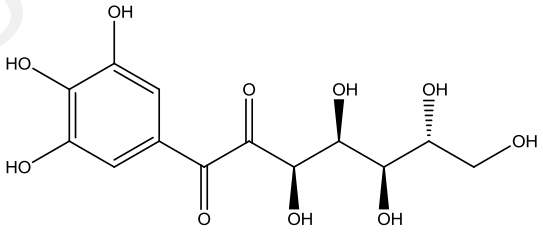
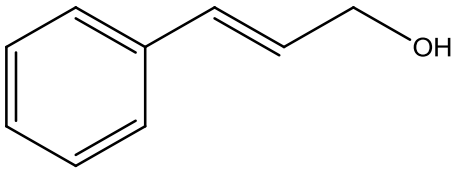
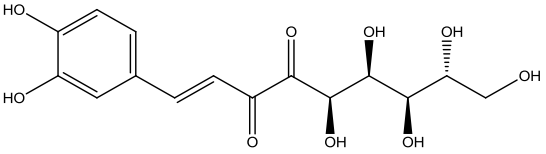
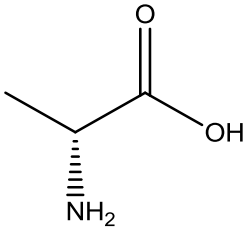
No.	m/z	Gano-component	Structure
1	108.0	Protocatechuic acid	
2	125.0	Gallic acid	
3	164.0	p-Hydroxy cinnamic acid	
4	192.0	Caffeic acid	
5	332.0	Monogalloyl glucose	 Monogalloyl glucose
6	338.0	Cinnamic acid	 Cinnamic acid
7	341.1	Caffeoyl glucose	 Caffeoyl glucose
8	349.0	D-Alanine	 D Alanine

Table 4.5 (continued): Gano - components identified in the HWE of *G. lucidum*

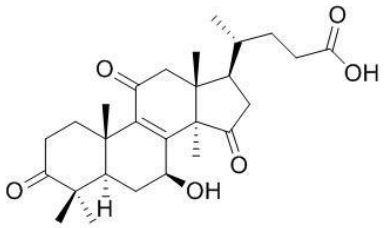
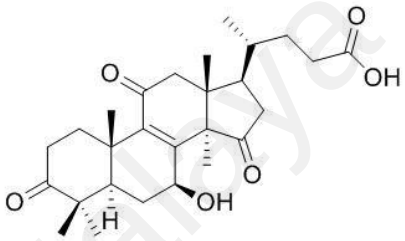
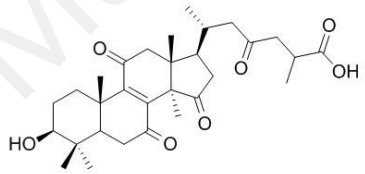
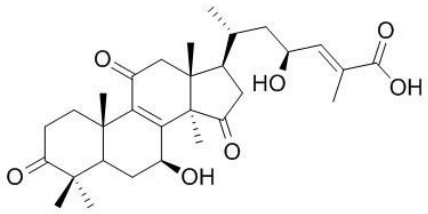
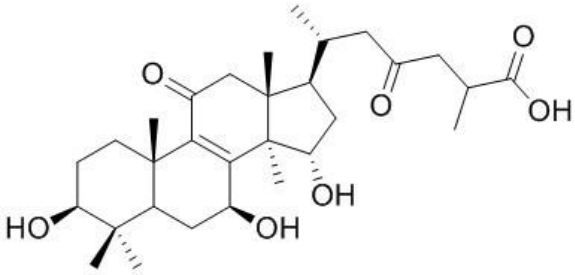
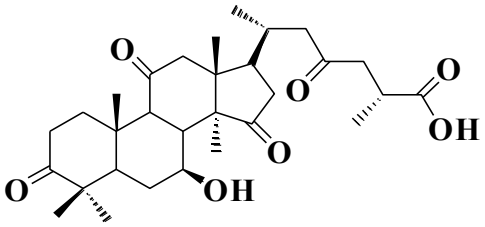
No.	m/z	Gano-component	Structure
9	496.2	Lucidenic acid A	
10	510.2	Ganoderic acid A	
11	513.2	Ganoderic Acid AM1	
		Ganoderic acid LM2	
12	517.3	Ganoderic acid B	
13	529.2	Ganoderic acid C6	

Table 4.5 (continued): Gano - components identified in the HWE of *G. lucidum*

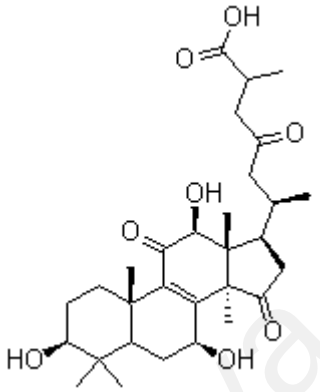
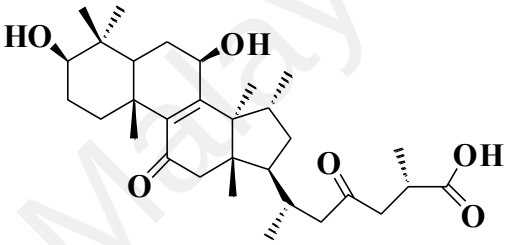
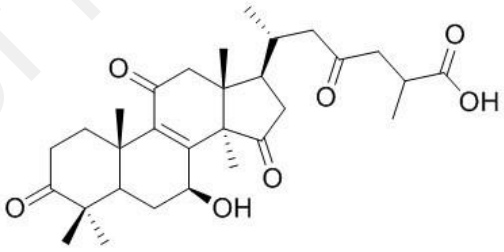
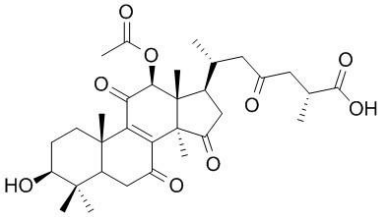
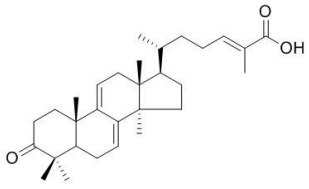
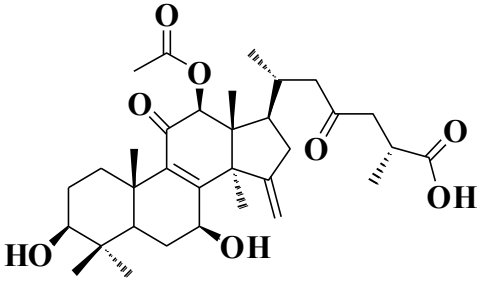
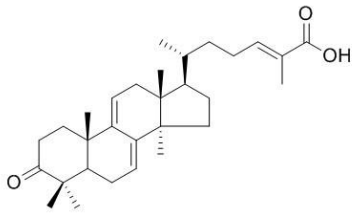
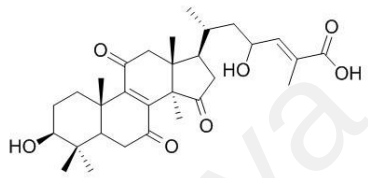
No.	m/z	Gano-component	Structure
14	533.2	Ganoderic acid G	
15	535.2	12-Hydroxyganoderic acid C2	
16	549.2	Ganoderic Acid D	
17	552	Ganoderic Acid H	
18	554.3	Ganoderic Acid J	
19	555.3	Ganoderic Acid K	

Table 4.5 (continued): Gano - components identified in the HWE of *G. lucidum*

No.	m/z	Gano-component	Structure
20	559	Ganoderic Acid S	
21	571.2	Ganoderic acid Z	
22	834.0	Unidentified	
23	835.0	Unidentified	
24	838.5	Unidentified	
25	839.0	Unidentified	
26	1034.6	Unidentified	

4.3.6 Determination of β-D-Glucan Level in the HWE of *G. lucidum*

The best *in vitro* anti-oxidative performance and content of the highest anti-oxidative and other bio-components in the HWE of *G. lucidum* intrigued the determination of its β-D-glucan content. Beyond its anti-oxidative performance, β-D-glucan has been revered for anti-hyerprlipidemia, anti-tumor, immunomodulating and other health giving potentials (Kanagasabapathy *et al.,* 2013; Wasser, 2002) . In the present study, as much as 44.15% (w/w) β-D glucan was detected in the HWE of *G. lucidum* (Raw data: Appendix A, Figure A 5.8, calculation using K-YBGL_CALC, Megazyme, USA).

4.4 Discussion

4.4.1 GC-MS/MS analysis of the hexane fraction of *G. lucidum*

11-Octadecenoic acid also called vaccenic acid (VA) was among the most available gano-components present in the hexane fraction (Table 4.1). It is an omega-7 fatty acid and finding from this study is novel in respect of being available in the mushroom

world, specifically in *G. lucidum*. Its cholesterol lowering, anti-atherosclerotic and anti-carcinogenic effects have been reported (Kritchevsky *et al.,* 2000; Lock *et al.,* 2004). Next to VA, oleic acid (9-Octadecenoic acid) was the second most abundant gano-component in the hexane fraction of *G. lucidum*. Anti-oxidative and anti-atherosclerotic functionality of its monoacylglycerol (MAG) moieties had been noticed both *in vitro* and in cellular models (Cho *et al.,* 2010). It is much potent than the palmitic-MAG in scavengeing free radicals, inhibiting Cu^{2+}- induced LDL oxidation, inhibited lowering the activity against LDL-associated phospholipase A(2) along with potent activation of paraoxonase activity, that maintains cellular anti-oxidative defense arsenal (Cho *et al.,* 2010). Similar mode of free-radical scavenging and anti-oxidative protection might have been employed in the present study. Diet rich-in oleic acid aids in lowering blood LDL level (Allman-Farinelli *et al.,* 2005). Besides, incorporation of oleic acid in the culture media had been reported lowering the uptake of oxidized LDL molecules into the THP-1 cells as well as decreased generation of malondialdehyde and lipid hydroperoxide (Cho *et al.,* 2010). At the molecular level, oleic acid reduces the expression of vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ECAM-1) and E-selectin and interrupts the activation of nuclear factor-kappa B (NFκB) and thus aids in controlling atherogenesis (Carluccio *et al.,* 1999; Cho *et al.,* 2010). Epidemiological studies also reveal that oleic acid, through an alternative mode of action, modulates vascular response to atherogenic precursors (Carluccio *et al.,* 1999).

n - Hexadecanoic acid (palmitic acid) had been found as the third most abundant gano-component (Table 4.1). It is a saturated fatty acid having anti-oxidant activities (Keawsa-Ard *et al.,* 2012). Another novel anti-oxidant present in the hexane fraction of *G. lucidum* was the tetradecanoic acid/myristic acid (Keawsa-Ard *et al.,* 2012). Among others, linoleic acid (LA, cis-9, cis-12 octadecadienoic acid) and its different

constitutional and stereoisomers had been detected that belong to the group "conjugated linoleic acid (CLA)". Both *in vitro* and *in vivo* anti-oxidative performance of CLA had been found (da Silva Marineli *et al.,* 2012; Palacios *et al.,* 2003). In rat plasma, CLA had been found increasing catalase level by 3.7 fold and in liver, CLA supplemented with phytosterol, decreased lipid peroxidation by 58% (da Silva Marineli *et al.,* 2012). Its anti-oxidative potential against mitochondrial and peroxisomal lipid peroxidation had been found better than that of vitamin A (Palacios *et al.,* 2003). Multiple strands of research had demonstrated hypocholesterolemic, LDL lowering and anti-atherosclerotic effect of CLA (Kritchevsky *et al.,* Lee *et al.,* 1994; 2004; Toomey *et al.,* 2003). Proposed anti-atherosclerotic effect of CLA involves stimulation of the nuclear transcription factor peroxisome proliferator activated receptor γ (PPARγ) expression in the vascular smooth muscle cells (Toomey *et al.,* 2003). Reduced risk of coronary heart diseases (CHD) has been linked with increased consumption of linoleic acid (Rudel, 1999).

5, 6 - Dihydro ergosterol was another important gano-component detected in the present study (Table 4.1). Ergosterol is the most common fungal sterol and among numerous bio-functionalities, its role as the membrane antioxidant has also been regarded (Wiseman, 1993; Yaoita *et al.,* 2002; Zhang *et al.,* 2003). Its level varies depending on the cultivation and extraction techniques of mushrooms (Barreira *et al.,* 2013; Lv *et al.,* 2012; Stojković *et al.,* 2014). Kim *et al.* (1999) extracted ergosterol peroxide from the mushroom *Armillariella mellea* and reported its anti-oxidant capacity to be far exceeding that of α-tocopherol and thiourea. Supplementation of ergosterol and oleic acid in the medium containing *Saccharomyces cerevisiae* had been reported to lower the production of reactive oxygen species, oxidative stress and the resultant damage towards cellular and biomolecular levels (Landolfo *et al.,* 2010). Sterols from *G. lucidum* had been found inhibitory towards generation of reactive oxygen species

(ROS) and protective against hypoxia/reoxygenation-mediated oxidative stress (Zhao *et al.,* 2005).

Mushrooms' anti-oxidant effects relating to DPPH free radical scavenging and Folin-Ciocalteu assay (reducing capacity test) has been attributed towards distinct groups of bio-components (Kalogeropoulos *et al.,* 2013). Phenolic and polyphenolic group of reducing substances have been regarded providing DPPH free-radical scavenging and Folin-Ciocalteu assay performance while triterpenoids have been linked with metal chelating activities (Kalogeropoulos *et al.,* 2013). Thus, 5,6-dihydroxy ergosterol and structurally relevant components such as 7, 22 - ergostadienol, ergosta 7, 22- dien-3-ol present in the hexane fraction might have been involved in chelating transition metal ion, Fe^{2+} and thus mediated reduced oxidative stress and consequent lowered LDL oxidation.

4.4.2 GC-MS/MS analysis of the hexane fraction of *H. erinaceus*

Ergosterol was among the most abundant bio-components present in the hexane fraction of *H. erinaceus* (Table 4.2). Indeed, ergosterol is the principal sterol in the edible mushrooms that provide anti-oxidative defense (Yaoita *et al.,* 2002; Zhang *et al.,* 2003). 5,6-Dihydroergosterol has potent inhibitory role against the expression of iNOs and production of nitric oxide (NO) that manifest it as an OS retarding and inflammation preventing agent (Park *et al.,* 2013). Total sterol extracted from mushrooms had been suggested to have ameliorating effect on hypoxia/reoxygenation – induced oxidative stress and inhibitory effect on the formation of reactive oxygen species (ROS) (Zhao *et al.,* 2005).

Fatty acids of both saturated (octadecanoic acid/stearic acid, n-heptadecanoic acid/margaric acid, n-hexadecanoic acid/palmitic acid, pentadecanoic acid and tetradecanoic acid/myristic acid methyl ester) and unsaturated types (9, 12-

octadecadienoic acid/Z,Z/*cis*-linoleic acid) were also detected (Table 4.2). These components had been reported having preventive role against oxidative stress induced physiological complications (Guillamón *et al.,* 2010; Kalač, 2009). For instance, compared to other saturated fatty acids, the LDL lowering capacity of octadecanoic acid is higher and the anti-oxidative effect of 9, 12-octadecadienoic acid (linoleic acid) is praiseworthy (Hunter *et al.,* 2010; Peyrat-Maillard *et al.,* 2003).

4.4.3 GC-MS/MS analysis of the hexane fraction of *L. edodes*

The most abundant bio-component in the hexane fraction of *L. edodes* was α-tocopherol (Table 4.3). Indeed, α-tocopherol is a chain breaking anti-oxidant whose participation might be related towards the LDL oxidation inhibitory effect of this mushroom. Its free radical scavenging mode involves hydrogen atom transfer (HAT). Reacting with a lipid peroxyl molecule, it becomes converted to a radical form of α-tocopherol with concomitant generation of a hydroperoxide and thus halts the propagation of peroxidative reaction (Figure 4.1). The intermediates produced are either unreactive or less harmful than the lipid peroxyl molecules. In the next phase, the α-tocopherol radical form can either revert back to its previous form or combine with a new peroxyl molecule to be converted into an inactive form (Figure 4.1).

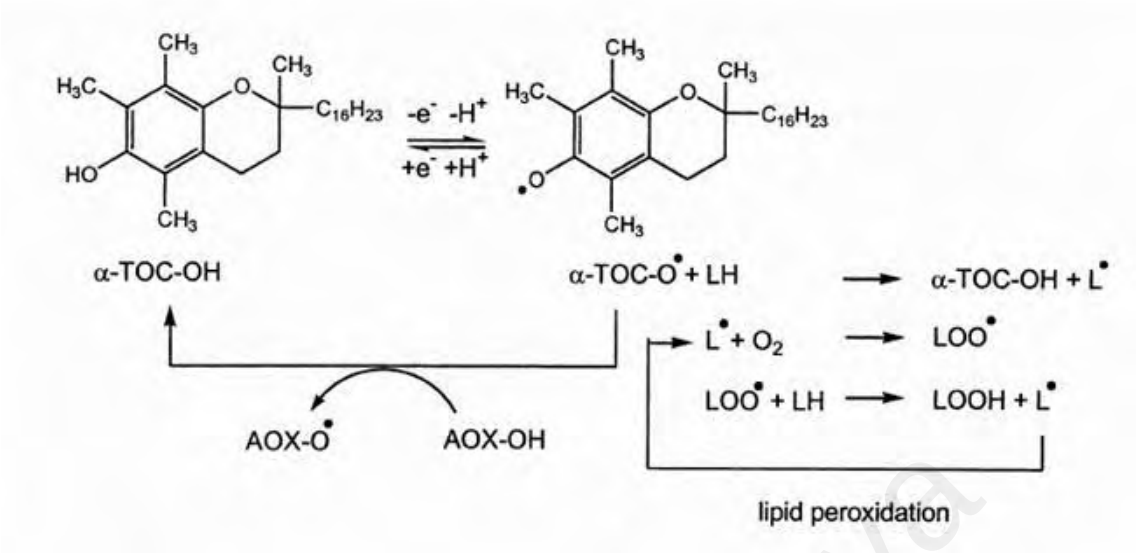


Figure 4.1: Lipid peroxidation inhibitory effect of α-tocopherol

In the physiological system, α-tocopherol is apt in reconstituting the free radicals into inactive molecules based on its capacity to recycle from the chromanoxyl form, interaction with lipid radicals and its distribution pattern in the membrane (Serbinova *et al.,* 1991). α-tocopherol had been found lessening OS in both human plasma and *in vitro* LDL oxidation generated through Cu^{2+} and/or 2,2'-azobis-(2-amidinopropane) hydrochloride (AAPH) supplementation (Kontush *et al.,* 1996). However, its *in vivo* anti-oxidative potency depends on the presence of co-antioxidants such as ascorbate that aid in its regeneration (Kontush *et al.,* 1996). Intake of α-tocopherol (400 IU/day) in human had caused decreased LDL oxidation, increased lag time of LDL oxidation and increased serum oxygen radical scavenging capacity (O'Byrne *et al.,* 2002). α-tocopherol – mediated increament of lag time from 59.6 to 98.9 minutes had been reported at its physiological level of 10–30 μM (Baldi *et al.,* 2003). Besides, its anti-oxidative turned anti-atherosclerotic effect had been found in several animal studies (Peluzio *et al.,* 2001; Schwenke *et al.,* 2002). Reis *et al.* (2012) reported the content of highest amount of α-tocopherol in *L. edodes* (0.92 μg/100 g) whole powder than those

of *P. ostreatus* (0.59 µg/100 g), *P. eryngii* (0.25 µg/100 g) and *F. velutipes* (0.19 µg/100 g) (Reis *et al.,* 2012).

Like that of the hexane fraction of *G. lucidum,* ergosterol was found in the hexane fraction of *L. edodes.* Its anti-oxidative effect had been reported by several studies and even better than those of thiourea and α-tocopherol (Kim *et al.,* 1999; Wiseman *et al.,* 1993). Its inhibitory activity towards phosphorylation of MAPK, C/EBPb transcriptional activity and NF-kB and thus suppression of inflammatory responses had also been documented (Kim *et al.,* 1999).

Anti-oxidative performance of mushrooms varies from species to species and nature of the extracting solvent and procedure applied highly affects the respective mushroom's potency. When extraction process involves polar solvents such as methanol, phenolic and polyphenolics provide scavenging effect towards DPPH free-radicals and reducing capacities in Folin - Ciocalteu assay while triterpenoids participate in metal ion chelation (Kobori *et al.,* 2007; Landolfo *et al.,* 2010). In case of non-polar solvents such as hexane, ergosterol dominates over other anti-oxidative mushroom bio-components (Kalogeropoulos *et al.,* 2013). Thus, in congruence with the previous findings, ergosterol; 5, 6 - dihydroxy ergosterol; 7, 22 – ergostadienol; ergosta 7, 22- dien-3-ol present in the hexane fraction might be implicated in chelating Fe^{2+}. This may be the putative mechanism of mushroom-mediated reduced LDL oxidation as observed in the present study. Anti-oxidative performance of ergosterol is so much prominent that in the edible mushroom *Grifola frondosa,* it stands as the second-most anti-oxidant among a number of bio-components (Nieto & Chegwin, 2008).

Among fatty acids present in the hexane fraction of *L. edodes,* were oleic, linoleic and butyric acid. Anti-oxidative and anti-atherosclerotic excellence of oleic acid had been observed both *in vitro* and in cellular models (Cho *et al.,* 2010). Its free radical

scavenging and inhibitory potency towards LDL oxidation far exceeds that of the palmitic acide moiety (Cho *et al.,* 2010). Its importance also lies in maintenance of cellular anti-oxidative state through activation of paraoxonase activity (Cho *et al.,* 2010). In addition to reduced LDL oxidation, oleic acid has blood LDL lowering effect (Allman-Farinelli *et al.,* 2005). Its anti-atherosclerotic mode of action involves reduced expression of VCAM-1, ECAM-1, E-selectin and interrupted activaty of NFκB resulting in lowered adherence of monocytes to endothelial wall and dampened pro-inflammatory and pro-atherogenic activity (Carluccio *et al.,* 1999; Massaro *et al.,* 1999). Similar mode of action had been reported for butyric acid, another short chain fatty acid found in the hexane fraction of *L. edodes* (Massaro *et al.,* 1999; Zapolska-Downar *et al.,* 2004). Linoleic acid (LA, cis-9, cis-12 octadecadienoic acid) stands among the most abundant unsaturated fatty acids present in culinary-medicinal mushrooms (Kalogeropoulos *et al.,* 2013). Linoleic acid in its conjugated form had been found having anti-oxidative as well as blood LDL and triacylaglyceride lowering effects (Carluccio *et al.,* 1999; Palacios *et al.,* 2003). CLA alone had been found increasing plasma level of catalase by 3.7 fold and decreasing lipid peroxidation by 58% in rat hepatic tissues (da Silva Marineli *et al.,* 2012). Its protective role against oxidation of mitochondrial and peroxisomal PUFAs had been found far exceeding that of vitamin A (Palacios *et al.,* 2003). Compared to *P. ostreatus, P. eryngii* and *F. velutipes,* increased level of PUFA (82%) and lowered level of saturated fatty acids (SFAs) (15.1%) in the whole powder of *L. edodes* had been detected by Reis *et al.* (2012). In terms of most abundancy, linoleic acid was top among the PUFAs while oleic acid among the mono-unsaturated fatty acids (MUFAs) and palmitic acid among the SFAs (Reis *et al.,* 2012).

As discussed in case of the hexane fraction of *G. lucidum* (section 4.3.1), content of ergosterol in the same fraction of *L. edodes* might contribute to the HMGR inhibitory effects (Gil-Ramírez *et al.,* 2013; Gil-Ramirez *et al.,* 2011; Gil-Ramírez *et al.,* 2013).

Among 26 edible-medicinal mushrooms, *L. edodes* had been reported to be the best in inhibiting the activity of HMGR (Gil-Ramírez *et al.,* 2013).

4.4.4 LC-MS/MS analysis of the MD fraction of *F. velutipes*

Protocatechuic acid, p-coumaric acid and ellagic acid were the most abundant polyphenolics present in the MD fraction of *F. velutipes* (Table 4.5).

A. Protocatechuic acid

Protocatechuic acid (PCA), an important member of the polyphenolic anti-oxidants possesses numerous health benefits (Kakkar & Bais, 2014). Its presence in both wild and edible-medicinal mushrooms have been documented (Barros *et al.,* 2009; Mattila *et al.,* 2002). Its *in vitro* free radical scavenging (DPPH, ABTS, superoxide and hydroxyl ion), metal ion chelating (Fe^{2+}) capacity and content of reducing power had been found better than those of the synthetic anti-oxidant and vitamin E analogue, trolox. Its *in vitro* anti-oxidative excellence remains unaltered in both aqueous and lipid media (Li *et al.,* 2011). Its anti-oxidative mode of action entails either free-radical scavenging and/or metal ion chelation.

a. PCA- mediated scavenging of DPPH free-radical involves the following steps (Figure 4.2):

i. Acceptance of a hydrogen atom (H•) by the PCA from DPPH free radical (DPPH•) and formation of the unstable anion PCA• (A) and stable DPPH-H.

ii. Formation of a stable quinone by withdrawing a hydrogen atom (H•) from PCA• (A).

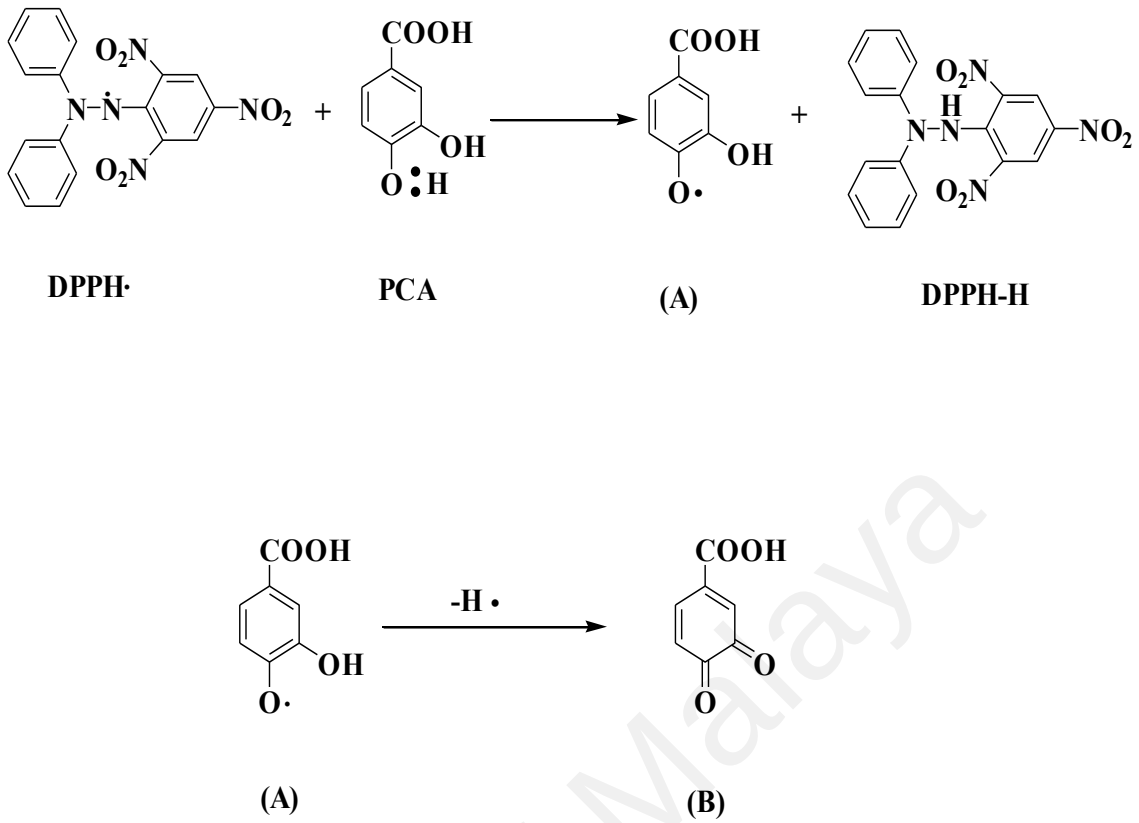


Figure 4.2: Protocatechuic acid (PCA) – mediated scavenging mechanism of DPPH free radical

b. Metal ion chelation by PCA: Metal ion (Fe^{2+}) is chelated by the ortho-dihydroxyl group of PCA (Figure 4.3).

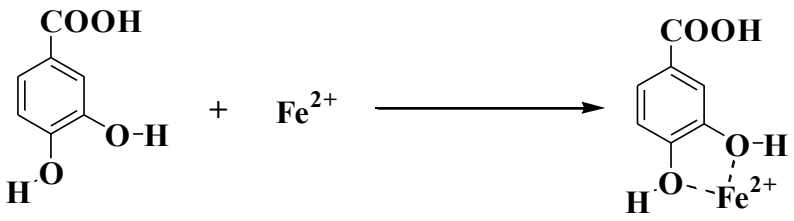


Figure 4.3: Protocatechuic acid – mediated metal ion chelating mechanism

B. p-Coumaric (p-Hydroxy cinnamic acid moeiey)

Prior to our findings, identification and quantification of p-coumaric acid had been performed in wild mushrooms collected from northeast Portugal (Barros *et al.,* 2009; Vaz *et al.,* 2011). Inhibitory effect towards both *in vitro* and *in vivo* LDL oxidation by p-coumaric acid had been found (Zang *et al.,* 2000). The probable mechanism is ˙OH scavenging (Zang *et al.,* 2000). It has been found superior to curcumin in decreasing oxidative stress in the human colon cell culture (HT-29) (Ferguson *et al.,* 2005).

C. Ellagic acid

Ellagic acid, a flavonoid possesses anti-oxidant capacity better than that of melatonin and ascorbic acid (Atta-Ur-Rahman *et al.,* 2001; Festa *et al.,* 2000). Its anti-oxidative protection against colon cell lines (HCT 16) had also been found (Seeram *et al.,* 2005).

Lipid hydroperoxides are generated from PUFA peroxidation during LDL oxidation. When hydroperoxides decompose, the process becomes a chain reaction. Phenolic anti-oxidants, through chanin breaking mechanism, reduce the alkoxyl and/or peroxyl radicals to alkoxides and/or hydroperoxides, respectively (Rice-Evans *et al.,* 1997). Potency of the phenolic antioxidants depends on the position of the phenolic group in the structure, ability to stabilize the unstable phenoxyl radical and redox potential which is inversely related to the anti-oxidative capacity (Rice-Evans *et al.,* 1997; Teixeira *et al.,* 2013). Other factors such as presence of pro- and anti-oxidative enzymes and other anti-oxidants also affect the anti-oxidative performance of the phenolic anti-oxidants (Rice-Evans *et al.,* 1997; Teixeira *et al.,* 2013).

4.4.5 LC-MS/MS analysis of the HWE of *G. lucidum*

Content of phenolics and their contribution towards anti-oxidative potentiality of *G. lucidum* had been reported previously (Abdullah *et al.,* 2011; Hasnat *et al.,* 2013; Mau

et al., 2002a; Stojković *et al.,* 2014). Phenolics stand among the most potent anti-oxidative mushroom bio-components. Their content vary from species to species, part to part of the same mushroom species and their activities depend on the process of extraction and mode of application as well as to the oxidant they encounter (Heleno *et al.,* 2012; Mau *et al.,* 2002b; Stojković *et al.,* 2014). Protocatechuic acid, cinnamic acid, p-hydroxy cinnamic acid and p-hydroxy benzoic acid are among the most commonly available phenolics in the *Ganoderma* mushrooms (Heleno *et al.,* 2012; Mau *et al.,* 2005). Ganoderic acids of more than 140 categories had been reported to be present *G. lucidum* (Ye *et al.,* 2010). Anti-oxidative performance of the ganoderic acids stand among their immense bio-functionalities (Smina *et al.,* 2011).

Based on the source of mushroom production, extraction procedures and anti-oxidant content, different anti-oxidative performance have been observed among different varieties of *Ganoderma* and *G. lucidum* itself. For example, *G. lucidum* collected from China, Korea, Portugal and Serbia all had shown anti-oxidative performance but their extent differed from each other with respect to reducing capacity and free radical scavenging (Heleno *et al.,* 2012; Kim *et al.,* 2008; Stojković *et al.,* 2014). Phenolic extracts of the fruiting bodies of *G. lucidum* had higher anti-oxidant performance and higher content of total phenolics while the polysaccharide extracts of the spores had higher anti-oxidative capacity (Heleno *et al.,* 2012). Similarly, content of ganoderic acids were found to be variable among 36 different samples of *Ganoderma* isolated from different parts of China and content of *G. lucidum* was 10 times higher than those of *G. tsugae* (Wang *et al.,* 2006).

In the present study, the HWE containing the polyphenolic and tri-terpenoids outperformed in annihilating the DPPH free-radicals and possessing the total phenolics while the non-polar fractions surpassed the polar fractions in demolition of lipid

peroxidation and LDL oxidation activities. The *modus operandi* might be the higher content and availability of the phenolics in the HWE of the respective mushroom species. Total phenolic assay measures the reducing capacity of the Folin reagent in the alkaline media. Anti-oxidants containing higher phenolics are supposed to reduce the oxidant at higher extent based on the higher reducing power (hydrogen atom) provided by the hydroxyl group(s) attached with the benzene structure. The positive control quercetin contains five hydroxyl groups (at 3, 3′, 4′, 5, 7 positions) and one carbonyl group (at 4′position) and showed the highest reducing capacity. Compared to quercetin, single or multiple hydroxyl groups present in the phenolic or polyphenolic acids (protocatechuic acid, p-hydroxy coumaric acid, ellagic acid, gallic acid, caffeic acid, cinnamic acid, p-hydorxy cinnamic acid) contained in the HWE of the respective mushroom species conferred its best reducing capacity and DPPH free radical annihilating efficiency. Since DPPH and Folin-Ciocalteu reactions were performed in aqueous microenvironment, perhaps the lipo-philic anti-oxidants (ergosterol, saturated and unsaturated fatty acids) could not access at all or very little to exert their free radical annihilating and Folin reagent reducing actions. Besides, complexity of the total phenolics, nature of individual phenolic component, steric hindrance and accessibility, sample preparation, ionic potentiality of the reagents used and color interference highly affect the relationship between DPPH free radical scavenging and Folin-Ciocalteu assays (Apak *et al.,* 2013; Huang *et al.,* 2005). This assumption is supported by the previous finding that the extent of anti-oxidative performance of the mushrooms depends not only on its content of phenolics but also on the nature of the phenolics (free or bound to polysaccharide) (Heleno *et al.,* 2012).

In case of lipid peroxidation and LDL oxidation inhibition, the higher performance of the HEX of the mushroom species might be attributed to their content of lipophilic (ergosterol and fatty acids) and/or amphiphilic anti-oxidants (α-tocopherol).

Supposedly, suitable alignment of α-tocopherol in the lipid peroxidation solvent facilitated its superb anti-lipid peroxidation effect than those of the phenolics of the HWE (Maqsood & Benjakul, 2010). Another apprehension is that the antioxidant activities of mushrooms determined by chemical tests surpass those of the biochemical assays (Barros *et al.,* 2008). As inhibition of lipid peroxidation of the buffered egg yolk is a bio-chemical test, the discrepancy in the anti-oxidative performance between the hexane and HWE is natural and might be mediated by the different bio-components present in different amount and differing functionality in the respective mushroom fractions. Similar proposition might be applied in case of the LDL inhibitory effect of the hexane extracts of different mushrooms.

4.4.6 Determination of β-D glucan level in the HWE of *G. lucidum*

Anti-oxidative potentiality of the HWE of *G. lucidum* described in the previous chapter might partly be attributed to the content of this higher amount (44.15%) of β-D glucan in the extract (Raw data: Appendix A, Figure 5.8, calculation using K-YBGL_CALC, Megazyme, USA). This claim is supported by some other observations that have linked this polysaccharide's free radical scavenging potential even at lower level than that of us (Khan *et al.,* 2014; Kofuji *et al.,* 2012; Kogan *et al.,* 2005; Kozarski *et al.,* 2011; Maity *et al.,* 2014; Patra *et al.,* 2013; Şener *et al.,* 2006). Hydroxyl radical scavenging capacity of β-D-glucan had been found intermediary between the hydrophilic anti-oxidant mannitol and the lipo-philic anti-oxidant α-tocopherol (Babincova *et al.,* 2002). Its mode of action involves hydrogen atom transfer mechanism and termination of the chain reaction of free radical generation. Its level varies from mushroom species to species and also based on the extraction process (Ahmad *et al.,* 2014). Its protective role against OS induced neurotoxicity has also been linked with its anti-oxidative properties (Kaya *et al.,* 2016). In conjunction with its *in vivo* anti-oxidative effect through improving CAT, GPX and SOD, lipid lowering

efficiency of β-D glucan from *P. sajor-caju* has been found to be achieved through stimulating the expression of hormone sensitive lipase and adipose triglyceride lipase along with lowering the expression of lipoprotein lipase (LPL), peroxisome proliferator-activated receptor gamma (PPAR-γ) and sterol regulatory binding protein-1c (SREBP-1c) (Kanagasabapathy *et al.,* 2013). As the present study is aimed at evaluating the anti-oxidative, cholesterol lowering and AD ameliorating effect of selected culinary-medicinal mushrooms, the observed anti-oxidative effect of the *G. lucidum* HWE along with its content of higher amount of β-D glucan has been of greater importance in choosing this mushroom and extract for furthering *in vivo* studies.

4.5 Conclusion

Both culinary *(H. erinaceus, L. edodes* and *F. velutipes)* and medicinal *(G. lucidum)* mushrooms possess anti-oxidative potentialities. Anti-oxidative performance of different solvent-partitioned fractions varies from each other. The reason might be differing content of bio-active components in different fractions. Among four mushroom species *(G. lucidum, H. erinaceus, L. edodes* and *F. velutipes)* of the present study, the *G. lucidum* showed the top-notch *in vitro* anti-oxidative performance. Under the current experimental set up, the HWE of *G. lucidum* outperformed the solvent-partitioned fractions. Phenolics and tri-terpenoids gano-components identified in the HWE of *G. lucidum* might contribute to this superb performance that beacons towards this mushroom extract's therapeutic potentiality. Based on these, *in vivo* studies of *G. lucidum* HWE had been outlined in the following chapters.

CHAPTER 5: *IN VIVO* ANTI - OXIDATIVE AND HYPOCHOLESTEROLEMIC EFFECT OF THE HOT WATER EXTRACT OF *G. LUCIDUM*

5.1 Introduction

As the HWE of *G. lucidum* showed the best *in vitro* anti-oxidative potentiality (chapter 3) and contained higher amount of bio-active components (chapter 4), it was selected in the present study for assessing its *in vivo* anti-oxidative and cholesterol lowering performance *en route* to deciphering its AD ameliorating effect. The experimental paradigm was based on the fact that the brain is much vulnerabale to OS due to its higher rate of oxygen utilization (one-fourth of total respired oxygen) and lipid content in contrast to possession of lower anti-oxidative defense mechanism (Ansari & Scheff, 2010; Mazzetti *et al.,* 2015; Reed, 2011; Sottero *et al.,* 2009).

Oxidative stress directly affects the the neuronal cells and Aβ itself exacerbates the condition (McLellan *et al.,* 2003). Even partial defect in the SOD (Mn) could not provide anti-oxidative support to the Tg19959 AD model mice (Dumont *et al.,* 2009). On the other hand, only 2 - 3 times overexpression of this enzyme in the same animals could lower OS and also decrease Aβ deposition and improve memory (Dumont *et al.,* 2009). Similarly, enhanced AD symptoms such as excellerated rate of OS - driven Aβ oligomerization and memory loss have been observed in case of the Tg2576 AD model mice deficient of SOD1(Cu/Zn) (Murakami *et al.,* 2011). High amount of redox - active metal ions (Cu^{2+} and Fe^{2+}), phospholipids and poly unsaturated fatty acids (PUFAs) also make the brain cells much susceptible to OS (Garbarino *et al.,* 2015; Patel, 2016). As a result, compromised *in vivo* anti-oxidative defense and abrupt lipid peroxidation greatly affect AD pathogenesis (Chen *et al.,* 2008; Resende *et al.,* 2008; Su *et al.,* 2010). Even, Aβ itself acts as a pro-oxidative agent (Combs *et al.,* 2001; Ding *et al.,* 2007).

Proteomics observation reveals that methionine35 of the Aβ sequence raises its OS - causing prowess while its replacement with cysteine reduces the oxidative risk (Butterfield & Boyd-Kimball, 2005; Butterfield *et al.,* 2013). Besides, OS generated from mitochondrial dysfunction also adds injury to AD pathogenesis (Castellani *et al.,* 2002; Melov *et al.,* 2007; Reddy, 2011; Yao, Jia *et al.,* 2009). The situation is further exacerbated through APP and Aβ as they act as mitochondrial deregulators (Anandatheerthavarada *et al.,* 2003; Caspersen *et al.,* 2005; Manczak *et al.,* 2006).

Oxidative stress associated with hypercholesterolemia heightens the risks of AD (Oda *et al.,* 2010). Even, hypercholesterolemia itself is a risk factor of AD as it provides with the lipid rafts for Aβ generation (Björkhem, 2006; Heverin *et al.,* 2005; Sharma *et al.,* 2008). Hypercholesterolemic animals have been found vulnerable towards AD development including enhanced Aβ deposition in the hippocampus (Sparks *et al.,* 1994; Refolo *et al.,* 2000). On the other hand, cholesterol lowering approaches seem promising in reducing AD symptoms (Refolo *et al.,* 2001; Matthew *et al.,* 2011). Conceptually, AD therapeutic approaches entailing improved anti-oxidative defense along with cholesterol lowering potential seem imperative. Thus, the *in vivo* anti-oxidative and hypcholesterolemic effects of the selected HWE of *G. lucidum* have been determined whose description has been put forward in the following sections.

5.2 Materials and Methods

5.2.1 Animals

Ninety wistar male rats (weight range 120 ± 5 gm) were divided into six groups: control (C), *G. lucidum* HWE fed control (CE), hypercholesterolemic (H), *G. lucidum* HWE fed hypercholesterolemic (HE), Alzheimer's diseased (A in case of anti-oxidative, hypochoesterolemic, memory and learning related behavioral and biochemical and histopathological tests. AD in case of proteomics study) and *G. lucidum* HWE fed

Alzheimer's diseased (AE) each group containing 15 rats. *In vivo* anti-oxidative and hypocholesterolemic studies of each of the six groups were performed**.** Hypercholesterolemia to the H rats was evoked by adding 1% cholesterol and 1% cholic acid (for intestinal better absorption of cholesterol) with the basal diet. Alzheimer's diseased model rats were prepared through infusion of Aβ (1-42) in the right ventricle. The extract fed groups (CE, HE and AE) received 200 mg/kbw *G. lucidum* HWE. No specific dosage regimen of mushrooms had been found in the existing literature for *in vivo* experimentation. For the mushroom powder, the mostly utilized dosages range from 3% to 20% (w/w) (Hossain *et al.,* 2003; Mori *et al.,* 2008; Talpur *et al.,* 2002). In case of extracts, 100-400 mg/kbw of the animals had been found (Anandhi *et al.,* 2013; Jeong *et al.,* 2010; Omar *et al.,* 2015; Yamac *et al.,* 2010). As the species of mushrooms and the strain, type, age, sex and habitat of the experimental animals greatly influence the experimental output, the intermediary dosage regimen of the published literature (200 mg/kbw) was used for evaluating the hypocholesterolemic effect of *G. lucidum* HWE. Animals had been housed in a 12 hr day night cycle at 25±2 °C temperature. Twenty four hours following the last treatment and test, the rats were kept in fasting overnight. Then, the rats were anesthesized with intra-peritoneal injection of sodium pentobarbital (35 mg/kbw) and sacrificed. Blood was collected from the inferior vena cava immediately, centrifuged at 1000 rpm, collected plasma and serum and preserved at – 80 °C). All the experimental protocols had been approved by the ethical permission committee, University of Malaya Institutional Animal Care and Use Committee (UMIACUC) [Ethics reference no. ISB/25/04/2013/NA (R)] (Appendix G).

5.2.2 Measurement of *in vivo* anti-oxidative effect of *G. lucidum* HWE

In the present sudy, levels of catalase (CAT), glutathione peroxidase (GPX) and superoxide dismutase (SOD) in the plasma and liver homogenates of the controlled and

experimental rats were determined to evaluate th *in vivo* anti-oxidative potentiality of the *G. lucidum* HWE.

5.2.2.1 Catalase activity assay

a. Principle

Catalase (CAT) activity was assayed using the assay kit purchased from Cayman Chemical, USA (item no. 707002) and following the instruction provided. CAT possesses both catabolic (conversion of H_2O_2 into water and molecular oxygen) and peroxidative activities (conversion of H_2O_2 into water and formaldehyde in presence of low molecular weight alcohol such as methanol).

b. Reaction

$$2\ H_2O_2 \longrightarrow 2\ H_2O + O_2 \qquad \text{(catalytic activity)}$$

$$2\ H_2O_2 + CH_3OH \longrightarrow 2\ H_2O + HCOOH \text{ (peroxidative activity)}$$

The assay protocol utilizes the peroxidative action of CAT in presence of methanol and H_2O_2 that produces formaldehyde. Chromogen purpald (4-amino-3-hydrazino-5-mercapto-1, 2, 4-triazole) is allowed to react specifically with formaldehyde that produces a hetero-bicyclic compound whose subsequent oxidation is reflected through changing of the reaction mixture from colorless to purple and measurement of the absorbance at 540 nm.

c. Procedure

First of all, 100 µl assay buffer (100 mM potassium phosphate, pH 7.0) was added to the wells of 96-well ELISA plate. In the standard wells, methanol (30 µl), each standard diluted with sample buffer (20 µl) and formaldehyde (final concentration 0, 5, 15, 30,

45, 60, and 75 µM) were added. In the control wells, 20 µl of CAT control were added. Then, samples (20 µl) (five times diluted serum sample and ten times diluted tissue homogenate diluted with sample buffer containing 25 mM potassium phosphate, pH 7.5, having 1 mM EDTA and 0.1% BSA) were added to the sample wells. Addition of 20 µl of H_2O_2 to the wells started the peroxidative reaction of CAT and the initiation time was recorded. The plate, been covered, had undergone occasional shaking in a shaker for 20 minutes at room temperature. Addition of 30 µl of potassium hydroxide terminated the reaction. Then, 30 µl of purpald was added and incubated for 10 minutes at room temperature. To each well, 10 µl of potassium periodate was added and incubated for 5 minutes while covered and shaking. Finally, absorbance was read at 540 nm using a plate reader.

5.2.2.2 Glutathione peroxidase (GPx) activity assay

a. Principle

Glutathione peroxidase (GPx) assay was performed using the kit purchased from Cayman Chemical, USA (item no. 703102) and following the company's instructions. Indirect measurement of GPX activity was followed through a coupled reaction with glutathione reductase (GR) based on the principle that GPx is involved in catalyzing the reduction of H_2O_2 and other hydroperoxides. In the first stage of the experiment, GPx had been involved in reducing cumene hydroperoxide (ROOH) by reduced glutathione (GSH) and production of oxidized glutathione (GSSG). In the second phase, GSSG is converted to its reduced form (GSH) by NADPH with the catalysis of GR. As a result, NADPH itself is oxidized to $NADP^+$ along with decrease in absorbance at 340 nm. At rate limiting state, this absorbance change is directly proportional to the GPX activity of the sample and is calculated accordingly.

b. Reaction:

$$\text{Phase I : } 2\ GSH + ROOH \xrightarrow{GPx} GSSG + ROH + H_2O$$

$$\text{Phase II : } GSSG + NADPH^+ + H^+ \xrightarrow{GR} 2\ GSH + NADP^+$$

c. Procedure

For blank (non-enzymatic/background study, we used 3 wells of the 96-well ELISA plate and added 120 µL of assay buffer and 50 µL of co-substrate mixture (as provided by the manufacturer). Bovine erythrocyte GPx was used as the positive control. For sample and positive control, 20 µL of each was added with 100 µL of assay buffer and 50 µL of co-substrate mixture in triplicate. Reaction was initiated by adding 20 µL of cumene hydroperoxide in each of the wells and initiation time noted. The plate was shaken for 10 seconds inside the plate reader and the absorbance read at 340 nm at every one minute for total 5 time points. The decrease in absorbance was recorded and the obtained data used for calculating GPx activity.

5.2.2.3 Superoxide dismutase (SOD) activity assay

a. Principle

Using the superoxide dismutase (SOD) activity assay kit purchased from Cayman Chemical, USA (item no. 706002) and following the company's instructions, the activity of SOD was assessed. SOD usually dismutates superoxide anions into hydrogen peroxide and molecular oxygen.

$$2\ O_2^- + 2\ H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

In the first phase of the present experiment, superoxide radicals are generated from xanthine using xanthine oxidase (XO). In the second phase, superoxide radicals are

reduced by 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) and produces a formazen dye (red colored). Rate of reduction is directly proportional to XO activity and inhibited by SOD present in the sample. SOD activity is measured from the extent inhibition of reduction (1 unit of SOD causes 50% inhibition of reduction by INT). Thus, increased SOD level in the sample causes decreased superoxide production and lowered formazen generation.

b. Reaction

Phase I:

$$\text{Xanthine} + \text{Xanthine oxidase} \xrightarrow{(XO)} O_2^- + \text{Uric acid}$$

Phase II:

$$O_2^- + \text{INT} \longrightarrow \text{Formazen dye}$$

Or,

$$2\,O_2^- + 2\,H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

c. Procedure

In each well of the 96-well ELISA plate, 200 µL of INT was used followed by 10 µL of either diluted sample and/or standard. Reaction was initiated by adding 20 µL of xanthine oxidase in each of the wells and initiation time noted. The plate was covered, shaken for 10 seconds and incubated for 30 minutes at room temperature. Then, absorbance was read at 450 nm, values plotted and calculated to obtain the SOD activity.

5.2.3 Hypocholesterolemic study

5.2.3.1 Measurement of body weight

Using a digital scale (Kent scientific, SCL 66110, USA) body weight of each of the rats was measured at every 24 hours and recorded up to the end of the experiment. Weekly average body weight (gm) of rats belonging to different groups was compared with each other to find the effect of *G. lucidum* HWE feeding up on hypercholesterolemia and hyperlipidemia.

5.2.3.2 Measurement of triacylglycerol

a. Principle

Following the method of Werner *et al.,* (1981), the level of triacylglycerol (TG) was measured through a couple of enzymatic reactions. Firstly, lipase action generated fatty acid and glycerol from TG. Secondly, glycerokinase with ATP converts glycerol to glycerol-3-phosphate. Thirdly, glycerol-3-phosphate is oxidized by glycerophosphate oxidase to dihydroxyacetone phosphate and hydrogen peroxide. In the fourth step, peroxidase mediated reaction of hydrogen peroxide with 4-aminoantipyrine and N-Ethyl-N-sulfopropyl-n-anisidine (ESPAS) forms a coloured complex whose intensity is proportional to the TG concentration and is measured spectrophotometrically at 546 nm.

b. Reaction:

Triglycerides + $3H_2O$ ------- Lipase -----> glycerol + fatty acids

Glycerol + ATP ---------- glycerokinase ---------> glycerol-3-phosphate + ADP

Glycerol-3-phosphate + O_2 --------- glycerophosphate oxidase ----------> dihydroxyacetone phosphate + H_2O_2

$$H_2O_2 + \text{ESPAS} + \text{4-Aminoantipyrine} \xrightarrow{\text{peroxidase}} \text{Red Quinone} + H_20$$

c. Reagents

Reagent 1: Pipes Buffer (pH 7.5) 50 mmol/L, ESPAS 1 mmol/L, Magnesium Salt 15 mmol/L, ATP 0.3 mmol/L.

Reagent 2: Enzymes such as Lipoprotein lipase (1100 U/L), Glycerokinase (800 U/L), Glycerol – 3 – Phosphate Oxidase (5000 U/L), Peroxidase (350 U/L).

Reagent 3: Standard Glycerol (Triglycerides equivalent) 200 mg/dl.

d. Method

10 µL of plasma and/or standards were mixed with 1 mL of TG working reagent. We mixed the contents well and incubated at 37 °C for 10 minutes. Absorbance of the standard and the samples were measured against a reagent blank at 546 nm.

e. Calculation

$$\text{TG concentration (mg/dL)} = \frac{\text{(OD of test sample – OD of blank)}}{\text{(OD of standard – OD of blank)}} \times 200$$

5.2.3.3 Measurement of total cholesterol

a. Principle

The enzymatic method of Allain *et al.,* (1974) was followed to determine the level of TC in the plasma and liver homogenates of the rats . Using cholesterol esterase, cholesterol esters were hydrolyzed into free cholesterol and fatty acids. Free cholesterol underwent oxidation by cholesterol oxidase and produced cholest-4en-3-one and hydrogen peroxide. A colored quinone complex was produced through

reaction of hydrogen peroxide with 4-amino antipyrene in presence of phenol. Intensity of the colored quinone complex is directly proportional to the concentration of cholesterol present and is measured at 500 nm spectro-photometrically.

b. Reaction

Cholesterol esters $\xrightarrow{\text{cholesterol esterase}}$ cholesterol + Fatty acids

Cholesterol $\xrightarrow{\text{cholesterol oxidase}}$ Cholest-4-en-3-one + H_2O_2

H_2O_2 + Phenol + 4-Aminoantipyrine $\xrightarrow{\text{peroxidase}}$ Red Quinone + H_2O

c. Reagents

A. 1. Buffer (50 mmol/L pH 6.7)

2. Cholesterol oxidase ³ 50 U/L

3. Cholesterol esterase ³ 100 U/L

4. Peroxidase ³ 3 IU/L

5. 4-amino antipyrine (0.4 mmol/L)

B. Cholesterol standard (200 mg/dL).

d. Method

10 µL of plasma and/or standards were mixed with 1 mL of cholesterol reagent. We mixed the contents well and incubated at 37°C for 10 minutes. Absorbance of the standard and the samples were measured against a reagent blank at 500 nm.

e. Calculation

$$\text{Cholesterol concentration (mg/dL)} = \frac{\text{(OD of test sample – OD of blank)}}{\text{(OD of standard – OD of blank)}} \times 200$$

5.2.3.4 Measurement of HDL-C

a. Principle

The method of Izzo *et al.,* (1981) was followed to determine the level of HDL-C in plasma and liver homogenates of the rats. Precipitation of the apo-B containing lipo-proteins (chylomicrons, VLDL and LDL) using polyethylene glycol and centrifugation let HDL-C free for measurement whose concentration is measured spectrophotometrically.

b. Reagents

A. Precipitating reagents: Glycine buffer pH 10.2, polyethylenge glycol (10 g/dL)

B. HDL-C standard: 50 mg/dL.

c. Procedure

Chylomicrons were precipitated, VLDL and LDL adding precipitating reagent (0.3 ml precipitating solution added to 0.3 mL of sample), mixing thoroughly and centrifuging at 4000 rpm for 10 minutes. We collected the supernatant and to 100 µL, 500 µL of HDL-C working solution added. Mixing them well we incubated for 5 minutes and then absorbance read at 500 nm.

d. Calculation

$$\text{HDL-C concentration (mg/dL)} = \frac{\text{(OD of test sample – OD of blank)}}{\text{(OD of standard – OD of blank)}} \times 200$$

5.2.3.5 Measurement of VLDL and LDL

The levels of VLDL and LDL were measured using the Friedewald equation (Friedewald *et al.,* 1972). Calculations were performed as follows

$$\text{VLDL-C (mg/dL)} = \text{TG}/5$$

$$\text{LDL-C (mg/dL)} = \text{TC} - [(\text{TG}/5) + \text{HDL-C}]$$

5.2.3.6 Measurement of atherogenic index (AI) and LDL/HDL ratio

Atherogenic index of plasma (AIP) was measured following the method of Harnafi *et al.* (2008), and the formula used was

$$\text{AIP} = (\text{TC} - \text{HDL-C}) / \text{HDL-C}.$$

We calculated the ratio of plasma LDL-C to HDL-C for measuring the LDL/HDL ratio.

5.2.4 Organ function tests (Cardiac, Liver and Kidney Function Tests)

5.2.4.1 Measurement of Aspartate aminotransferase/Glutamate oxalate transaminase (AST/GOT) activity

a. Principle

Based on the method of Reitman and Frankel (1957), the activity of AST was measured using AST assay kit (Sigma-Aldrich, MAK 055) . AST reversibly transfers an amino group from L-aspartate (an α amino acid) to 2-oxoglutarate/ α ketoglutarate (an α keto acid) and forms oxaloacetate (an α keto acid) and glutamate (an α amino acid).

L-Aspartate + α ketoglutarate -----AST----------Oxaloacetate + Glutamate

Generation of gluatamate is proportional to the activity of AST and is expressed as U/L (1 unit of AST is defined the amount of enzyme that generates 1.0 µmole of glutamate per minute at pH 8.0 at 37 °C). Rate of conversion was measured at 450 nm.

b. Procedure:

Glutamate standard curve was prepared using 10 µL of glutamate standards (concentration 0, 2, 4, 6, 8 and 10 nmole/well) in a 96-well ELISA plate. In the other wells, 10 µL of sample to the 100 µL of the AST master mix in the ELISA plate well, mixed thoroughly and took initial absorbance ($A450_{initial)}$ within 2 minutes ($T_{initial}$) at 37 °C. At every 5 minutes, we took the absorbance (A_{450}) until value of the sample surpassed the highest value of the standard (10 nmol / well). Penultimate value before that of the highest (nearest or crossing the most concentrated standard) corresponds to the final absorbance ($A450_{final)}$ and the corresponding penultimate time is the final time (T_{final}). It was observed whether both the $A450_{initial}$ and $A450_{final}$ remain within the range of the standard curve.

c. Calculation

Background values were corrected by subtracting the value obtained for 0 concetration of glutamate from each of the $A450_{final}$ of standard and sample value.

Change in measurement from $T_{initial}$ to T_{final} was calculated as follows:

$$\Delta A_{450} = A450_{final} - A450_{initial}$$

Comparing the ΔA_{450} of each sample with the standard curve, the amount of glutamate (nmole) produced was determined.

AST activity was calculated following the formula:

AST activity = (B × Dilution factor) / (T × V)

Where,

B = Amount of glutamate (nmole) produced

$T = T_{final} - T_{initial}$

V = Sample volume (mL) used.

5.2.4.2 Measurement of Alanine aminotransferase/Glutamate pyruvate transaminase (ALT/GPT) activity

a. Principle

Following the method of Reitman and Frankel (1957), the activity of ALT/GPT was measured using the kit purchased from Sigma-Aldrich (MAK 052) . Normally, ALT transfers an amino group from alanine to α ketoglutarate and forms oxaloacetate and pyruvate.

L-Alanine + α ketoglutarate -----ATL----------Oxaloacetate + Pyruvate

Generation of pyruvate is proportional to the activity of AST and is expressed as U/L (1 unit of ALT is defined as the amount of enzyme that generates 1.0 µmole of pyruvate per minute at 37 °C). Rate of conversion was measured at 570 nm.

b. Procedure

Pyruvate standard curve was prepared using 10 µL of pyruvate standards (concentration 0, 0.2, 0.4, 0.6, 0.8 and 1 nmole/well) in a 96-well ELISA plate. In the other wells, 10 µL of sample to the 100 µL of the ALT master mix in the ELISA plate well, mixed thoroughly and took initial absorbance ($A570_{initial)}$ within 2 minutes ($T_{initial}$) at 37 °C. At every 5 minutes, we took the absorbance (A_{570}) until value of the

sample surpassed the highest value of the standard (10 nmol/well). Penultimate value before that of the highest (nearest or crossing the most concentrated standard) corresponds to the final absorbance ($A570_{final)}$ and the corresponding penultimate time is the final time (T_{final}). It was observed whether both the $A570_{initial}$ and $A570_{final}$ remain within the range of the standard curve.

c. Calculation

Background values were corrected by subtracting the value obtained for 0 concetration of glutamate from each of the $A570_{final}$ of standard and sample value.

Change in measurement from $T_{initial}$ to T_{final} was calculated as follows:

$$\Delta A_{570} = A570_{final} - A570_{initial}$$

Comparing the ΔA_{570} of each sample with the standard curve, the amount of pyruvate (nmole) produced was determined.

ALT activity was calculated following the formula:

ALT activity = (B × Dilution factor) / (T × V)

Where,

B = Amount of pyruvate (nmole) produced

$T = T_{final} - T_{initial}$

V = Sample volume (mL) used.

5.2.4.3 Measurement of Alkaline phosphatase (ALP) activity

a. Principle

Following the principle that in alkaline medium, ALP hydrolyzes the organic monophosphate esters into phenol/alcohol and inorganic phosphate, the ALP activity was measured using the assay kit (Abcam, ab83369) (Tietz *et al.,* 1983). In the applied experimental condition, ALP dephosphoralizes p-nitrophenyl phosphate (pNPP) into p-nitrophenol and phosphate at pH 10. Intensity of the yellow colored p-nitrophenol is proportional to the ALP activity at 405 nm.

p-nitrophenyl phosphate (pNPP)----------ALP------- p-nitrophenol + phosphate

b. Procedure

20 µL of sample and/or standards were mixed with 20 µL of 0.67M pNPP and 960 µL of reaction buffer. Adding 20 µL of ALP, we mixed the contents well and read the increase in absorbance of the standard and the samples against a reagent blank at 405 nm.

c. Calculation

$$\text{ALP activity (U/L)} = \frac{(\text{OD of test sample} - \text{OD of blank})}{(\text{OD of standard} - \text{OD of blank})} \times \text{Dilution factor}$$

5.2.4.4 Measurement of serum total bilirubin

a. Principle

Following the modified method of Mori (1978), the level of total bilirubin in the rat serum serum was measured. According to the principle, total bilirubin reacts with diazotized sulfanilic acid in presence of caffeine to produce a colored complex

whose intensity at 530 nm is directly proportional to the bilirubin present in the sample.

Bilirubin + Diazotized sulfanilic acid ------ Azobilirubin

b. Procedure

At first, water/blank (250 µL) and reconstituted calibrator (200 µL water + 50 µL calibrator) was used in the ELISA plate wells. Then, in the separate wells, 50 µL of each of the samples were added with 200 µL of the working reagent and mixed well. Keeping the plate at 37°C for 10 minutes, absorbance was read at 530 nm.

c. Calculation

$$\text{Bilirubin concentration (mg/dL)} = \frac{(\text{OD of test sample} - \text{OD of blank})}{(\text{OD of calibrator} - \text{OD of blank})} \times 5$$

Where, 5 mg/dL = equivalent bilirubin concentration of the calibrator.

5.2.4.5 Measurement of serum creatinine level

a. Principle

Based on the principle of Jaffe reaction, the level of rat serum creatinine level was measured (Bonsnes & Taussky, 1945; Schirmeister *et al.,* 1964). A coupled enzymatic reaction converts creatinine into creatine which in presence of alkaline picrate solution forms a yellow/orange colored complex and intensity of the complex at 570 nm is proportional to the creatinine present.

b. Procedure

In ELISA plate well, creatinine standards of 0, 0.2, 0.4, 0.6, 0.8 and 1.0 nmole/well were prepared (total volume 50 µL/well, using the creatinine assay buffer). Aliquot of sample (2-50 µL) were added in the respective wells and total volume made up to 50 µL/well using the creatinine assay buffer. Then, 50 µL of the reaction master mix (44/42 µL creatinine assay buffer + 2 µL creatininase + 2 µL creatinase + 2 µL creatinine enzyme mix + 2 µL creatinine probe) were added to each well. After mixing thoroughly and keeping the plate at 37°C for 10 minutes, absorbance was read at 530 nm.

c. Calculation

$$\text{Creatinine concentration (mg/dL)} = \frac{\text{(OD of test sample – OD of blank)}}{\text{(OD of standard – OD of blank)}}$$

Creatinine concentration (mg/dL) = Sa / Sv

Where, Sa = Amount of Creatinine in unknown sample (nmole) from standard curve

Sv = Sample volume (µL).

5.2.4.6 Measurement of blood urea level

a. Principle

Based on the principle of Jung *et al.* (1975), the level of rat blood urea was measured. A coupled enzymatic reaction converts urea into a colored complex whose intensity at 570 nm is proportional to the urea present.

b. Procedure

In ELISA plate well, urea standards of 1, 2, 3, 4 and 5 nmole/well were prepared (total volume 50 µL/well, using the urea assay buffer). Aliquot of sample (2-50 µL) were added in the respective wells and total volume made up to 50 µL/well using the urea assay buffer. Then, 50 µL of the reaction master mix (44/42 µL urea assay buffer + 2 µL peroxidase substrate + 2 µL developers + 2 µL enzyme mixes + 2 µL convertase) were added to each well. After mixing thoroughly, we kept the plate at 37 °C for 60 minutes and read absorbance at 570 nm.

c. Calculation

$$\text{Urea concentration (mg/dL)} = \frac{\text{(OD of test sample – OD of blank)}}{\text{(OD of standard – OD of blank)}}$$

Urea concentration (mg/dL) = Sa / Sv

Where, Sa = Amount of Creatinine in unknown sample (nmole) from standard curve

Sv = Sample volume (µL).

5.2.4.7 Measurement of Protein concentration

a. Principle

Based on the principle of the biuret reaction that in alkaline media, proteins (actually the amino acids cysteine, cystine, tyrosine and tryptophan) reduce Cu^{2+} into Cu^{+}, total protein concentration in the samples was estimated using the Pierce BCA protein assay kit (Thermo scientific, 23225). Bicinchoninic acid present in the sample chelates Cu^{+1} and produces purple colored product whose absorbance at 562 nm is proportional to the protein concentration present in the sample.

b. Materials

Pierce BCA protein assay kit, BCA working reagent, bovine serum albumin (BSA) as the standard.

c. Procedure

BSA standards were prepared maintaining the concentration range of 20-2000 µg/mL and working reagent with mixing BCA reagents A and B in the ratio of 50:1. 2 mL of working reagent and 100 µL each of the sample and/or standard were added, mixed well and incubated at 37 °C for 30 minutes. Then, absorbance was measured at 562 nm.

5.2.5 Inhibition of HMG Co-A reductase (HMGR) activity

a. Principle

The *in vitro* inhibitory effect of the HWE of *G. lucidum* was measured following the methods of Gholamhoseinian *et al* (2010). Commercially available HMGR assay kit was utilized in the experimentation (Sigma-Aldrich, CS 1090, St. Louis, USA). HMGR catalyzes the conversion of HMG Co-A into mevalonate and Co-ASH with concomitant oxidation of NADPH into $NADP^+$ and decrease in absorption at 340 nm. Spectrophotometric measurement of absorbance related to the inhibition of the *G. lucidum* HWE up on HMGR activity against the pravastatin.

HMG Co-A + 2 NADPH + H^+ -------HMGR----- Mevalonate + CoA-SH + 2$NADP^+$

b. Procedure

Following the manufacturer's recommendation, in the 96-well ELISA plate well, mixing of 181 µL assay buffer, 1 µL *G. lucidum* HWE/pravastatin, 4 µL NADPH, 12 µL HMG Co-A and finally 2 µL HMGR were performed. The plates were mixed gently

and subjected to absorbance measurement (Biotek H1 multimode plate reader associated with Gen 5 software) at every 20 seconds for a period of 20 minutes. Percentage inhibition of the HMGR activity was calculated following the equation below:

c. Calculation

$$\text{Inhibition of HMGR activity (\%)} = (A0\text{-}As) \times 100/A0$$

Where, A0 is the absorbance of the control, and As is the absorbance of the reaction mixture containing the extract.

5.3 Results

5.3.1 Effect on enzymatic anti-oxidative defense

As shown in Tables 5.1 and 5.2, feeding of *G. lucidum* HWE increased enzymatic anti-oxidative defense of the rats (Raw data: Appendix A, Tables A - 4.10 – 4.47 ; Figures A - 4.1, 4.2 and 4.3; Statistical data: Appendix C, Tables C - 5.1-5.4) .

Table 5.1: Plasma anti-oxidant enzyme levels

Plasma anti-oxidant enzyme level (nmol/mL)	C	CE	H	HE	A	AE
Catalase (CAT)	9.83 ± 0.10^{a}	14.66 ± 0.30^{b}	7.34 ± 0.30^{c,e}	11.78 ± 0.23^{d}	7.33 ± 0.20^{c,e}	10.57 ± 0.15^{f}
Glutathione peroxidase (GPX)	29.53 ± 0.70^{a}	42.23 ± 0.22^{a}	26.03 ± 0.5^{c,e}	32.37 ± 0.34^{d,f}	25.40 ± 0.50^{c,e}	31.20 ± 0.30^{d,f}
Superoxide dismutase (SOD)	59.96 ± 0.80^{a}	67.42 ± 0.42^{b}	48.20 ± 0.6^{c,f}	50.27 ± 0.40^{d,f}	44.80 ± 0.95^{a}	49.42 ± 0.75^{c,d,f}

Data are expressed as mean±SE. Mean values with different lower case superscripts are indicators of statistically significant difference at P≤0.05 level with one-way ANOVA and post-hoc Tukey's HSD test (n=3). Where, C stands for the control group; CE for the *G. lucidum* HWE fed control group; H for the hypercholesterolemic group; HE for *G. lucidum* HWE fed H group; A for the AD group and AE for the *G. lucidum* HWE fed AD group.

Table 5.2: Liver anti-oxidant enzyme levels

Liver anti-oxidant enzyme level (nmol/mL)	C	CE	H	HE	A	AE
Catalase (CAT)	59.64 ± 0.53^{a}	67.88 ± 0.37^{b}	46.55 ± 0.52^{c,e}	52.4 ± 0.54^{d}	46.81 ± 0.45^{c,e}	53.89 ± 0.47^{f}
Glutathione peroxidase (GPX)	55.80 ± 0.32^{a}	59.93 ± 0.12^{b}	49.26 ± 0.68^{c}	54.15 ± 0.37^{d,f}	50.93 ± 0.65^{e}	53.07 ± 0.28^{d,f}
Superoxide dismutase (SOD)	85.46 ± 0.53^{a}	90.55 ± 0.16^{b}	78.92 ± 1.19^{c,e}	82.34 ± 0.54^{d,f}	77.73 ± 0.96^{c,e}	80.80 ± 0.42^{d,f}

Data are expressed as mean±SE. Mean values with different lower case superscripts are indicators of statistically significant difference at P≤0.05 level with one-way ANOVA and post-hoc Tukey's HSD test (n=3). Where, MD stands for methanol:dichloromethane; HEX for hexane; DCM for dichloromethane, EA for ethylacetate, AQ for aqueous and HWE for hot water extract.

5.3.2 Hypocholesterolemic effect of *G. lucidum* HWE

5.3.2.1 Effect of *G. lucidum* HWE on body weight change

During the eight week long observation, gradual increase in body weight (ranging from 140 ± 5 g up to 280 ±2 g) of the rats of all the groups was noticed (Figure 5.1) (Raw data: Appendix A, Table A 5.1; Statistical data: Appendix C, Tables C - 6.1 and 6.2). However, there was distinct body weight change among different groups. The hypercholesterolemic rats (H) gained the maximum weight and their rate of becoming weighty surpassed that of the others.

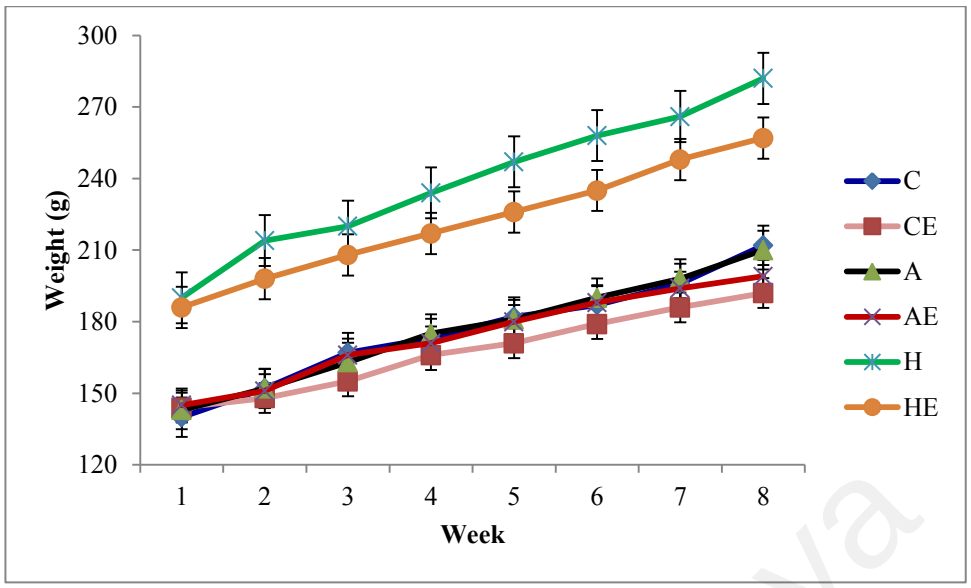


Figure 5.1: Effect of *G. lucidum* HWE upon body weight change of the rats.
Data are expressed as mean±SE (n=3). Data were analyzed with one-way ANOVA and post-hoc Tukey's HSD test (P≤0.05). Where, C=Control, CE= *G. lucidum* HWE fed control, H=hypercholesterolemic, HE=*G. lucidum* HWE fed hypercholesterolemic, A=AD model rats and AE= *G. lucidum* HWE fed AD rats, respectively.

Weight gain tendency of the normo-cholesterolemic rats was moderate and feeding of *G. lucidum* HWE resulted in decreased weight gain as time passed by. Similarly, *G. lucidum* HWE fed AD rats experienced relatively lower body weight growth than their non-fed counterparts. These findings indicate towards body weight lowering potencies of the *G. lucidum* HWE up on the experimental animals.

5.3.2.2 Effect on plasma TG level

Feeding of hypercholesterolemic diet to the rats resulted in their increased plasma TG levels (Table 5.3) (Raw data: Appendix A, Table A - 5.2; Statistical data: Appendix C, Tables C - 6.3 and 6.4) . The hypercholesterolemic (H) rats had 1.58 times higher plasma TG level compared to the controlled (C) rats indicating the increased atherogenic propensity of the H rats. Feeding of *G. lucidum* HWE lowered plasma TG level in both the control, hypercholesterolemic and AD rats. Plasma TG lowering effect of the *G. lucidum* HWE was in the order of controlled (15%) > hypercholesterolemic

(13%) > AD (5%) rats (Table 5.3). TG lowering effect of *G. lucidum* HWE was statistically significant in each group compared to their respective controls (Table 5.3).

Table 5.3 Lipid profile, plasma atherogenic index and LDL/HDL ratio

Plasma parameter (mg/dL)	C	CE	H	HE	A	AE
TG	122.73 ± 0.63^{a}	103.93 ± 0.85^{b}	192.0 ± 0.880^{c}	167.33 ± 0.90^{d}	140.93 ± 0.70^{e}	133.33 ± 0.52^{f}
TC	90.6 ± 0.74^{a}	81.9 ± 0.91^{b}	135.2 ± 0.84^{c}	109.4 ± 1.0^{d}	114.67 ± 0.85^{e}	101.87 ± 0.56^{f}
HDL – C	29.06 ± 0.42^{a}	36.47 ± 0.55^{b}	26.94 ± 0.58^{c,e}	33.33 ± 0.40^{d,f}	26.73 ± 0.40^{c,e}	32.4 ± 1.35^{d,f}
LDL – C	36.97 ± 0.58^{a}	24.68 ± 0.10^{b}	69.86 ± 0.90^{c}	42.60 ± 1.34^{d,f}	59.75 ± 0.91^{e}	42.8 ± 0.57^{d,f}
VLDL – C	24.54 ± 0.13^{a}	20.78 ± 0.17^{b}	38.40 ± 0.18^{c}	33.47 ± 0.18^{d}	28.19 ± 0.14^{e}	26.67 ± 0.10^{f}
AI	2.12 ± 0.04^{a,d}	1.25 ± 0.04^{b}	4.05 ± 0.10^{c}	2.29 ± 0.05^{a,d,e}	2.4 ± 0.05^{d,e}	1.54 ± 0.05^{f}
LDL/HDL	1.28 ± 0.02^{a,d,f}	0.68 ± 0.04^{b}	2.6 ± 0.08^{c}	1.29 ± 0.05^{a,d,f}	2.25 ± 0.06^{e}	1.32 ± 0.027^{a,d,f}

Where, C=Control, CE= G. lucidum HWE fed control, H=hypercholesterolemic, HE=*G. lucidum* HWE fed hypercholesterolemic, A=AD model rats and AE= *G. lucidum* HWE fed AD rats, respectively. Data are expressed as mean±SE. Mean values containing different lower case superscripts are statistically significant at P≤0.05 level with one-way ANOVA and post-hoc Tukey's HSD test (n=3).

5.3.2.3 Effect on plasma TC level

Feeding of hypercholesterolemic diet to the rats resulted in 1.5 times increased plasma TC levels (Table 5.3) (Raw data: Appendix A, Table A 5.3; Statistical data: Appendix C, Tables C - 6.3 and 6.4) . Later on, treating the rats with *G. lucidum* HWE lowered plasma TC level significantly in all the rat groups (Table 5.3). Unlike that of TG lowering pattern, plasma TC lowering effect *G. lucidum* HWE on the hypercholesterolemic rats was higher than those of the normo-cholesterolemics and the pattern was H (19.08%) > A (11.16%) > C (9.60%) rats (Table 5.3).

5.3.2.4 Effect on plasma HDL-C level

In the present study, the hypercholesterolemic and AD rats had lowered plasma HDL levels compared to the normo-cholesterolemic controls (Table 5.3) (Raw data: Appendix A, Table A 5.4; Statistical data: Appendix C, Tables C - 6.3 and 6.4) . Feeding of *G. lucidum* HWE increased plasma HDL-C levels significantly ($P \leq 0.05$) in all the rat groups (Table 5.3). However, the rate of increasing was highest in the normocholesterolemics and the increasing trend was C (25.5%) > H (23.80%) > A (21.20%) rats (Table 5.3).

5.3.2.5 Effect on plasma LDL-C and VLDL-C level

Plasma LDL-C level increased 1.89 times in the hypercholesterolemic and 1.62 times in the AD rats, compared to the controlled ones (Table 5.3) (Raw data: Appendix A, Tables A - 5.5 and 5.6; Statistical data: Appendix C, Tables C - 6.3 and 6.4) . Similarly, plasma VLDL-C level increased by 1.56 times in the H and by 1.15 times in the A rats (Table 5.3).

5.3.2.6 Effect on atherogenic index (AI) and LDL/HDL ratio

As depicted in Table 5.3 (Raw data: Appendix A, Tables A - 5.7 and 5.8; Statistical data: Appendix C, Tables C - 6.3 and 6.4) , the hypercholesterolemic rats had increased atherogenic index (AI) compared to their normo-cholesterolemic counterparts. However, the *G. lucidum* HWE fed rats had lowered atherogenic index of plasma (AIP). On the other hand, the hypercholesterolemic and the AD rats showed higher LDL/HDL ratio compared to the *G. lucidum* HWE fed rats (Table 5.3).

5.3.2.7 Inhibition of HMG Co-A reductase (HMGR) activity

As shown in the Figure 5.2, the HEX and the HWE of *G. lucidum* showed the most potent inhibitory effect towards HMGR activity. HMGR inhibitory effect of both of the fractions was very close to each other (52.12 for HWE and 53.3 for HEX) and

statistically not significant ($p \leq 0.05$) (Raw data: Appendix A, Tables A - 6.1-6.4; Statistical data: Appendix C, Tables C - 6.5 and 6.6).

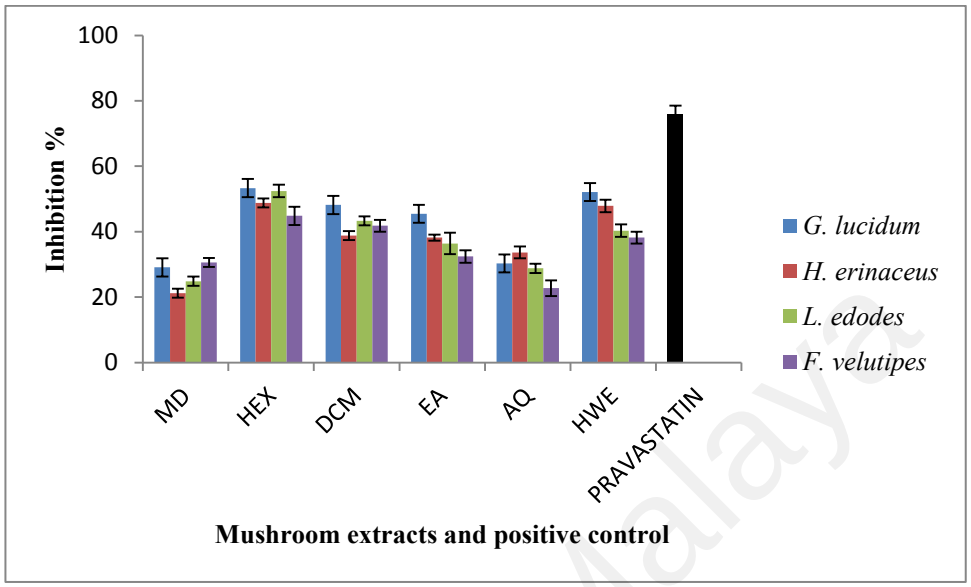


Figure 5.2 HMG Co-A reductase inhibitory effect of mushrooms

Where, C=Control, CE= *G. lucidum* HWE fed control, H=hypercholesterolemic, HE=*G. lucidum* HWE fed hypercholesterolemic, A=AD model rats and AE= *G. lucidum* HWE fed AD rats, respectively. Data are expressed as mean±SE. Bar charts with different lower case superscripts are statistically significant at P≤0.05 level with one-way ANOVA and post-hoc Tukey's HSD test (n=3).

5.3.3 Organ function tests (Cardiac, liver and kidney function tests)

Feeding of *G. lucidum* HWE at the dosage of 200 mg/kbw did cause neither any visible adverse effect nor death of the experimental animals. Rather, the rats' body weight increased and their demand for intaking *G. lucidum* HWE increased as they grew up. On the other hand, significantly ($P \leq 0.05$) increased levels of serum AST/GOT, ALT/GPT and ALP enzymes in the hypercholesterolemic and AD rats compared with their controlled counterparts were observed (Table 5.4) (Raw data: Appendix A, Tables A - 7.1-7.8, Figures A - 4.1-4.3 and 5.1; Statistical data: Appendix C, Tables C - 6.7 and 6.8).

Table 5.4 *Ganoderma lucidum* HWE fed rats' organ function tests

Organ function tests	C	CE	H	HE	A	AE
SGOT/AST (U/L)	50.8 ± 0.42[a]	52.33 ± 0.44[b]	74.67 ± 0.73[c]	63.33 ± 0.40[d,f]	69.4 ± 0.55[e]	64.13 ± 0.58[d,f]
SGPT/ALT (U/L)	19.93 ± 0.50[a]	21.66 ± 0.37[b]	28.67 ± 0.5[c,e]	22.87 ± 0.276[d]	29.6 ± 0.36[c,e]	24.8 ± 0.38[f]
ALP (U/L)	65.73 ± 0.63[a]	60.06 ± 0.52[b]	106.07 ± 0.51[c]	94.06 ± 0.61[d]	87.73 ± 0.54[e]	81.33 ± 0.43[f]
Bilirubin (mg/dL)	0.21 ± 0.006[a,b]	0.22 ± 0.005[a,b]	0.53 ± 0.01[c]	0.33 ± 0.005[d]	0.48 ± 0.008[e]	0.40 ± 0.008[f]
Creatinine (mg/dL)	0.25 ± 0.005[a,b]	0.27 ± 0.023[a,b]	0.66 ± 0.01[c]	0.53 ± 0.01[d]	0.5 ± 0.007[e]	0.35 ± 0.007[f]
Urea (mg/dL)	15.80 ± 0.48[a,b,f]	15.67 ± 0.31[a,b]	24.80 ± 0.44[c]	19.87 ± 0.73[d,e,f]	20.67 ± 0.30[d,e,f]	17.53 ± 0.33[a,d,e,f]
LPO (nmol/mg protein)	1.33 ± 0.044[a,b]	1.42 ± 0.05[a,b]	2.67 ± 0.04[c]	1.85 ± 0.06[d]	2.46 ± 0.009[e]	2.27 ± 0.02[f]

Where, C=Control, CE= *G. lucidum* HWE fed control, H=hypercholesterolemic, HE=*G. lucidum* HWE fed hypercholesterolemic, A=AD model rats and AE= *G. lucidum* HWE fed AD rats, respectively. Data are expressed as mean±SE. Mean values containing different lower case superscripts are statistically significant at P≤0.05 level with one-way ANOVA and post-hoc Tukey's HSD test (n=3).

5.4 Discussion

5.4.1 Effect on enzymatic anti-oxidative defense

Compared to the controlled rats, the hypercholesterolemics had lowered levels of plasma and liver anti-oxidant enzymatic (CAT, GPX and SOD) activities (Tables 5.1 and 5.2). Lowered levels of the anti-oxidative enzymes indicate deleterious effects of hypercholesterolemia such as increased OS generation and loss of cell membrane function and integrity. Hypercholesterolemia led to increased OS generation and associated reduced anti-oxidative defense had been noticed by multiple studies. This is due to increased lipid peroxidation, increased LDL oxidation in the arterial wall and decreased anti-oxidative enzymatic activity (Stocker & Keaney, 2004). Importantly, elevated cholesterol can stimulate superoxide production and inhibit nitric oxide generation (Landmesser *et al.,* 1999). Feeding of *G. lucidum* HWE increased enzymatic anti-oxidative defense of the rats as shown in Tables 5.1 and 5.2. Current findings are also in compatible with those of Wang *et al.* (2004) who showed that addition of 0.3%,

0.6% and 1.8% of *G. lucidum* powder with the basal diet significantly increased the levels of anti-oxidative enzymes (glutathione reductase, GPX and SOD) in different organs of the mice. Like the present *in vitro* (chapter 3) and *in vivo* anti-oxidative tests, Hasnat *et al* (2013) also reported the *in vitro* and *in vivo* anti – oxidative defense uplifting effect of the aqueous extract of *G. lucidum* grown on germinated brown rice.

Administration of *Ganoderma* tri-terpenoids and polysaccharide (PSG-1) to animals had been found to increase blood and liver CAT, GPX and SOD activity (Jang *et al.,* 2014; Lakshmi *et al.,* 2006; Smina *et al.,* 2011; Sudheesh *et al.,* 2012; Zhu *et al.,* 2016). Actually the anti-oxidative enzymes (CAT, GPX and SOD) participate in mutual anti-oxidative defense of the organisms. In the mitochondria of the eukaryotes, MnSOD is the first anti-oxidative enzyme that lowers the steady state of superoxide (O_2^-). GPX acts mainly in the liver and reduce the hydroperoxides. Polyphenolics, tri-terpenes and polysaccharide present in the *G. lucidum* HWE might be involved in providing anti-oxidative defenses (Smina *et al.,* 2011; Zhu *et al.,* 2016).

5.4.2 Hypocholesterolemic effect of G. lucidum HWE

5.4.2.1 Effect on body weight change

In the present study, *G. lucidum* HWE fed rats experienced lowered rate of body weight (Figure 5.1). Previous studies concerning effect of food intake on the animal body weight had presented conflicting results (Harnafi *et al.,* 2009; Ramachandran *et al.,* 2003). Supposedly, hypercholesterolemic diet had hindering effect upon intestinal absorption of proteins and other nutrients whose net effect was negative weight balance in those findings. This may be the cause why Wang *et al*. (2004) had not noticed change in body weight of the mice whose basal diet was supplanted with 0.3%, 0.6% and 1.8% of *G. lucidum* powder. Current findings are in agreement with several other findings

who reported a gradual increased body weight in the cholesterol fed rats (Amin *et al.,* 2011; Nwozo *et al.,* 2011; Otunola *et al.,* 2010).

Body weight change of the experimental animals occurs in two successive stages that overlap with each other to some extent. Firstly, during the maturing period, all the body parts (head, trunk, tail, internal organs) grow and gain relative weight. Secondly, during post-maturation, the organs do not gain relative weight so much and increased body fat corresponds towards increased body weight. As the present experimental animals were almost matured from their beginning of the experiment, decreased body weight gain of the *G. lucidum* HWE fed rats might occur due to decreased fat (triacylglycerol) deposition, decreased cholesterol biosynthesis or increased lipolysis and/or the combined effect of all.

5.4.2.2 Effect on plasma TG level

TG regulates lipoprotein interactions and thus plays important role in maintaining lipid homeostasis. Its own level also varies depending on the lipoproteins especially LDL-C. Increased TG levels in the hypercholesterolemic rats might be due to the decreased clearance of TG owing to the lowered lipoprotein lipase (LPL) activity or due to increased deposition of LDL-C (Nofer *et al.,* 2002) (Table 5.3). Previously, Kabir and Kimura (1988) reported the TG lowering effect in the 5% dried *G. lucidum* fed spontaneously hypertensive rats. Human intervention studies supplemented with *G. lucidum* had also documented its TG lowering effect (Chu *et al.,* 2012; Wachtel-Galor *et al.,* 2004).

TG lowering effect of the HWE *G. lucidum* might be mediated by different strategies. First of all, decreased cholesterol biosynthesis through increased inhibition of the HMGR might be involved. Secondly, the phenolic content of the HWE of *G. lucidum* might form complexes with micelles and impair further metabolism (Bose *et*

al., 2008). Thirdly, the phenolics might inhibit pancreatic lipase activity resulting in lowered TG biosynthesis or stimulate the plasma lipoprotein lipase that restored the catabolism of TG (Ikeda *et al.,* 2005). Overall, the HWE of *G. lucidum* could withstand plasma elevation of TG level and could aid in guarding against hypercholesterolemia and atherogenic propensity.

5.4.2.3 Effect on plasma TC level

Significant reduction ($P \leq 0.05$) in TC levels of the *G. lucidum* HWE-treated rats were observed in the present study (Table 5.3). The reason for increased rate of TC lowering in the hypercholesterolemic rats compared to those of the normo-cholesterolemics might be that the basal rate of cholesterol metabolism remained unaffected with the mushroom dosage applied during the experimental time-course (Hossain *et al.,* 2003). Controlled human intervention studies had also documented such effect (Chu *et al.,* 2012; Wachtel-Galor *et al.,* 2004).

TC lowering effect of *G. lucidum* might be mediated by several mechanisms. Importantly, the ganoderic acids share the structural features of the active site of HMG Co-A and might bind in the active site of HMGR that contributed its competitive inhibition of HMGR and lowered cholesterol biosynthesis. Besides, ganoderic acids might inhibit 14α demethylase and inhibit the conversion of lanosterol to lathosterol and thus inhibit the last step of cholesterol biosynthesis (Hajjaj *et al.,* 2005; Li *et al.,* 2006).

Also, gano-polyphenolics might bind with bile acids and lower solubility of cholesterol in micelles (Ngamukote *et al.,* 2011). Gano-polyphenolics might also inhibit the activity of pancreatic cholesterol esterase and thus reduced intestinal cholesterol absorption (Heidrich *et al.,* 2004; Ngamukote *et al.,* 2011). In addition, β-D glucan present in the *G. lucidum* HWE might also form viscous gel and impair the formation of micelles that would lower intestinal cholesterol absorption (Cheung, 2013). Similarly,

chitin and chitosan might also aid in this concert (Neyrinck *et al.,* 2009). Moreover, increased fecal excretion of cholesterol in the form of bile acids might also be involved in the TC lowering performance of *G. lucidum*. In a similar study, Berger *et al* (2004) reported that feeding of the dried powder of *G. lucidum* to the hamsters at 2.5 % dosage, increase fecal total bile acids, chenodeoxycholate and corpostanol-3-one.

5.4.2.4 Effect on plasma HDL-C level

Hyperlipidemia is associated with decreased HDL level and increased susceptibility towards OS (Hansel *et al.,* 2004). Lowered level of HDL-C in the hypercholesterolemic rats of the present study might be due to the accelerated clearance of apo A1 from the plasma following hypercholesterolemia in the H and A rats (Baba *et al.,* 2007; Sorci-Thomas *et al.,* 1989). On the other hand, significantly increased ($P \leq 0.05$) plasma HDL-C level in the *G. lucidum* HWE fed rats indicates increased clearance of TC from the peripheral tissue to the liver for excretion that points towards CVD ameliorating effect of *G. lucidum* HWE. Among multiple strategies, decreased biosynthesis and increased catabolism of TG and TC might result in increased plasma HDL-C level of the rats.

5.4.2.5 Effect on plasma LDL-C and VLDL-C

Increased levels of LDL-C and VLDL-C in the hypercholesterolemic rats might arise from their intake of hypercholesterolemic diet that might have downregulated the hepatic LDL receptors of the H rats (Roach *et al.,* 1993). *Ganoderma lucidum* HWE supplementation caused significant lowering effect upon plasma level of both LDL-C and VLDL-C in all the rat groups (Table 5.3). LDL-C lowering effect was highest for the hypercholesterolemic rats (39.02%), moderate in the controlled (33.27%) and lowest in the AD rats (28.36%) (Table 5.3). *Ganoderma lucidum* HWE caused significantly lowering effect upon the plasma LDL-C level of both H and AD rats as compared to the controlled (Table 5.3). *Ganoderma lucidum* HWE mediated decreased cholesterol

absorption and biosynthesis might cause decreased availability of hepatic cholesterol for lipo-protein biosynthesis in the extract fed rats (CE, HE, AE). As a consequence, decreased VLDL secretion in the plasma along with decreased conversion of VLDL into LDL may end into lowered plasma LDL level (Burnett *et al.,* 1997, 1999). Mechanistically, *G. lucidum* HWE induced increased LDL receptor in the rat hepatocytes may contribute to the LDL lowering effect that resulted in lowered secretion of LDL in the rat plasma (Bursill *et al.,* 2007). Increased clearance of LDL from the blood of the mushroom-fed rats may also have been involved (Burnett *et al.,* 1999). In case of *P. ostreatus* fed hypercholesterolemic rats, increased plasma clearance of VLDL had been noticed (Bobek & Ozdin, 1993). Feeding of *G. lucidum* HWE might have increased the fractional turnover of VLDL-C and lipoprotein lipase activity that resulted in decreased VLDL-C and TG level in the mushroom fed rats (Bobek *et al.,* 1993; Bobek & Ozdin, 1993; Kimball *et al.,* 1983).

5.4.2.6 Effect on AI and LDL/HDL ratio

The increased atherogenic index (AI) of plasma of the hypercholesterolemic rats observed in the present study (Table 5.3) might be emanated from the increased level of the atherogenic lipoprotein fractions (LDL-C) associated with hyperlipidemia (Dobiasova, 2006; Dobiášová & Frohlich, 2001). Like other hypolipidemic natural products, lowered AI values in the *G. lucidum* HWE fed rats also was expected (Acay *et al.,* 2014). Significantly lowered AI values in the *G. lucidum* HWE fed rats was observed in the present study (Table 5.3). Lowered AI in the *G. lucidum* HWE fed rats corresponds towards this mushroom's lipid lowering, anti-atherogenic, cardioprotective and AD ameliorating performance (Dobiasova, 2006; Dobiášová & Frohlich, 2001).

LDL/HDL ratio is another indicator of hyperlipidemia led physiological complications including atherosclerosis and AD (Granholm *et al.,* 2008). Higher HDL

level, lower LDL level and lowered LDL/HDL ratio is expected for the hyperlipidemia modulating agents (Granholm *et al.,* 2008). *Ganoderma lucidum* HWE feeding could lower the LDL/HDL ratio in every rat group (Table 5.3). These results further substantiate the hypolipidemic, hypocholesterolemic, anti-atherogenic and AD modulating potentiality of the *G. lucidum* HWE (Granholm *et al.,* 2008).

5.4.2.7 Inhibition of HMGR

All the mushroom fractions and extracts that underwent *in vitro* anti-oxidative assesments in chapter 3, also were analyzed for theing inhibitory effect towards HMGR. Compared to others, the hexane and the HWE of *G. lucidum* showed better inhibitory effects towards HMGR activity (Figure 5.2). As the presence of statin in the *G. lucidum* fractions had not been searched in the present study, it is difficult to convincingly indicate that natural statins were present in the respective fractions. However, ergosterol present in the hexane fraction might be regarded as the inhibitor of HMGR activity. Our proposition is supported by Gil-Ramirez *et al* (2013a, 2013b) who reported the HMGR inhibitory effects of edible mushrooms to be mediated by sterol (ergosterol) and polysaccharide (β-D glucan) (Gil-Ramírez *et al.,* 2013; Gil-Ramirez *et al.,* 2011; Gil-Ramírez *et al.,* 2013). Thus, contrary to statins, a novel *in vitro* inhibitory mechanism of HMGR activity might have been functional in case of *G. lucidum* fractions (Gil-Ramírez *et al.,* 2013; Gil-Ramírez *et al.,* 2013). Gano-components (especially ergosterol) might have scavenged the HMGR substrate, HMG Co-A and itself bound to the enzyme that resulted in lowered HMGR activity. Ergosterol peroxide from the edible mushroom *Sarcodon aspratus* had been found to down-regulate the expression of low-density lipoprotein receptor (LDLR) and HMG-CoA reductase genes in RAW264.7 cells (Kobori *et al.,* 2007). Feeding of 5% *G. lucidum* dried powder to the hamsters resulted in 1.5-fold decreased HMGR activity in the hepatic microsomes (Berger *et al.,* 2004). Berger *et al.* (2004) also identified sterols and triterpenes, rather

than statins in *G. lucidum* fractions, as inhibitors to the HMGR activity *ex vivo* (Berger *et al.,* 2004). Also, 26-oxyenosterols (ganoderols A, B, ganoderal A and ganoderic acid Y) derived from *G. lucidum* have been found inhibitory towards cholesterol biosynthesis in human hepatic cell lines T9A4 (Hajjaj *et al.,* 2005).

Ganoderic acids (7-oxo-ganoderic acid Z and 15-hydroxy-ganoderic acid S) isolated from the lipophilic fraction of *G. lucidum* had been found inhibitory towards HMGR activity (Li *et al.,* 2006) . Ganoderol A at 7 μM concentration, lowered cholesterol biosynthesis up to 40% in the T9A4 cell lines and the mechanism of inhibition was either through conversion of acetate and mevalonate as the cholesterol biosynthetic precursors or by inhibiting 14α demethylase in the conversion of lanosterol to lathosterol (Hajjaj *et al.,* 2005). As outlined in chapter 4, different ganoderic acids detected in the HWE of *G. lucidum* might also have contributed towards HMGR inhibitory effects (Li *et al.,* 2006).

5.4.3 Organ function tests

Compared with the control and the *G. lucidum* HWE fed rats, the AD and the hypercholesterolemic rats were detected with elevated levels of serum AST/GOT, ALT/GPT and ALP enzymes in the present study. Usually, these are the enzymes of diagnostic importance whose level rise in blood as they leak out of the damaged cell membranes following CVD complications. Thus, cholesterol affected the membrane integrity and permeability of the rats. In the similar fashion, significantly ($p \leq 0.05$) increased bilirubin, creatinine and urea level in the hypercholesterolemic rats also indicate the cholesterol-mediated perturbed metabolism of the respective animals (Table 5.4). Elevated bilirubin and creatinine levels in the hypercholesterolemic rats indicate their abnormal hepatic and renal functions, respectively (Muntner *et al.,* 2000). Besides, increased serum bilirubin remains as a individual risk factor of CVD (de Sauvage

Nolting *et al.,* 2011). Cholesterol-rich diet might cause excessive protein catabolism and amino acid deamination for gluconeogenesis that resulted in elevated urea level. A number of studies also reported high fat diet induced impaired organ function and lipid profile in the experimental animals (Frantz *et al.,* 2012; Saki *et al.,* 2011; Vaziri, 2003). ALT is much specific for hepatocellular injury than AST as ALT is predominantly present in the hepatic cytosol and AST is available in liver, heart and in kidney (Nikolaou *et al.,* 2012).

Hypercholesterolemia-induced oxidative stress leads towards hepatocyte membrane lipid peroxidation and disrupts the membrane integrity allowing the AST, ALT and ALP to be leaked into the serum (Arhan *et al.,* 2009). However, feeding of *G. lucidum* HWE had ameliorating effect upon both the enzymatic and non-enzymatic biomarkers (Table 5.4). These findings indicate the cellular membrane integrity maintaining and leakage restoring capability of the *G. lucidum.* Our findings are in agreement with those of Lakshmi *et al.,* (2006), who reported that *G. lucidum* was able in reverting the AST, ALT and ALP levels back to their normal levels in the benzo[a]pyrene-induced hepatic injured rats. Similar protective role of *G. lucidum* had been reported for the ethanol and carbon tetrachloride-injured hepatotoxicity (Ali *et al.,* 2014; El Shawi *et al.,* 2015; Jang *et al.,* 2014; Lin & Lin, 2006; Sudheesh *et al.,* 2012). Free-radical scavenging effect of *G. lucidum* extract had been suggested providing hepato-protective support against hepatocellular necrosis (Lin & Lin, 2006). In case of *G. atrum*, polysaccharide (PSG-1) had been found providing hepato-protective effect through lowering AST and ALT levels along with increasing anti-oxidative potentiality and stimulating increased synthesis of short chain fatty acids (SCFA) by the liver (Zhu *et al.,* 2016). SCFAs (acetic acid, propionic acid, butyric acid) are fermented products of complex carbohydrates. *Ganoderma lucidum* β-D glucan might be fermented by the rat gut microbiota into SCFAs that later on might be reabsorbed and provide cholesterol

lowering effect in the liver (Bhakta & Kumar, 2013; Hara *et al.,* 1999; Hu *et al.,* 2013) . Peptide isolated from *G. lucidum* had also been found potent in ameliorating carbon tetrachloride induced hepatotoxicity through anti-oxidative mode with corresponding lowered AST, ALT levels (He *et al.,* 2008). Even, tri-terpenes isolated from *G. lucidum* had been found safe and having no adverse effect upon hepatic and renal functioning as evidenced by the maintenance of the normal value of AST, ALT, ALP, urea and creatinine in the treated rats (Smina *et al.,* 2011). As evidenced from the current findings, feeding of *G. lucidum* HWE did not cause any adverse effect to the rats rather evoked anti-oxidative defense and plasma hypolipidemic status (Tables 5.1 – 5.4). Thus, *G. lucidum* HWE could be considered as safe and non-toxic to the experimental animals.

5.5 Conclusion

Feeding of the HWE of *G. lucidum* enhanced *in vivo* anti-oxidative defenses and also provided hypocholesterolemic effects to the rats. Heightened anti-oxidant status of the animals had been evidenced by the increased plasma levels of the anti-oxidant enzymes (CAT, GPX and SOD) and by the decreased plasma levels of TG, TC, LDL-C, LDL/HDL ratio, AI and increased HDL-C. The hypocholesterolemic effect of the HWE of *G. lucidum* has further been proved through its lowering effect upon the cholesterol biosynthetic regulatory enzyme, HMG Co-A reductase. Moreover, feeding of the HWE of *G. lucidum* did not cause any detrimental effect to the experimental animals (Table 5.4). In conjunction with the previous chapter's (chapter 3) *in vitro* anti-oxidative findings with those of current chapter's *in vivo* anti-oxidative and hypocholesterolemic effects, the HWE of *G. lucidum* could be interpreted in AD amelioration.

CHAPTER 6: ALZHEIMER'S DISEASE AMELIORATING EFFECT OF *G. LUCIDUM*

6.1 Introduction

Alzheimer's disease (AD) is neuro-degenerative disorder affecting mainly the elderly people. People afflicted with AD suffer from declined memory and learning abilities, problem in speaking and performing daily activities by him or her. The patient becomes solely dependent on the family members and the care-givers and ultimately bed-bound. This occurs because the neurons of the AD patients become damaged due to the formation of Aβ plaques and/or NFTs. Currently, more than 40 million people are suffering with AD round the globe and the alarmingly increased rate of this fatal disorder adds extra burden to the ever increasing economical and societal sectors and demands immediate medico-healthcare oriented management (Alzheimer's Association, 2016).

Despite tremendous efforts in combating this global epidemic, there is hardly any AD medication available upto present date. The available therapeutic strategies are aimed at symptom – modifying targets rather than preventing the neurons from damages. For example, the six drugs approved by the United States Food and Drug Administration (USFDA) can improve AD symptoms only through modulating brain neurotransmitter release. The failure to achieve the ultimate goal in slowing AD progression might remain hidden into multiple causative factors encompassing OS, metabolism, hypertension, genetics, epigenetics as well as adaptive response to some stressors. Thus, effective management of the co-existing factors of AD has been highly regarded in the most recent recommendation from the Alzheimer's association (Alzheimer's Association, 2016).

As the HWE of *G. lucidum* showed most potent *in vitro* (chapter 3) and *in vivo* anti-oxidative and cholesterol lowering effects (chapter 5) as well as possessed the highest amount of bio-components (chapter 4), this was selected for evaluating its AD ameliorating effect. Another rationale for choosing *G. lucidum* also emanates from the fact that despite its immense medicinal values, there is hardly any study available that incorporates anti-oxidative, hypocholesterolemic and AD ameliorating effect of this mushroom. Thus, the present chapter is aimed at determining the AD ameliorating effect of this mushroom through memory related learning and behavioral tests, neuro - biochemical and histopathological assessments.

6.2 Materials and methods

6.2.1 Animals

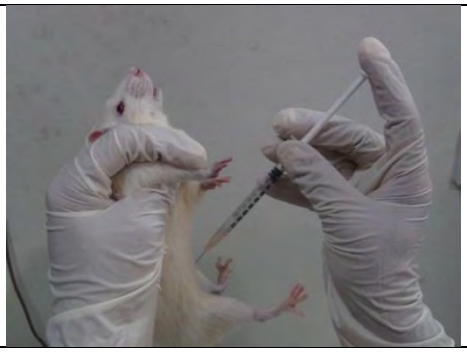
Ninety wistar male rats were divided into six groups, each group containing 15 rats, based on their weight range (120 ± 5 gm). The groups were control (C), *G. lucidum* HWE fed control (CE), hypercholesterolemic (H), *G. lucidum* HWE fed hypercholesterolemic (HE), Alzheimer's diseased (A in case of anti-oxidative, hypochoesterolemic, memory and learning related behavioral and biochemical and histopathological tests. AD in case of proteomics study) and *G. lucidum* HWE fed Alzheimer's diseased (AE). The AE group received 200 mg/kg body weight *G. lucidum* HWE. All the experimental protocols had been approved by the ethical permission committee, University of Malaya Institutional Animal Care and Use Committee (UMIACUC) [Ethics reference no. ISB/25/04/2013/NA (R)] (Appendix G).

6.2.2 Preparation of AD model rats

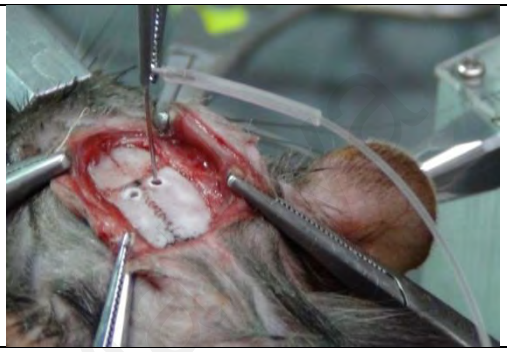
Male wistar rats were prepared by injecting $A\beta_{1-42}$ (ab120959, abcam, USA) in the right cerebral ventricles (Figure 6.1). Compared with the transgenic AD model rats, this

process is economical and the progression of AD symptoms and amyloidogenesis have been reported to be like those of human subjects (Lecanu & Papadopoulos, 2013). Also, Drugs found effective in rat models have already been translated to human therapeutic purposes and rat models provide opportunities towards pharmacodynamic studies of candidate drug or bio-component involved in AD therapeutics (Shineman *et al.,* 2011). Anesthetizing the rats with intra-peritoneal injection of sodium pentobarbital (40 mg/kg body weight), hair on the rats' heads was shaved and fixed the rats in the stereotaxic frame (SR-5R-HT, Narishige, Japan) by using the locks associated with it. Povidone-iodine (6%, USP) was used as antiseptic to rub the shaved portion of the head and placed the head at the midpoint of the stereotaxic frame. The skull was opened through incision, clipped the skin and sterile cotton and ice cold saline (0.9% NaCl) were used to wash bleeding. Removing skull-linked muscles, the skull was kept open and dried for a while so that the bregma became visible. Paralleling the skull to the frame, the ventricular points were stereotaxically spotted at 1.2 mm lateral and 0.8 mm posterior distant from the bregma following the brain atlas of Paxinos and Watson (1998). Marking of the spotted points followed microdrilling to make two pin holes at the two ventricles. For infusion of $A\beta_{1\text{-}42}$, a vehicle consisting of 35% acetonitrile (v/v) and 0.1% trifluoroacetic acid (v/v, pH 2.0) was used. $A\beta_{1\text{-}42}$ (5 µL, 2.5 nmol at 1 µL/min flow rate) was infused in the right ventricle and 5 µL of 1% $Alcl_3$ in the left ventricle (for $A\beta_{1\text{-}42}$ aggregation) using a Hamilton microsyringe (Neuros model 7001 KH, Hamilton, USA). After infusion, the rat was left untouched for a while so that adequate absorption could take place and then sealed the pin holes with sterile bone foam and cement. After drying of the bone cement, the skin clips were removed, wetted skin with saline (0.9% saline) and stitched the skin using sterile needle and thread. Thus, the AD positive controls were prepared. A sham control group was prepared using the same solvent injection. In order to be certain about $A\beta_{1\text{-}42}$ infusion in the cerebral ventricles,

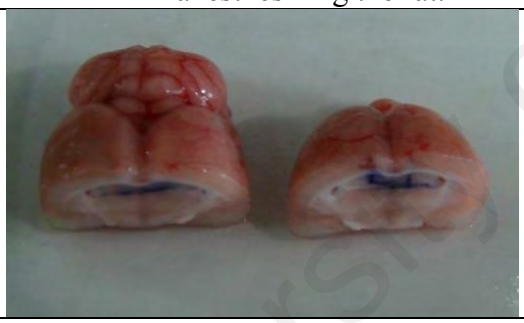
another trial surgery of rats maintaining the same procedure and using coomassie brilliant blue instead of $A\beta_{1-42}$ was performed in parallel with the original. After about 1 hour, rats of the trial group were sacrificed, the brains collected and the ventricles observed. Oberving the dye in the right cerebral ventricle following the same procedure, reaching of $A\beta_{1-42}$ to the right cerebral ventricles could be ascertained (Figure 6.1).



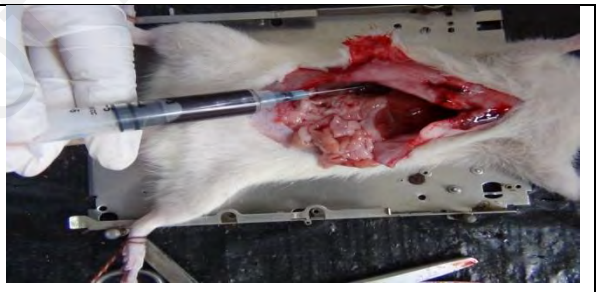
A. Intra-peritoneal injection of sodium pentobarbital for anesthesizing the rat.



B. Aβ infusion to rat's right cerebral ventricle.



C. Coomassie brilliant blue showing the evidence of Aβ reaching to the right cerebral ventricle.



D. Collection of blood.

Figure 6.1: Preparation of AD model rats and collection of blood

6.2.3 Post-operative care and recovery of the rats

As post-operative care to the rats, antibiotic ciprofloxacin was injected intramuscularly to avoid infection and put the rats in a temperature-regulatory chamber for maintaining their body temperature at 37ºC. After their anesthesia period was over, food and saline were provided to the rats and transferred those to the sterile bedding for recovering of the surgical stress. During recovery phase (about 7 days), repeated

injection of antibiotic (cephalexin, 15 mg/kg body weight, thrice daily) associated with careful feeding and drinking assistance to the rats. After the rats recovered from surgical stress and became healthy, they were adapted for the behavioral tests. All the experimental protocols had been approved by the ethical permission committee, University of Malaya Institutional Animal Care and Use Committee (UMIACUC) [Ethics reference no. ISB/25/04/2013/NA (R)] (Appendix G).

6.2.4 Memory and learning related behavioral tests

Evolutionarily, humans and rodents (mice, rats, guinea pigs, hamsters) have very close root of origin and they share much common behavioral and survival strategies. Thus, behavioral studies on rodents exemplify human behavioral traits whose analyses aid greatly in identifying and managing pathophysiological alterations as well for establishing therapeutic approaches. Animal models of behavioral abnormalities, especially those for neurodegenerative diseases such as AD have been *in vogue* for the last few decades. Capacity of an animal to orient itself in its environment is essential for its very existence as its feeding, mating and escaping from the enemies depend on this feature. Interestingly, orientation is an important criterion of spatial memory and AD patients gradually suffer from disorientation of time and place. At extreme cases, the AD patients cannot even locate their own houses though they had been living there since their birth. In memory and learning related behavior tests, both spatial and non-spatial performances of experimental animals are studied and through comparison with the controlled ones, alteration of the affected animals and amelioration of the drug treated ones are measured. In the present study, behavioral tests for both spatial and non-spatial memory and learning were performed whose descriptions have been appended below (Table 6.1).

Table 6.1: Memory and learning related behavior tests performed

Tests for spatial memory and learning	Tests for non-spatial memory and learning
Eight-armed radial maze test.	Novel object recognition (NOR) test.

6.2.4.1 Eight-armed radial maze test

A. Principle

Eight-armed radial maze test is based on the principle of win-shift task: the experimental animal is supposed to achieve the reward (food pellet, as it is kept hungry) from different locations within the environment and it must forage among different goals. While foraging, it uses landmarks and visual cues for memorizing spatial location of the reward and later on utilizes its acquired memory for further navigation. Its repetitive search in the same arm of the maze and failure to achieve the reward in a single trial are considered as poor or impaired memory. Developed by Olton and Samuelson (1976), the eight armed radial maze had successfully been used in deciphering the spatial learning abilities of the rats. There are different procedural attempts for data collection and memory measurement in radial arm maze (RAM). Matching-to-sample (MTS) free-choice system measures the long-term memory, where only a few arms contain food always and all the arms are kept open. In non-match to sample (NMTS) free-choice strategy, the animals are initially allowed to enter all the arms and receive food without delay. As trial progresses, delay in receiving food is applied and short-term memory is measured. In delayed match to sample (DMTS) free-choice process, the animals are allowed to receive food from the baited arms, they are provided with delay to visit the baited arms. Subsequent trials involve alteration of the baited arms. Long-term memory performance is the resultant effect of animals' visiting

to the baited and un-baited arms, food pellet receiving and maze latency (delay). All these are free-choice selection processes and there is also forced-choice type of DMTS, where some of the arms of the RAM are blocked and the animals are forced to explore the un-blocked ones only. In the present study, the DMTS free-choice procedure was performed to measure the memory and learning related behavior ameliorating effect of *G. lucidum* HWE upon the rats.

B. Eight-armed maze set up

An eight - armed radial maze made of polyvinyl chloride (0.5 cm width) was used in the present experiment (Figure 6.2). The maze was placed in a closed room and raised 38 inches above from the floor with arms prolonged from a central octagonal arena with a diameter of 30 cm. Each arm of the maze was of 50 cm long, 11 cm wide and 4 cm height. Additional arms (12 cm height) extended from the octagonal arena prevented rats jumping from one arm to another. Two centimeter before the end of each arm, there were food cups of 2.5 cm diameter and 1.5 cm depth (Figure 6.2). Visual cues (wall posters, chair and tables) and the position of the experimenter behind a canopy were kept constant during the entire period of the experiment.

C. Behavioral experiment

Behavioral experiments of 8-armed radial maze consisted of three phases: conditioning, acquisition and final experimentation

I. Conditioning: Each rat was handled for 5 minutes 7 days to habituate them to the experimenter and to the maze environment. The went up to gradual deprivation of food up to 15% of normal food intake to induce them to search for food as the food pellets (10 mg) were placed in the food cups of the maze.

II. Acquisition: Rats placed at the center of the maze explored the arms to find the food reward (Figure 6.2). They were trained for 7 days with one trial per day. Food pellets placed in any arm was kept constant across the whole session and trial.

A. 8-armed radial maze. B. Rat in search of food pellet. C. Rat approaching food pellet.

D. Rat found food pellet and looks at visual cue for memory formation. E. Rat uses visual cues and memory to find the baited arm. F. Improved memory leads the rat to the baited arm.

Figure 6.2: Eight armed radial maze test

III. Experimentation phase: Each rat was examined to collect food reward from the food cups of the four of the eight arms (Figure 6.2). A trial was terminated after either all the bait had been consumed or after 5 minutes had elapsed. Following the memory error definition of Jarrard *et al.* (1984), we measured three parameters of memory function:

a. Reference memory error (RME): entry into the unbaited arms (arms containing no foods).

b. Working memory error (WME): repeated entry into the arms that had already been visited.

c. Maze latency: time required for completing the entry into the four baited arms.

6.2.4.2 Novel object recognition (NOR) test

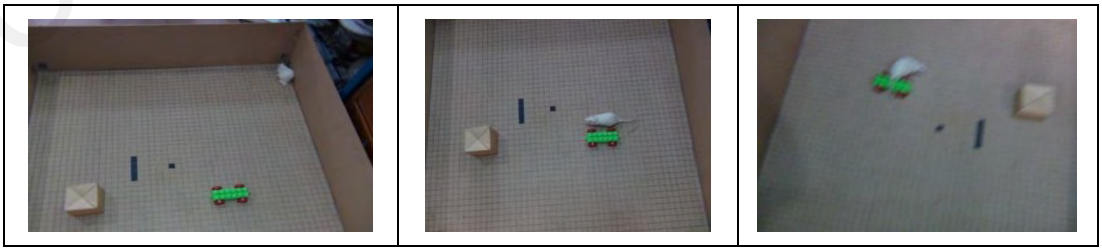
Novel object recognition (NOR) test is used to test the recognition (working, short- and/or long-term) memory of the animals. The test is based on the natural tendency of the rodents to explore the new objects. Thus, animals with better recognition memory will explore the newly oriented objects for long time compared with the exploration timing of the familiar objects (Bevins & Besheer, 2006). A polypropylene box with dimension of 120cm × 120cm × 120cm and plastic toys were used and followed the procedure described by Bevins and Besheer (2006) (Figure 6.7). The processes involved habituation (2 days), training (2 days) and testing (4 days). Each rat was allowed to explore two identical objects for 5 minutes followed by recognition and exploration of the novel object for 5 minutes. Activities of the rats were monitored using video camera (HDR CX130E, Sony, Japan) and arcsoft showbiz software and the video files were tracked with the tracking software kinovea.

The results are expressed as percentage of preference for novel object exploration:

$$T\ (\%) = (T_{novel} / [T_{novel} + T_{familiar}] \text{ X } 100).$$

Where, $T_{familiar}$ = Time spent in exploring the familiar object.

T_{novel} = Time spent in exploring the novel object.



A. Starting phase. B. Approaching phase. C. Recognition phase.

Figure 6.3: Novel object recognition test

6.2.5 Neuro - biochemical and neuro - immunological tests of memory and learning related parameters

6.2.5.1 Plasma and brain lysate preparation

Twenty four hours following the last treatment and behavioral test, the rats were kept in fasting overnight. Then, the rats were anesthesized with intra-peritoneal injection of sodium pentobarbital (35 mg/kg body weight) and sacrificed. The brains were perfused through the left ventricle with ice-cold saline immediately after blood collection. Heads were separated from the body followed by immediate collection of the brains from the skulls and preserved at – 80 °C. However, rats whose brains were preserved in glutaraldehyde and/or formalin for histopathological studies were not perfused. Hippocampus was separated from the cerebral cortex followed by homogenization of the hippocampus using Dounce glass homogenizer (D8938, Sigma-Aldrich, USA) and ice cold phosphate buffer (25mM, pH 7.4). Tissue remnants were removed by centrifuging the homogenates at 800 g and collected the supernatant. An aliquot of the supernatant was used for preparing cytosolic fraction (through centrifuging at 1000 rpm), detergent soluble fraction (DSF) and detergent insoluble fraction (DIF) of hippocampus. DSF was prepared following the modified method of Hashimoto *et al.* (2005). Briefly, homogenization of the hippocampus was performed in 1% Triton X-100 containing ice cold phosphate buffer (25 mM, pH 7.4), followed by centrifugation at 800 g for 10 min and collection of the supernatant. Agitation of the supernatant for 2 hr over ice-cold temperature followed by centrifugation at 10, 000 rpm for 1 hr produced the supernatant denoted as DSF and the pellet denoted as the DIF.

6.2.5.2 Antibodies and enzyme linked immunosorbent assays (ELISAs)

A. Aβ (1-42) oligomers

The levels of $A\beta_{(1-42)}$ oligomers in DSF of hippocampus homogenates and brain derived neurotrophic factor (BDNF), synaptosomal associated protein 25 KD (SNAP 25), post-

synaptic density protein 95 KD (PSD 95), vesicular acetylcholine transporter (VAchT) and tumor necrosis factor α (TNFα) in the cytosolic fractions of the rat hippocampus homogenates were detected utilizing sandwich ELISA. Briefly, ELISA microplates (Cellstar, Greiner-bio one, USA) were coated with 20 µl of the DSF and or cytoplasmic fraction of hippocampus using 0.1M carbonate buffer (pH 9.6). Blocking was done using 3% BSA in PBS followed by addition of primary antibody [rabbit polyclonal anti-Aβ, Abcam, ab 10148; mouse monoclonal anti-BDNF, Abcam, ab 10505; mouse anti-SNAP 25, Abcam, ab 31281; mouse anti-PSD 95, Abcam, ab 18258; rabbit polyclonal anti-VAchT, Abcam, ab 68984 and mouse monoclonal anti-TNFα, Abcam, ab 1793, USA, respectively] with dilution of 1:1000 and incubated at room temperature for 2 h. HRP-coupled anti-rabbit IgG (Abcam, ab 6721, USA) was used as the secondary antibody and incubated for 2 h at 37°C. Then, tetramethyl benzidine substrate (TBS) (Sigma-Aldrich, USA) was added and incubated in dark for 30 min at 25°C followed by addition of stop solution (0.1 N HCl). Wells containing 0.1 M carbonate buffer (pH 9.6) only were used as the negative controls. The wells were analyzed with a multiwell plate reader (Synergy H1, Gen 5 BioTek multimode plate reader, USA) at 450 nm.

B. Aβ (1-42) anti-fibrillating assay of *G. lucidum* HWE

As the conversion of oligomeric Aβ (1-42) into its fibrillar form is an important aspect of AD pathogenesis and withstanding the fibrillation process stands as an important AD preventing stratagem, the inhibitory effect of the *G. lucidum* HWE upon the *in vitro* fibrillation of Aβ (1-42) oligomers was measured (Ahmed *et al.,* 2010). Thioflavin T test was used following the modified method of Ma *et al*. (2014). Briefly, Aβ(1-42) was dissolved in the aggregation buffer (20 mM Tris, 100 mM NaCl, 1 mM EDTA, pH 7.4) and final concentration of Aβ(1-42) made into 50 µM with or without the addition of 20 µM *G. lucidum* HWE and incubated at 37 °C for 24 h. Then, 40 µL of the solution was mixed with 210 µL of 10 µM ThT and glycine-NaOH buffer

(50mM, pH 8.0) and fluorescence measured with excitation wavelength of 448 nm and emission wavelength of 487 nm (FLx 800 fluorescence reader, Gen 5 Biotek, USA).

6.2.5.3 Transmission electron microscopy (TEM) studies

A. Brain sample preparation for transmission electron microscope (TEM) study

As hippocampus is involved in memory and learning effects mostly and degenerative hippocampal neurons are hallmarks of AD and impaired memory, hippocampi of the experimental animals were chosen for TEM studies to view the effect of *G. lucidum* HWE upon the experimental animals. Following procedures have been performed to view the hippocampal neurons through TEM:

a. Primary fixation, trimming and post - fixation: In order to preserve the structures in their native state, chemical and irreversible fixation of the hippocampus were performed by immersing them in 2.5 % glutaraldehyde immediately after collection, kept overnight and trimmed the tissues into 1-2 mm sizes on the second day. Washing with cacodylate buffer 2-3 times, the trimmed tissues were post-fixed with the osmium tetroxide: cacodylate buffer (1:1) for 2 hours and then stored O/N at 4 °C after washing with cacodylate buffer.
b. Washing and dehydration: After a series of washing 2 – 3 times each with doubled distilled water (5 min), 35% ethanol (10 min), 50% ethanol (10 min), 70% ethanol (10 min), 95% ethanol (15 min), 100% ethanol (2 times, each for 15 min), propylene oxide (15 min), propylene oxide : Epon [containing agar, dodecenyl succinic acid anhydride, methyl nadic anhydride and 2,4,6 tri-dimethyl amino ethyl phenol] (1 :1, 1 hour) and propylene oxide : Epon (1 : 3) (2 hour), we kept the sampls in Epon O/N.

c. Embedding: The samples were embedded using fresh resins and allowed to polymerize first at 37 °C for 5 hours and then at 60 °C O/N.

d. Block trimming: By clamping the block firmly into the block holder and placing under the stereomicroscope (Leica, M 80), the excess embedding media was trimmed until the tissue was exposed. Through trimming, the block was made into a trapezium shape.

e. Semithin sectioning: The trimmed block was clamped into a microtome chuck, adjusted the angle of the glass knife (prepared using Leica EM KMR3 and Reichert knifemaker) to use its "E" edge and trimmed the block surface (Leica ICC50 HD). Removing the debris, fine sections were collected with forceps and transferred them onto a drop of double distilled water on a glass slide. Glass slides containing the sections were collected upon bench top to let the section fixed.

f. Toluidene blue staining: Putting a drop of filtered toluidine blue stain onto dried semithin sections, the slides were kept on the hot plate for 1 minute. Washing out the stain into a beaker, differentiation was performed with 95% ethanol. After washing two times with water, the slides were dried by putting on the hot plate (Electrochemical slide drying bench).

g. Ultra-thin sectioning: Clamping the trimmed block into a ultramicrotome chuck securely and looking through microscope (Leica EM UC6), ultra-thin sections of 75-85 nm were prepared using a diamond knife and then collected onto copper grids.

h. Uranyl acetate staining: The ultra-thin sections were stained facing side of the copper grids with millpore filtered and centrifuged uranyl acetate solution (on a piece of dental wax put on a petri dish) for 10 minutes while remaining it covered. Washing three times with doubled distilled water and wiping with filter

paper, the dental wax was surrounded with a pellet of NaOH and added a drop of millpore filtered and centrifuged lead citrate solution to the ultrathin sections facing side of the copper grids. After 10 minutes, the grids were washed three times and let the grid drying with a filter paper so that the grid was ready for viewing.

B. Viewing of the prepared samples by TEM

The prepared samples were viewed using the TEM (Leon Libra 120, Zeiss, Germany) equipped with the soft imaging viewer v5.0 software, images captured and produced for reporting. All the experimental protocols had been approved by the ethical permission committee, University of Malaya Institutional Animal Care and Use Committee (UMIACUC) [Ethics reference no. ISB/25/04/2013/NA (R)] (Appendix G).

6.3 Results

6.3.1 Effect of intra-cerebroventricular infusion of Aβ1-42

Infusion of soluble Aβ1-42 to the rat cerebral ventricles affected their memory and learning related behavioral tasks indicating the effectiveness of the current model of AD studies. In addition to the quantitative data described below under specific sub-sections, distinct behavioral discrepancies in the AD model rats compared with those of the age-matched controls were observed. Among the mostly noted altered behavioral responses were such as the AD rats did not explore the rearing cages frequently, they remained aloof and rarely social and rarely tried to cross-over the cages.

6.3.1.1 Memory and learning related behavioral tests

6.3.1.2 Eight-armed radial maze study

As depicted in Figure 6.4 and 6.5, feeding of *G. lucidum* HWE lowered RME [$F_{(5,42)}$ = 11.31, P≤ 0.05] and WME [$F_{(5,42)}$ = 16.78, P≤ 0.05] in all the rat groups (Raw data: Appendix B, Tables B 1.1 - 1.15; Statistical data: Appendix C, Tables C -7.1, 7.2, 7.3 and 7.4). Tukey post-hoc multiple comparison tests revealed that WME lowering effect of *G. lucidum* HWE was statistically significant (P ≤ 0.05). Also the maze latency (time required to explore the baited arms) gradually decreased in the extract fed rats (Figure 6.6). In addition to the extract fed AD (AE) rats, the extract fed hypercholesterolemics (HE) showed better performance in the RAM test (Figures 6.5). These findings correspond towards this extract's memory and learning enhancement as well as AD ameliorating effect.

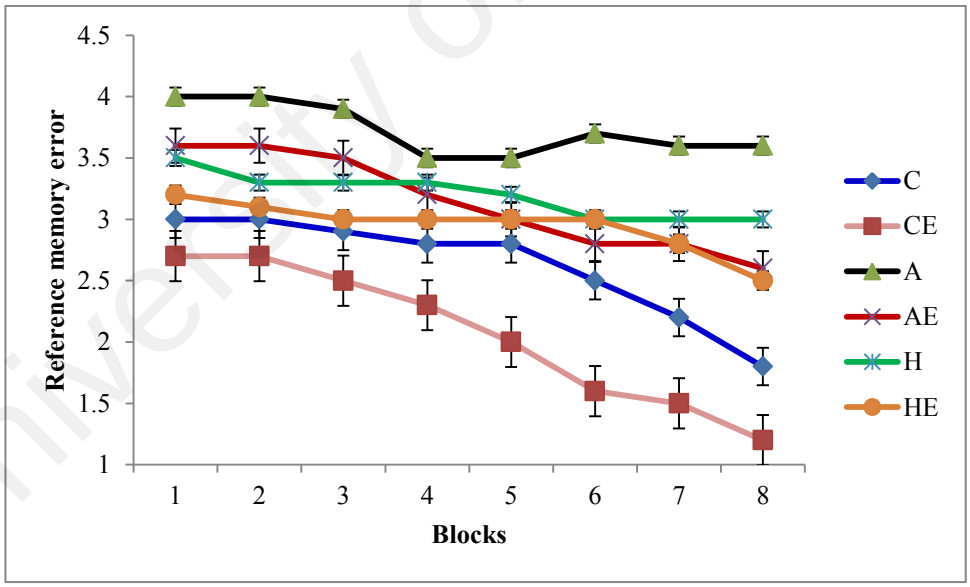


Figure 6.4: Reference memory error of the rats

Data of every 6 trials have been averaged over a block and expressed as mean±SE (n=3). Data were analyzed with one-way ANOVA and post-hoc Tukey's HSD test (P≤0.05). Here, C=Control, CE= *G. lucidum* HWE fed control, H=hypercholesterolemic, HE=*G. lucidum* HWE fed hypercholesterolemic, A=AD model rats and AE= *G. lucidum* HWE fed AD rats, respectively.

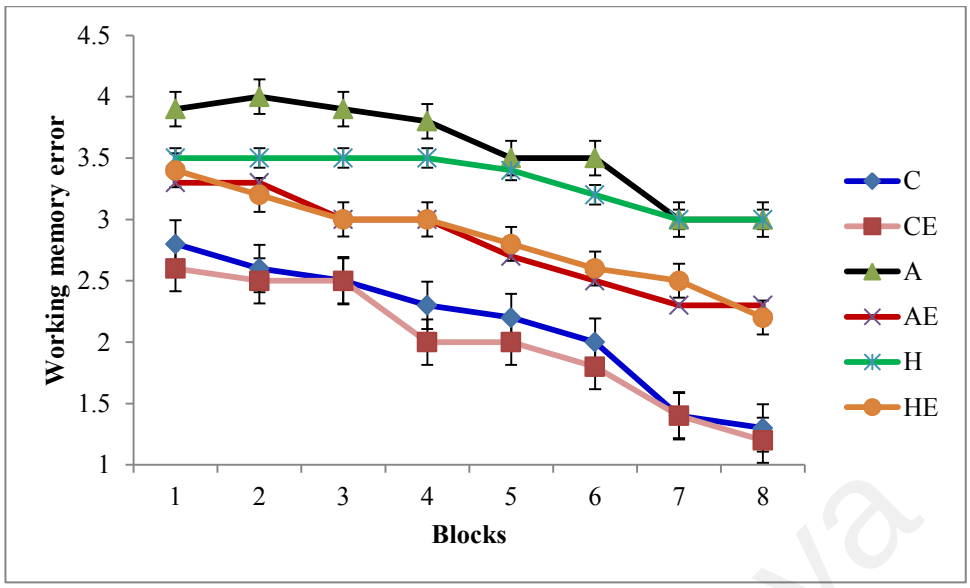


Figure 6.5: Working memory error of the rats

Data of every 6 trials have been averaged over a block and expressed as mean±SE (n=3). Data were analyzed with one-way ANOVA and post-hoc Tukey's HSD test (P≤0.05). Here, C=Control, CE= *G. lucidum* HWE fed control, H=hypercholesterolemic, HE=*G. lucidum* HWE fed hypercholesterolemic, A=AD model rats and AE= *G. lucidum* HWE fed AD rats, respectively.

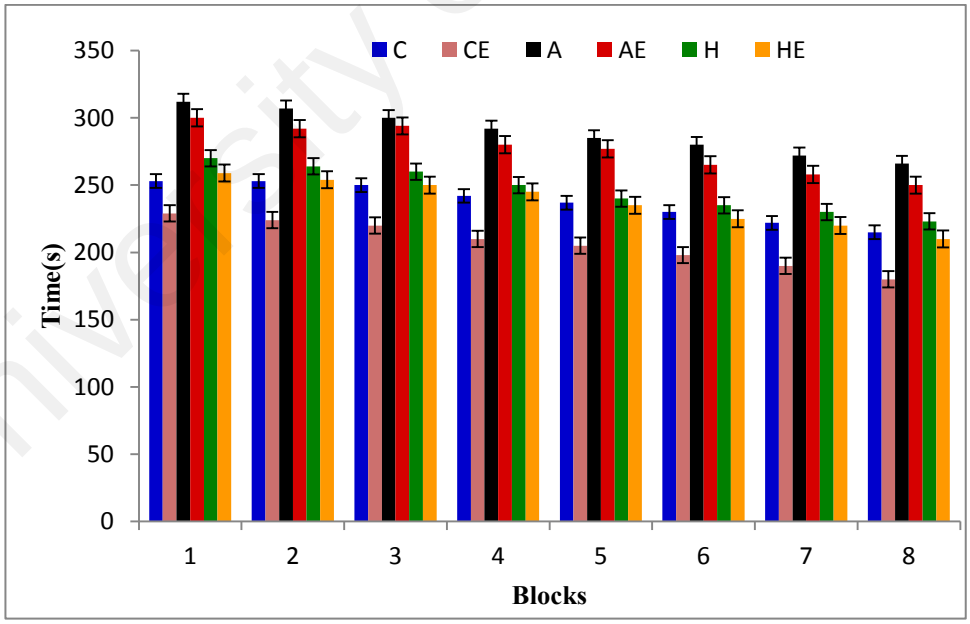


Figure 6.6: Time spent in exploring the baited arms

Data of every 6 trials have been averaged over a block and expressed as mean±SE (n=3). Data were analyzed with one-way ANOVA and post-hoc Tukey's HSD test (P≤0.05). Here, C=Control, CE= *G. lucidum* HWE fed control, H=hypercholesterolemic, HE=*G. lucidum* HWE fed hypercholesterolemic, A=AD model rats and AE= *G. lucidum* HWE fed AD rats, respectively.

1.1.1.1 Novel object recognition (NOR) test

In novel object recognition test, the *G. lucidum* HWE fed rats showed higher preference for recognizing and exploring the novel objects than each of the controlled counterparts (Figure 6.7) (Raw data: Appendix B, Table B 1.16 – B 1.21; Statistical data: Appendix C, Tables C - 7.5 and 7.6). Preference of the extract fed controlled rats (CE) was the highest (84.83%), followed by those of the extract fed hypercholesterolemic (HE, 71.67%) and extract fed AD rats (AE, 64%) (Figure 6.7). One way ANOVA with Tukey post-hoc test showed that although the values of each extract fed group did not significantly differ from those of the non-fed rats, there was significant group difference [$F_{(5,30)} = 19.27$, $P \leq 0.05$].

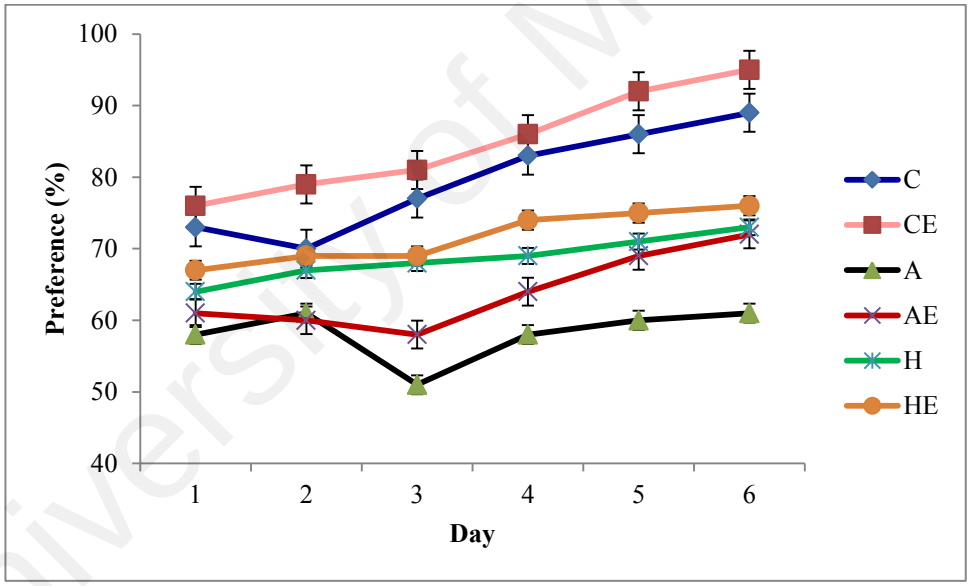


Figure 6.7: Preference for novel object

Data are expressed as mean±SE (n=3). Data were analyzed with one-way ANOVA and post-hoc Tukey's HSD test (P≤0.05). Here, C=Control, CE= *G. lucidum* HWE fed control, H=hypercholesterolemic, HE=*G. lucidum* HWE fed hypercholesterolemic, A=AD model rats and AE= *G. lucidum* HWE fed AD rats, respectively.

6.3.2 Neuro - biochemical and neuro - immunological tests of memory and learning related parameters

As depicted in the Table 6.2, the HWE of *G. lucidum* had ameliorating effect upon the memory and learning related neuro-biochemical and neuro-immunological markers of the rats such as BDNF, SNAP 25, PSD 95 and VAchT. On the other hand, decreased levels of anti-Aβ (1-42) oligomers and TNFα levels were observed in all the rat groups treated with *G. lucidum* HWE (Table 6.2) (Raw data: Appendix B, Table B 8.1; Statistical data: Appendix C, Tables C - 8.1 and 8.2).

Table 6.2: Antibodies and enzyme linked immunosorbent assays (ELISAs)

% of control	CE	A	AE	H	HE
Anti-Aβ (1-42) oligomer	85.30 ± 0.49^{a}	141.85 ± 0.60^{b}	119.80 ± 0.44^{c}	107.03 ± 0.35^{d,e}	102.56 ± 0.50^{d,e}
BDNF	109.57 ± 0.91^{a,e}	84.00 ± 1.78^{b,c}	90.43 ± 1.74^{b,c,d}	95.72 ± 1.03^{b,c,d}	103.60 ± 0.29^{a,e}
SNAP 25	104.80 ± 0.71^{a}	85.33 ± 0.69^{b}	89.16 ± 0.57^{c}	93.25 ± 0.63^{d,e}	94.09 ± 0.78^{d,e}
PSD 95	102.44 ± 1.25^{a}	91.56 ± 0.94^{b,c,d,e}	94.23 ± 0.42^{b,c,d,e}	94.83 ± 0.83^{b,c,d,e}	95.57 ± 0.98^{b,c,d,e}
TNFα	90.02 ± 0.80^{a}	120.50 ± 1.34^{b}	111.05 ± 0.83^{c}	103.62 ± 1.00^{d}	97.56 ± 0.90^{e}
VAchT	109.12 ± 1.27^{a,c}	91.98 ± 0.68^{b,d}	106.04 ± 0.57^{a,c,e}	96.48 ± 0.87^{b,d}	111.89 ± 1.13^{a,c,e}

Data are expressed as mean±SE (n=3). Data were analyzed with one-way ANOVA and post-hoc Tukey's HSD test (P≤0.05). Here, C=Control, CE= *G. lucidum* HWE fed control, H=hypercholesterolemic, HE=*G. lucidum* HWE fed hypercholesterolemic, A=AD model rats and AE= *G. lucidum* HWE fed AD rats, respectively. BDNF stands for brain derived neurotrophic factor, SNAP 25 for synaptosomal associated protein 25 KD, PSD 95 for post-synaptic density protein 95 KD, TNFα for tumor necrosis factor α and VachT for vesicular acetylcholine transporter, respectively.

6.3.3 Effect of *G. lucidum* HWE on inhibition of Aβ (1-42) fibril formation

As depicted in the Figure 6.8, addition of 20 µM of *G. lucidum* HWE could significantly (one sample student's t test, $P \leq 0.05$) lower the Aβ (1-42) fibrillation process *in vitro* (Statistical data: Appendix C, Table C 9.1).

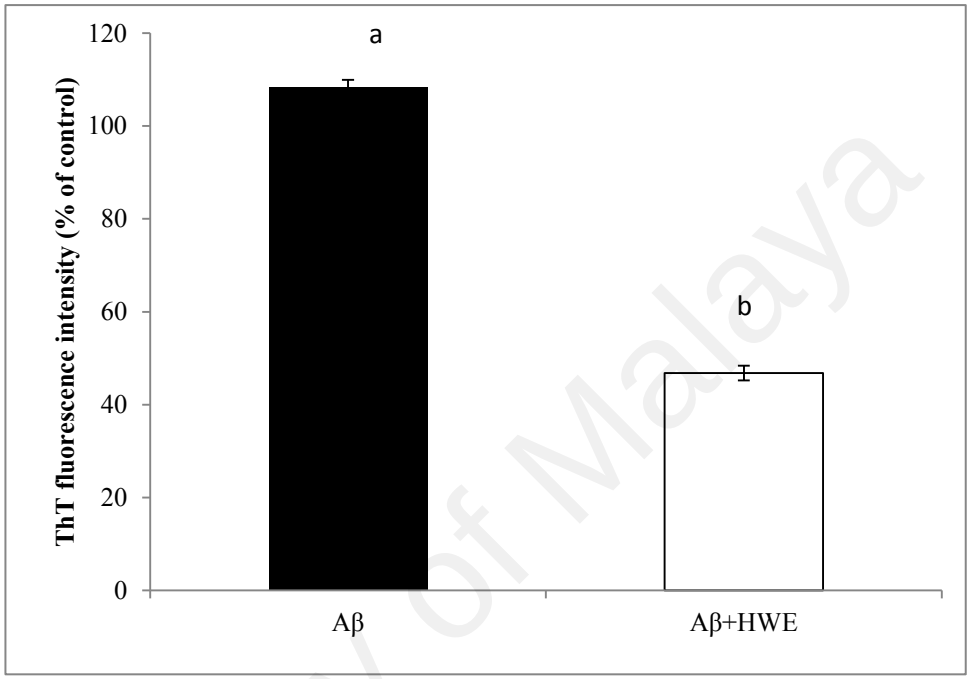


Figure 6.8: Fibrillation inhibitory effect of *G. lucidum* HWE

Data are expressed as mean±SE (n=3). Data were analyzed with one sample t test ($P \leq 0.05$). Here, Aβ= Aβ oligomers and Aβ+HWE= *G. lucidum* HWE treated Aβ oligomers, respectively.

6.3.3.1 TEM studies of the hippocampus

As depicted in Figure 6.9, infusion of Aβ (1-42) caused profuse alteration in the AD rats' hippocampal neuronal structure and organization as compared with the controls. Prolonged dendritic projections in the hippocampal neurons of the control and those of the *G. lucidum* HWE fed rats (AE) had been observed while the AD rats had mostly degenerative, plaque like structures.

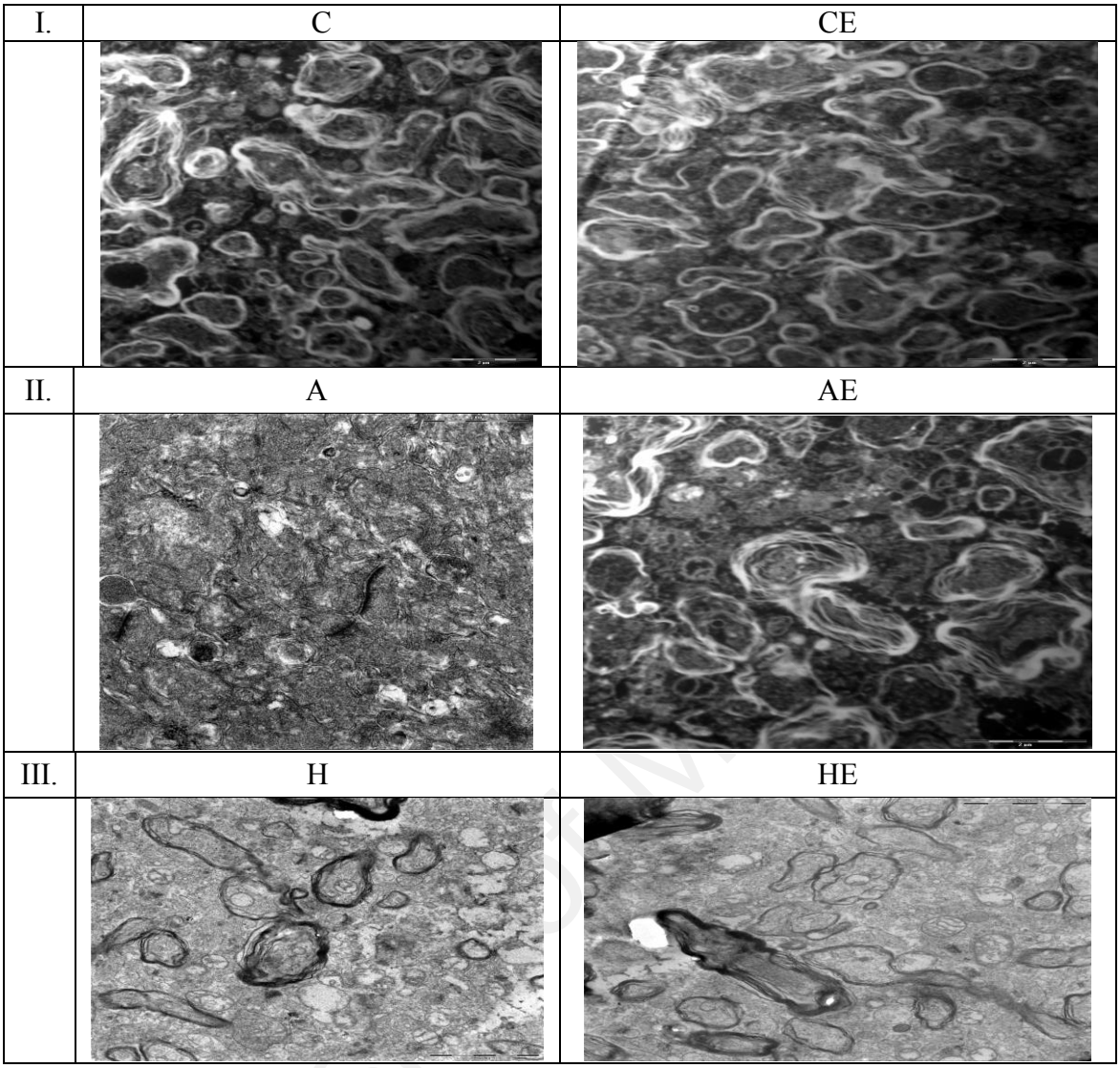


Figure 6.9: TEM view of the CA1 region of the rat hippocampus

Where, C=Control, CE= *G. lucidum* HWE fed control, H=hypercholesterolemic, HE=*G. lucidum* HWE fed hypercholesterolemic, A=AD model rats and AE= *G. lucidum* HWE fed AD rats, respectively.

6.4 Discussion

6.4.1 Effect of intra-cerebroventricula $A\beta_{1-42}$ infusion

Impaired behavioral responses of the AD rats demonstrate that Aβ1-42, even at soluble form *apriori* towards aggregation, plaque formation and neurodegneration. May be, Aβ1-42 disrupted short term and/or long-term potentiation (LTP) in the hippocampus and caused impaired memory and learning functions (Freir *et al.,* 2001; Stepanichev *et al.,* 2003; Stepanichev *et al.,* 2006). Also, the soluble form of $A\beta_{1-42}$ itself might evoke impaired neuronal activities and neurodegeneration that negatively

affected the $A\beta_{1-42}$ infused rats' learning and memory related behavioral performance (Townsend *et al.,* 2006).

6.4.2 Enhancement of memory and learning by *G. lucidum* HWE

6.4.2.1 Eight-armed radial maze study

Feeding of *G. lucidum* HWE to the AD rats improved their memory and learnin abilities (Figures 6.4, 6.5 and 6.6).The stated claim is substantiated by the fact that DMTS free-choice is an important aspect of memory performance in the RAM (Stolberg, 2005). Gradual increased win-shift behavior of the *G. lucidum* HWE fed rats while the win-stay behavior in their non-fed counterparts was noted. These findings represent the improved associative memory of the extract fed rats as they preferred the food pellet associated arms to those of non-associated. Perhaps, the extract fed rats developed their cognitive maps better and extrapolated the location swifter and much precisely than those of the non-fed ones. As a whole, ingestion of *G. lucidum* HWE effected in improving spatial memory of the rats as evidenced by the decreased rate of RME, WME and maze latency in the extract fed rats effect (Figures 6.4, 6.5 and 6.6).

Perhaps, this is the first RAM report entailing *G. lucidum* HWE mediated memory and learning related behavioral amelioration of the AD model rats. Even, RAM reports involving other mushroom species are also not available so far. Thus, comparative discussion with other RAM studies involving *G. lucidum* and/or edible-medicinal mushrooms could not be incorporated in this section. However, RAM based AD ameliorating studies of multiple natural products have been conducted in different laboratories of the world.

Sweeney *et al*. (2007) conducted the delayed matching-to-sample (DMTS) free-choice type of RAM studies on intra-hippocampal $A\beta_{1-40}$ infused rats and found that the animals' short-term memory and other memory related performance had been disrupted.

Non-matching to sample (NMTS) type of RAM studies by Stepanichev *et al*. (2003) reported that intra-cerebrovascular infusion of $A\beta_{25-35}$ impairs short-term memory of the rats while the long-term memory remains unaffected. Their subsequent studies linked $A\beta_{25-35}$ driven neuro-degeneration in the CA1 region of the hippocampus with impaired memory in the experimental animals (Stepanichev *et al.,* 2006). Matching-to-sample (MTS) type of RAM-based memory tests by Holscher *et al*. (2007) had identified the memory related behavioral disruption in the $A\beta_{25-35}$-infused rats. This group remarked that though intra - cerbroventricular infusion of $A\beta_{25-35}$ disrupted short-term memory and working memory in the rats, this status could be ameliorated and even reverted back to the normal (Holscher *et al.,* 2007). RAM based studies of AD amelioration by docosahexaenoic acid (DHA) and madecassoside had been reported by Hashimoto *et al*, (2005) and Hossain *et al*. (2014), respectively.

Hypercholesterolemia decreases cholinergic neurons and acetylcholine in the basal nucleus of brain and stimulates Aβ formation and plaque deposition (Ullrich *et al.,* 2010). Hypercholesterolemia has also been implicated with neuro-inflammation and abnormal APP processing along with increased WME in mice (Thirumangalakudi *et al.,* 2008). In the current study, increased RME and WME in the hypercholesterolemic (H) rats might be due to this reason and compatible with other RAM studies (Ehrlich *et al.,* 2012; Granholm *et al.,* 2008). In line with this, gradual decreased rate of RME and WME in the *G. lucidum* HWE fed hypercholesterolemic (HE) rats indicate this mushroom's potentiality in reducing hypercholesterolemia - led memory dysfunction. In this connection, observed *in vitro* and *in vivo* hypocholesterolemic effect of this mushroom (chapter 5), in combination with memory enhancing effects, conform to the AD ameliorating potency of *G. lucidum.*

6.4.2.2 Novel object recognition test

Novel object recognition (NOR) is an important test for evaluating both short – and long-term memory. Present findings of higher preference and exploration of the *G. lucidum* HWE fed rats towards the novel objects indicate this mushroom's memory and learning ability improving effects (Figure 6.7). To the best of our knowledge, this is the first study incorporating *G. lucidum* in NOR test. However, studies incorporating *H. erinaceus* upon the AD mice models has reported improved spatial recognition and visual memory of the AD mice models prepared like those of the present study (Mori *et al.,* 2011). Memory improving effect has been deduced to be *inter alia* through increasing mossy fiber CA3 hippocampal neurons in the mice (Brandalise *et al.,* 2017). Similar mode of action might have been prevalent in case of the *G. lucidum* HWE fed rats showing higher preference for the novel objects and higher exploration of the environment. NOR is analogous to human declarative (spatial or episodic) memory (Figure 2.1) and based on the current memory improving findings, therapeutic potentiality of G. *lucidum* HWE seems promising in treating human AD subjects.

6.4.3 Antibodies and enzyme linked immunosorbent assays (ELISAs)

6.4.3.1 Aβ $_{(1-42)}$ oligomer lowering effect of *G. lucidum* HWE

As $A\beta_{(1-42)}$ is the culprit for memory disruption, its measurement in the brain homogenate is apparent. Thus, the level of $A\beta_{(1-42)}$ oligomer was measured using antibody against it in the DSF of the rat hippocampus as $A\beta_{(1-42)}$ oligomer is a soluble protein. Significantly increased level of $A\beta_{(1-42)}$ oligomer in the AD rats (41% higher than those of the controls) were found in the present study (Table 6.2) (Raw data: Appendix B, Table 8.1; Statistical data: Appendix C, Tables 8.1 and 8.2). This may be due to the combined effect of increased generation *per se* and deposition of those of through cerebro-ventricular infusion. On the contrary, significantly decreased ($P \leq 0.05$) level of $A\beta_{(1-42)}$ oligomer was observed in the *G. lucidum* HWE fed rats (lowered up to

19%) (Table 6.2). Although, hypercholesterolemia led 7% increased generation of $A\beta_{(1-42)}$ oligomer, feeding of the *G. lucidum* HWE could lower this level up to 2% (Table 6.2). This lowering of $A\beta_{(1-42)}$ oligomer burden in the AD and hypercholesterolemic rats' brain are indication of AD ameliorating effect of *G. lucidum*. Among multiple strategy, *G. lucidum* mediated decreased biosynthesis of cholestersol might be responsible for generating comparatively lowered level of $A\beta_{(1-42)}$. Content of phenolics in the HWE of *G. lucidum* might also contribute to this effect through their inhibitory effect upon BACE1 (Leow *et al.,* 2013; Wu *et al.,* 2015). *Ganoderma lucidum* mediated stimulation of the P13K and ERK signaling cascades resulting in stimulation of the non-amyloidogenic pathway and enhanced secretion of sAPPα might also have been involved (Pinweha *et al.,* 2008). Current findings are also compatible with those of Wang *et al* (2004), who found that *G. lucidum* powder at 0.3%, 0.6% and 1.8% of diet can significantly lower Aβ burden in the mouse brain, improve memory and learning abilites along with enhanced anti - oxidative enzymatic levels. Thus, lowering of brain Aβ burden along with increasing anti – oxidative defense might confer the AD ameliorating effect of the *G. lucidum* HWE.

6.4.3.2 Neurotransmission maintenance effect of *G. lucidum* HWE

Brain derived neurotrophic factor (BDNF) is a neuroprotectin group of growth factor involved in neuronal survival and functioning. Significantly lowered level of BDNF in the AD rats compared to the controls (84% compared with 100% of control value) was found in the present study (Table 6.2) (Raw data: Appendix B, Table 8.2; Statistical data: Appendix C, Tables 8.1 and 8.2). Feeding of *G. lucidum* HWE resulted in increased BDNF level both in the AD (from 84%, raised up to 90.43%) and the hypercholesterolemic (from 95.72%, incrased up to 103.60%) rats and the increasing effect was significantly high in the latter ($P \leq 0.05$) (Table 6.2).

BDNF participates in both pre- and post-synaptic neurotransmission by binding with the tyrosine kinase receptor B (TrkB) and thus plays important role in memory and learning activities (Lu, 2003). Its impairment has been found to affect memory related learning and behavioral performances in different organisms and in different conditions (Tyler *et al.,* 2002). AD patients possess considerably lower amount of BDNF (Shin *et al.,* 2015) . Deteriorated memory emanating from intra-cerebroventricular infusion of Aβ had been reported to be due to the lowering level of BDNF (Balducci *et al.,* 2010). Learning activities increases BDNF level in the rodent hippocampus and cortex (Cotman & Berchtold, 2002). As described in the behavioral test section (6.4.2), the *G. lucidum* HWE fed rats performed better than the non-fed ones in the learning tests and the mushroom fed rats were supposed to contain higher level of BDNF. Observed neuro – biochemical findings are compatible with those of the behavioral test findings of the present study. This claim is substantiated by the finding that BDNF not only forms spatial memory but also regulates retention and recalling of it (Mizuno *et al.,* 2000). Previous studies had shown that the triterpenoids present in *G. lucidum* provide BDNF enhancing effect (Zhang *et al.,* 2011). In the current study, several tri-terpenoids have been detected in the HWE of *G. lucidum* (chapter 4, Table 4.5) that might have been involved in increasing the BDNF level of the mushroom fed rats' hippocampus and thus improved their memory and learning abilities. In addition, phenolics present in the HWE of *G. lucidum* might also confer effects towards increasing the hippocampal BDNF level (Leow *et al.,* 2013; Zhao *et al.,* 2013).

6.4.3.3 Maintenance of pre-synaptic membrane and long-term potentiation by *G. lucidum* HWE

Synaptosomal - associated protein 25 KD (SNAP 25) is a pre-synaptic membrane protein necessary for long – term potentiation (LTP) and working memory (Söderqvist *et al.,* 2010). Its decreased level had been noticed in AD subjects (Greber *et al.,* 1999).

Compared with the controls, significantly lowered ($P \leq 0.05$) level of SNAP in the hippocampus of the AD rats was observed (Table 6.2) (Raw data: Appendix B, Table 8.3; Statistical data: Appendix C, Tables 8.1 and 8.2). In case of *the G. lucidum* HWE fed rats, SNAP 25 level increased significantly ($P \leq 0.05$). Previous studies indicate that spatial recognition is mediated by SNAP 25 and rats' performance in MWM had been found to be negatively affected with the anti-SNAP 25 antisense oligonucleotide (Hou *et al.,* 2004). Aβ leads towards loss of synaptophysin, another pre – synaptic protein, in the caspase – dependent way (Liu *et al.,* 2010). Thus, improved spatial recognition of the *G. lucidum* HWE fed rats in different spatial memory and learning related behavior tests (RAM and NOR) in the present study, might be attributed by the enhanced SNAP 25 level in the respective rats.

6.4.3.4 Maintenance of post – synaptic density by *G. lucidum* HWE

Post-synaptic density protein 95 KD (PSD – 95) is involved in maturation of the excitatory synapses and in maintenance of post – synaptic density (Chen *et al.,* 2011). It is a scaffold protein that takes part in regulating NMDA receptor mediated signaling processes (Sheng, 2001). Increased Aβ accumulation and impaired memory have been reported to be associated with its disruption and AD patients exhibit lowered level of PSD 95 in the hippocampus (Sultana *et al.,* 2010a). Decreased level of PSD 95 in the AD and hypercholesterolemic rats (94.43% and 95.57%, respectively) compared with the control (100%) were noted in the present study (Table 6.2) (Raw data: Appendix B, Table 8.4; Statistical data: Appendix C, Tables 8.1 and 8.2). This might be due to Aβ guided loss of PSD 95 in the caspase – dependent way (Liu *et al.,* 2010). However, feeding of *G. lucidum* HWE to the rats resulted in increased level of PSD-95 in the hippocampus. Thus, the improved memory and learning abilities of the *G. lucidum* HWE fed rats in the present study might be accrued from *inter alia* increased PSD 95

level in the hippocampi of the mushroom fed rats. Among multiple strategies, the anti-oxidative defense mechanism might be involved (Gray *et al.,* 2016).

6.4.3.5 Neuro-inflammation lowering effect of *G. lucidum* HWE

Neuro-inflammatory mode of AD pathogenesis involves increased level of tumor necrosis factor alpha (TNFα) as evidenced by cellular and animal model studies as well as in AD patients (Álvarez *et al.,* 2007; Bhaskar *et al.,* 2014; Lourenco *et al.,* 2013). TNFα acts as a neurotoxin and thus AD amelioration strategy points towards its reduced level (McAlpine *et al.,* 2009). As depicted in Table 6.2, the AD rats had significantly ($P \leq 0.05$) higher level of TNFα (120.5% compared to 100% of the controls) (Raw data: Appendix B, Table 8.5; Statistical data: Appendix C, Tables 8.1 and 8.2). Feeding of *G. lucidum* HWE significantly decreased (up to 1116.05%) TNFα level. Similar effect was observed in case of the mushroom fed hypercholesterolemic rats (lowered from 103.62% to 97.60%). At 400 µg/mL, the methanolic extract of *G. lucidum* had been found to significantly lower the production of microglial TNFα and prevents the dopaminergic neurons (Zhang *et al.,* 2011) . At the same dosage, it could lower the expression of TNFα mRNA up to 90% (Zhang *et al.,* 2011) . Presence of phenolic anti-inflammatory and anti-oxidant substances in the *G. lucidum* HWE might also cause TNFα lowering effect (Frautschy *et al.,* 2001). Content of β-D glucan, triterpenoids and polysaccharides had been supposed to confer this protecting effect of *G. lucidum* (Zhang *et al.,* 2011) . *Ganoderma lucidum* polysaccharide (GLPS) administered to the traumatic spinal cord injured rats also showed neuroprotective effect through lowering TNFα level along with reduced production of MDA (Gokce *et al.,* 2015). In addition to the TNFα lowering effect, MDA lowering effect of the HWE of *G. lucidum* was also observed in the present investigation (chapter 3, section 3.2.6 and 3.2.7). Thus, the current findings are compatible with those published ones as similar lowering effect of

G. lucidum HWE upon TNFα level (Table 6.2) and presence of similar bio-components have been observed in the present study (Table 4.5).

6.4.3.6 Enhancement of cholinergic neurotransmission by *G. lucidum* HWE

Vesicular acetylcholine transporter (VAchT) transports acetylcholine (ACh) from the presynaptic membrane of the cholinergic neurons into the secretory vesicles (SV), from where ACh is released into the synaptic cleft (Chen *et al.,* 2011). Activity of VAChT highly regulates cholinergic neurotransmission and VAChT deficiency leads towards neuromuscular abnormalities (Rodrigues *et al.,* 2013). Impaired cognitive function in AD subjects had been linked with reduced VAChT level (Chen *et al.,* 2011). In the present study, significantly lower ($P \leq 0.05$) level of VAChT was noted in the AD model rats (91.98% in AD, compared to 100% for the controls) while the *G. lucidum* HWE fed rats had been observed with significantly higher level of VAChT (106.04%) (Table 6.2) (Raw data: Appendix B, Table 8.6; Statistical data: Appendix C, Tables 8.1 and 8.2). Reduced level of AChT in the hypercholesterolemic rats (96.48%) brings testimony towards hypercholesterolemia induced impairment of cholinergic neurons and VAChT activity (Ullrich *et al.,* 2010). Thus, memory and learning related poor performances of the AD and the hypercholesterolemic rats may be due to *inter alia* disrupted VAChT activity and diminished cholinergic neurotransmission (Prado *et al.,* 2006). In line with this, the ameliorated memory and learning related performances of the *G. lucidum* HWE fed rats might be the offshoot of ameliorated VAChT activity of the cholinergic neurons in the respective rats.

6.4.4 Inhibition of Aβ(1-42) oligomerization and fibrillation by *G. lucidum* HWE

Agents capable of lowering and/or inhibiting Aβ fibrillation seem promising as the therapeutics against AD pathogenesis (Stefani & Rigacci, 2013). As evidenced by the improved memory and learning abilities and neuro-biochemical and immunological

tests, it is plausible that the HWE of *G. lucidum* would also have inhibitory effect upon Aβ fibrillation. This surmise is based on the findings that the natural polyphenols are potent in withstanding Aβ fibrillation (Porat *et al.,* 2006; Stefani & Rigacci, 2013). The anti-oxidative mode of action has been assumed to be capable of lowering Aβ fibrillation process also (Porat *et al.,* 2006). In this context, the phenolics and polyphenolics present in the HWE of *G. lucidum* (chapter 4, Table 4.5) might have shielded Aβ (1-42) fibrillation in the present study through their anti-oxidative mechanism. In addition, structural similarities of the polyphenolics with that of the amyloid β sheet might also confer resistance towards fibrillation (Porat *et al.,* 2006). Catechol types of flavonoids (protocatechuic acid, p-hydroxy cinnamic acid, ellagic acid) (chapter 4, Table 4.5) present in the *G. lucidum* HWE might inhibit Aβ (1-42) fibrillation by site-specific inhibition of the lysine residues (Rawat *et al.,* 2013; Sato *et al.,* 2013). However, other biochemical processes might also have been involved whose cumulative effect bear testimony to inhibitory effect of *G. lucidum* HWE up on Aβ fibrillation *en route* to the amelioration of AD pathogenesis.

6.4.4.1 TEM studies of the hippocampus

TEM studies featuring hippocampal neurons in their native state and pronged dendritic projections as well as absence of degenerative neuronal plaque like structures in the *G. lucidum* HWE-fed rats compared with those of the control and AD subjects are of immense importance (Figure 6.9). This is because neuronal degeneration is an important hallmark of AD. The memory and learning related outcomes of the *G. lucidum* HWE presented in the current study might be attributed *inter alia* to the prevention of neuronal degeneration also. Previous studies have shown the *G. lucidum* spore could reduce neuronal apoptosis in the hippocampus and thus prevent neuronal loss and aid in cognitive performance (Zhou *et al.,* 2012). Antagonization of the Aβ – induced neurotoxicity of the aqueous extract of *G. lucidum* had also been reported (Lai

et al., 2008). Similar mode of action might have been engaged with the outcomes of the current experiment. Also, anti-oxidative mode of neural protection against Aβ and hypercholesterolemia – induced oxidative stress might be functional (Zhao *et al.,* 2004). Content of polyphenolic substances in the HWE of *G. lucidum* might be implicated in this function (chapter 4, Table 4.5) (Lakey-Beitia *et al.,* 2015). Besides, anti-inflammatory effect of *G. lucidum* tri-terpernoids might also confer the neuro-protective effect of this mushroom (De Silva *et al.,* 2013; Dudhgaonkar *et al.,* 2009). Present findings are also compatible with those of Yongpan *et al.* (2016), whose TEM based histopathological studies showed that the *G. lucidum* tri – terpenoids at 0.25, 0.5 and 1 g/kg body weight could improve neuronal degeneration in a dose dependent manner Yongpan *et al.* (2016).

6.5 Conclusion

Feeding of *G. lucidum* HWE to the rats improved their memory and learning abilities. Improved cognitive performance of the *G. lucidum* HWE fed rats in the behavioral (eight armed radial maze and novel object recognition tests) and antibody and ELISA based tests of neurotransmitters were observed. TEM studies also provide support towards neuro-protective effect of *G. lucidum* HWE. All of these findings and those described in the previous chapters bear testimony towards the AD ameliorating effects of the *G. lucidum* HWE. As diverse range of proteins are involved in the maintenance of memory and learning abilities of an organism, studies concerning the status (up regulated / down regulated) of different proteins seem imperative in deciphering its memory and learning related amelioration. Thus, proteomic analysis, study and protein-protein interaction of the relevant rat groups (controlled, AD and the *G. lucidum* HWE fed AD rats) were performed whose description has been put forward in the next chapter.

CHAPTER 7: PROTEOMICS-BASED ANALYSIS ON ALZHEIMER'S DISEASE AMELIORATING EFFECT OF *G. LUCIDUM*

7.1 Introduction

As proteins are involved in many aspects of memory and learning processes and their derangements affect Alzheimer's disease (AD) pathogenesis highly, proteomics approaches seem pertinent in elucidating the state of AD and also of its amelioration. Separation of proteins from the complex biological mixture is the foremost part of proteomics approaches. Later, comes the identification of the individual protein and/or peptide for establishing it as a biomarker. For the last few years, two dimensional gel electrophoresis (2DGE) had been most extensively used for separation of proteins (Görg *et al.,* 2004). Separation principle is based on the molecular weight (MW) and electric charge (pI) of individual protein. Under electric current, migration of each of the proteins in polyacrylamide gel depends on its MW and pI. Separated proteins are then visualized by staining with silver and identified using the mass spectrometry. Mass spectrometry identifies the mass (m) and electric charge (z) carried by each protein and peptide and produces mass-to-charge (m/z) data. Using standard software and databases, the obtained m/z values are compared and the protein and peptides are characterized and identified.

Currently, liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) is the most attractive technique for proteomics studies of human and animal organs and tissues. In this approach, after separating the proteins in SDS-PAGE, protease (very often with trypsin) catalyzed digestion produces peptides that are loaded onto capillary column for reverse phase liquid chromatography. Peptides are eluted sequentially with buffer of increasing gradient and high voltage applied (2.0-2.5 KV).

High temperature evaporates the acidic liquid into gas and H^+ ionizes the peptides (electrospray ionization, ESI) and the ionized peptides move in the electromagnetic field which is scanned and measured by the detector and their m/z ratio is measured. Besides ESI, matrix - assisted laser desorption/ionization (MALDI) is also applied that uses a dry matrix instead of the liquid solvent for peptide ionization by laser pulses. While MALDI is suitable for pure, large peptides and protein, ESI can be applied for complex peptides and has upper hand in usage (Cravatt *et al.,* 2007). In the present study, SDS-PAGE followed by LC-MS/MS strategy have been applied for identifying the hippocampal proteins associated with AD pathogenesis and amelioration in the control (C), AD and AE rat groups.

Proteomic approaches utilizing 2DE and western blots can trap only the intact proteins and the proteolytically cleaved peptides are left that affects entire analyses. Procedure applied in the current research can grab both the proteolytically cleaved peptides and the intact proteins and minimizes the loss of the low molecular weight peptides. For accuracy, precision and reliability of proteomics, incorporation of both biological and technical replicates are essential. However, in this study, the biological samples were pooled to average the protein level and thus control the sample-sample variation. Technical replicate was maintained at 3 and frequency at 9 to represent independent measurement of noise signal and instrument error as well as to substantiate the accuracy and confidence of protein identification and to minimize the sample variation.

7.2 Materials and methods

7.2.1 Brain sample preparation and protein quantification

7.2.1.1 Brain sample collection

Rats were anaesthesized with pentobarbital, sacrificed and head removed followed by collection of brain on ice bath. Brains were frozen in liquid nitrogen and stored at –80 °C. All the experimental protocols had been approved by the ethical permission committee, University of Malaya Institutional Animal Care and Use Committee (UMIACUC) [Ethics reference no. ISB/25/04/2013/NA (R)] (Appendix G).

7.2.1.2 Protein extraction

Protein extraction from the brain samples was performed following homogenization of the brain sample (50 mg) with lysis buffer (1ml) using a homogenizer (Polytron PT 1200, Kinematica). To avoid protein degradation, we added 10 µL of protease inhibitor cocktail during homogenization followed by centrifugation at 10000× g at 4 °C for 10 minutes. The supernatant was collected and proceeded towards delipidation.

7.2.1.3 Delipidation procedure

As the brain is a fatty tissue, lipids may hamper protein separation and quantification steps and thus delipidation is an essential prerequisite for brain tissue proteomics. Prior to delipidation, 100 µL of the brain supernatant was dried using speedvac. Delipidation procedures were conducted following the methods of Shevchenko *et al.* (2010) . Briefly, 1.4 mL of ice-cold tri-n butyl phosphate / acetone / methanol mixture (1:12:1) was mixed with 100 µL of extract and incubated for 90 min at 4°C. Centrifugation of the mixture at 2800× g at 4 °C was performed for 15 minutes that pelleted the precipitate. Later, sequential washing was performed with 1 ml each of TBP, acetone and methanol. Last of all, the pellet was air dried and proceeded for protein quantification and protein separation through SDS-PAGE.

7.2.1.4 Protein quantification

Content of total protein in the delipidated brain samples was determined using the Pierce BCA protein assay kit (Thermo scientific, 23225). Bovine serum albumin (BSA) was used as the standard and manufacturer's directions followed for protein quantification (Section 5.3.4.7 and 6.3.5.2 B; Appendix A: Figure A 5.1; Table A 7.8).

7.2.2 Protein separation through SDS-PAGE

In SDS-PAGE, proteins are separated based on their charge and molecular weight. Proteins become denatured by SDS and attain negative charges proportionately with their masses that render them to move to the anode end. Their movement also is imparted by their respective weight: the less weighty proteins moving faster than those of the higher. The mini-PROTEAN tetra cell (165-8000, BIO-RAD, USA) was used according to the manufacturer's instructions for running SDS-PAGE in the current study.

7.2.2.1 SDS-PAGE assemblage

A set of vertical glass plate gel units were assembled with 0.75 mm spacer strips at the edges. Solution leakage was prevented by clamping the glass plates tightly and poured separating gel solution into the space by micropipete. Ultra-pure water was overlaid the gel top with ultrapure water and allowed the gel to polymerize for about two hours. After polymerization, the overlaying water was discarded and the stacking gel was casted. The separating gel surface was cautiously rinsed with the stacking gel for removing unpolymerized and residuous gel and poured the stacking gel (Appendix D, section 1: Preparation of solutions, buffers and gels). A comb from top-side was inserted immediately into the gel. Care was taken not to trap any bubble at the inner tip of the comb and we let the gel to polymerize for about one hour. After polymerization, the gel was used immediately.

7.2.2.2 Loading sample preparation

The sample buffer (4×), previously prepared and stored at 4 °C, was allowed to equilibrate to room temperature (Appendix D, section 1: Preparation of solutions, buffers and gels). Mixing the sample buffer and rat brain homogenate (1:3) was followed by heating the mixture at 100 °C for 5 minutes adding about 5 µL of trypan blue.

7.2.2.3 Running of SDS-PAGE

The shorter glass plates had been clamped inward of the chamber and placed the glass units into the vertical electrophoretic tank. Running buffer was poured at both upper and lower chambers and loaded the (cooled down to room temperature and mixed well) samples (12 µL, 7 µg) and protein marker (4 µL) into the SDS-PAGE wells (Appendix D, section 1: Preparation of solutions, buffers and gels). Placing the lid onto the tank and plugging it properly, electrophoresis turned on. Electrophoresis was allowed at 80 V for the initial 20 minutes, at 100 V for the second 20 minutes and for the rest of the time at 120 V so that excess heat could be avoided. Movement of the blue dye was observed while electrophoresis going on and when the dye had crossed about 1 cm from the gel's bottom-layer, electrophoresis was turned off and the apparatus disassembled.

7.2.2.4 Staining of the gel

Removing from the glass plate, the gel was fixed by immersing it into fixing solution (mixture of 10% acetic acid and 40% methanol) and letting occasional stirring for 30 minutes. Separating the gel from the fixing solution, it was immersed in another container containing 0.1% coomassie brilliant blue with shaking for about 20 minutes (Appendix D, section 1: Preparation of solutions, buffers and gels).

7.2.2.5 Destaining of the gel

For destaining, 10% acetic acid solution (aqueous) was used. The gel immersed into the destaining solution contained in a covered box underwent occasional stiring until the entire gel was fully destained. Then, individual bands was cut and if any gel plug still contained stain, repeated shaking of the gel plugs in 50 µL of 50% acetonitrile (ACN) in 50 mM ammonium bicarbonate was continued (Appendix D, section 1: Preparation of solutions, buffers and gels).

7.2.3 Preparation of the gel for protein identification

7.2.3.1 In-gel tryptic digestion

Effective protein identification and characterization by MS requires several preparatory steps following separation of the proteins. Digestion of the large proteins into smaller fractions and peptides is the initial step that was performed for in-gel plugs. Digestion involved preparatory phases of reduction, alkylation and dehydration of the proteins in gel plugs.

7.2.3.2 Reduction and alkylation:

Under non-denaturing conditions, most of the proteins are resistant to enzymatic proteolysis. Thus, solubilization of the hydrophobic proteins is performed using detergent-chaotrope mixture added lysis buffer with sonication and vigorous vortexing. For disrupting the tertiary structures of the solubilized proteins, reduction and alkylation are applied to them so that the disulfide linkages are broken and cannot be re-formed. Reducing agents (dithiothreitol, 2-mercepatoethanol) convert disulfide bond of cysteine into free sulfhydryl groups. Reacting with the free sulfhydryl groups of cysteine, alkylating agents (iodoacetamide, iodoacetic acid) form S-carboxyamidomethyl-cysteine that cannot be reoxidized and disulfide bonds are not formed. For reduction, the gel plugs were incubated in 150 µL of 10 mM dithiothreitol (DTT) in 100 mM

ammonium bicarbonate buffer at 60 °C for 30 minutes (Appendix D, section 1: Preparation of solutions, buffers and gels). After cooling at room temperature, the gel plugs were alkylated by incubating in 150 µL of 55 mM iodoacetamide (IAA) in 100 mM ammonium bicarbonate for 20 minutes in the dark chamber. The gel plugs were washed in triplicate with 500 µL of 50% ACN in 100 mM ammonium bicarbonate for 20 minutes. For dehydration of the gel plugs, shaking in 50 µL of 100% ACN for 15 minutes was performed followed by drying the gel plugs in speed vacuum for 30 minutes at 4 °C.

7.2.3.3 Digestion

Usually, the denatured, reduced and alkylated proteins are subjected to enzymatic or chemical digestion. For enzymatic digestion, trypsin is most commonly used as it specifically cleaves at the carboxyl terminal end of arginine and lysine and produces peptide fragments containing suitable length and charge for MS analysis. The gel plugs were incubated with 25 µL of 6 ng/ µLtrypsin in 50 mM ammonium bicarbonate at 37 °C overnight.

7.2.3.4 Extraction

After overnight digestion, the digested products were spun down by vortexing and transferred liquid to the fresh tubes. Adding 50 µL 50% ACN to the tubes, shaking was continued for 15 minutes. Then, the gel plugs were incubated with 50 µL of 100% ACN and shook for another 15 minutes. Transferring the liquid to the previous tubes, the digested samples were completely dried using the speed vacuum at 1000 rpm. The dried tubes were stored at - 80 °C for desalting and zip tip procedures (Appendix D, section 1: Preparation of solutions, buffers and gels).

7.2.3.5 Desalting and Zip tipping procedure

Desalting and Zip-tipping of the samples were performed using the respective solutions mentioned in (Appendix D, Section 2: Preparation of desalting and zipping solutions) and followed the steps as follows

Reconstitution of protein samples: The protein samples were reconstituted adding 10 µL of 0.1% formic acid, vortexing and spinning down the samples.

Wetting: 10 µL of wetting solution was aspirated and dispensed three times.

Equilibration: For equilibrating the tips, 10 µL of equilibrium solution was aspirated and dispensed for three times.

Protein binding: By pipetting in and out, 10 µL of protein samples was passed through the zip-tips for 10 cycles. Repeatation of the step was performed without letting the tip to be dried.

Washing: For three cycles, 10 µL of washing solution was aspirated and dispensed.

Elution: Adding 4 µL of elution solution into zip-tip and dispension was performed for three times. Finally, the content was dispensed in the protein sample solution.

7.2.4 LC-MS/MS Q-TOF quantification

All the MS/MS instruments and software used in the present section of the study were from Agilent (Agilent, Santa Clara, CA, USA). Eluted sample obtained from zip tip procedure was dried and 10µL of the lyophilized samples was reconstituted in the first LC mobile phase (0.1% formic acid) in triplicate. The peptides with a Nano-LC 1260 linked directly with an Accurate Mass Q-TOF 6550 containing a Chip-Cube interface Nano-ESI ion source. Polaris High Performance Chip was utilized and enriched the peptides using 360 nl enrichment column followed by their separation

using the separation column (C18 reverse phase, 150mm x 75ÂÎ¼m, 5 μm) with solvent A (0.2% formic acid in water) and a 5–80% gradient of solvent B (0.1% formic acid in acetonitrile) for 34 min with a flow rate of 0.35 μL/min. Mass data acquisition was undertaken at 8 spectra/second in the range of 100-200 m/z and subsequent collision induced dissociation (CID) of the twenty most intense ions. Setting the mass-tolerance of precursor and product ions at 20, MS/MS data acquisition was performed in the range of 200–3000m/z. In order to identify the proteins, the acquired MS/MS data were compared against the UniProtKB/Swiss Prot rat *(Rattus norvegicus)* database using the Spectrum Mill and X! Tandem. The differentially expressed proteins in the different groups were identified using their canonical sequence and proteins having fold change of at least 1.5 times were considered as the deregulated proteins. For validation of the identified proteins, the data were exported to the Scaffold database (version 4.5.1, Portland, USA). Proteins were grouped together if they would share at least two peptides and maintained their threshold level at 95.0% and <1% false discovery rate (FDR) by the Peptide Prophet *al*gorithm with Scaffold delta - mass correction for the matched peptide-spectra. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Proteins sharing significant peptide evidence were grouped into clusters. Proteins were annotated with GO terms from NCBI.

In the present experiment, label-free relative quantification was performed depending on the regulation of the peptides. For statistical analyses, the data were exported to the Mass Profiler Professional (MPP) software that analyzed depending on the MPP entities, the intensity of the total spectra of the proteins. Setting the baseline of the spectra to the median of the samples, frequencies of the entities were filtered minimally at all the replicates of each treatment. To overcome the complications of false discovery associated with multiple test analyses, ANOVA ($P<0.05$) was performed.

7.2.5 Bioinformatics and analysis of protein-protein interaction (PPI)

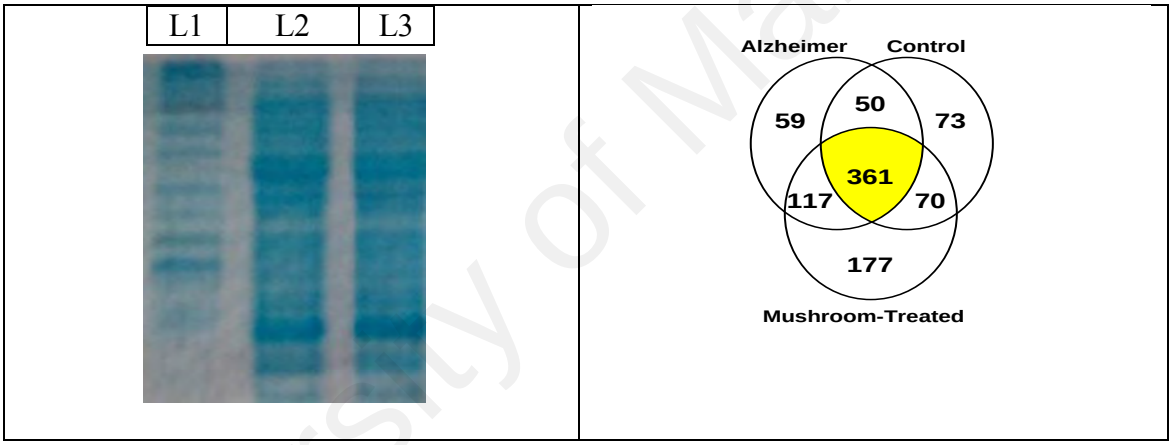
Most of the proteins do not work singly rather they participate in complex network or scaffold and interact with others. Thus, analysis of the relevant protein-protein networks provides important information in deciphering any bio-molecular system. Functional interaction networks of the proteins were identified using the STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) database (version 10.0; http://string-db.org/). STRING displays protein-protein interactions in a large network of connectivity and protein hubs. Active prediction methods that we used were experiments, neighborhood, databases, gene fusions, coexpression, cooccurrence and text mining, using high confidence (0.7).

For further identifying over-representing pathways and biological functions, the ingenuity pathway analysis (IPA), build version: 389077M, content version: 27821452, (Release date: 2016-06-14) was used (https://www.ingenuity.com/wp-content/themes/ingenuity-qiagen). Datasets of the proteins significantly expressed ($P<0.05$) and having log fold change of 1.5 and higher were uploaded (AD versus C, AD versus AE and C versus AE). Analysis setup was as follows

Criteria	Set up
Reference set	Ingenuity Knowledge Base (Genes Only)
Relationship to include	Direct and Indirect. Includes Endogenous Chemicals.
Optional Analyses	My Pathways My List
Filter Summary	Consider only molecules and/or relationships where (species = Human) and (data sources = ClinicalTrials.gov or ClinVar or Ingenuity Expert Findings or Ingenuity ExpertAssist Findings)

7.3 Results

Total 822 proteins with protein threshold at 95.0%, minimum peptide of 2 and peptide threshold at 0.1% FDR were identified in all the three groups (AD versus C, AD versus AE and AE versus C). Number of commonly expressed proteins among the three groups was 361 (Figure 7.1B). Among all the identified proteins (822), 329 were differentially expressed with statistical significance (P<0.05). Among the significantly regulated (P<0.05) 329 proteins, 289 met the criteria of fold change (LogFC of 1.5) cut off value (Appendix E, Table E 10.1). Number of proteins linked with AD, OS and hypercholesterolemia was 59, 20 and 12, respectively.



A. SDS-PAGE image: L1 - Control, L2 - AD and L3 – AE.

B. Overlap of the identified proteins in the hippocampus of the control (C), AD and mushroom treated (AE) rats.

Figure 7.1: A) SDS-PAGE image and B) Venn diagram of the identified proteins.

The differentially expressed (significantly up – and/or down – regulated at P<0.05) proteins were grouped with respect to their established functional links such as response to stimulus, memory and learning related neuro-synaptic function, metabolic processes, maintenance of cytoskeleton, immunological response, transportations and apoptosis.

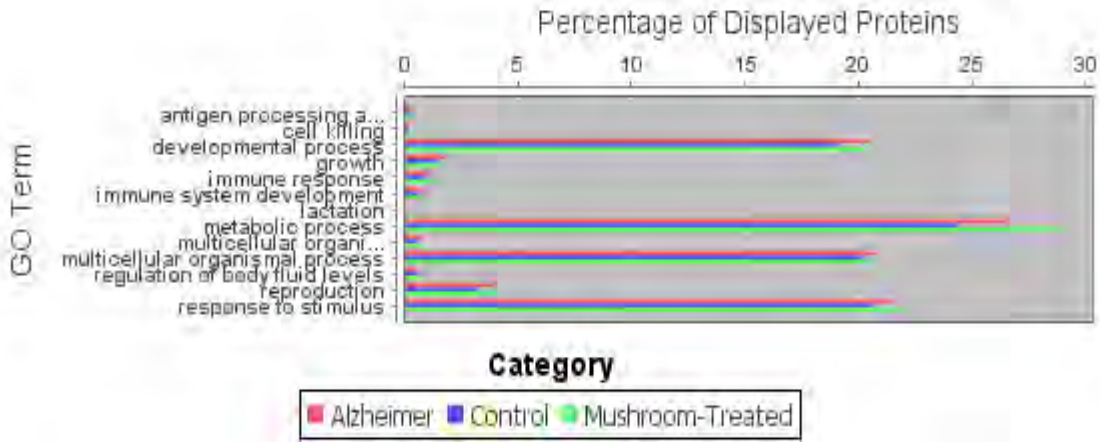


Figure 7.2: Gene ontology (GO) analysis featuring biological processes of the identified hippocampal proteins.

The highest amount of proteins differentially expressed in the AD and mushroom fed AD (AE) rats were those involved in metabolic processes (26% increase in AD and 29% in the AE rats) (Figure 7.2). Extent of differential expression in case of the proteins involved in response to stimulus, multicellular organismal processes and developmental processes was very close together (20±2%) (Figure 7.2). Molecular function based categorization showed that proteins involved in the transporter and enzyme regulatory activities were of the most differently expressed types followed by those of molecular transducing and anti-oxidant activities (Figure 7.3). Cellular component based analyses identified the proteins located at the cytoplasm, cellular organelle and membrane as the most differentially expressed ones (Figure 7.4).

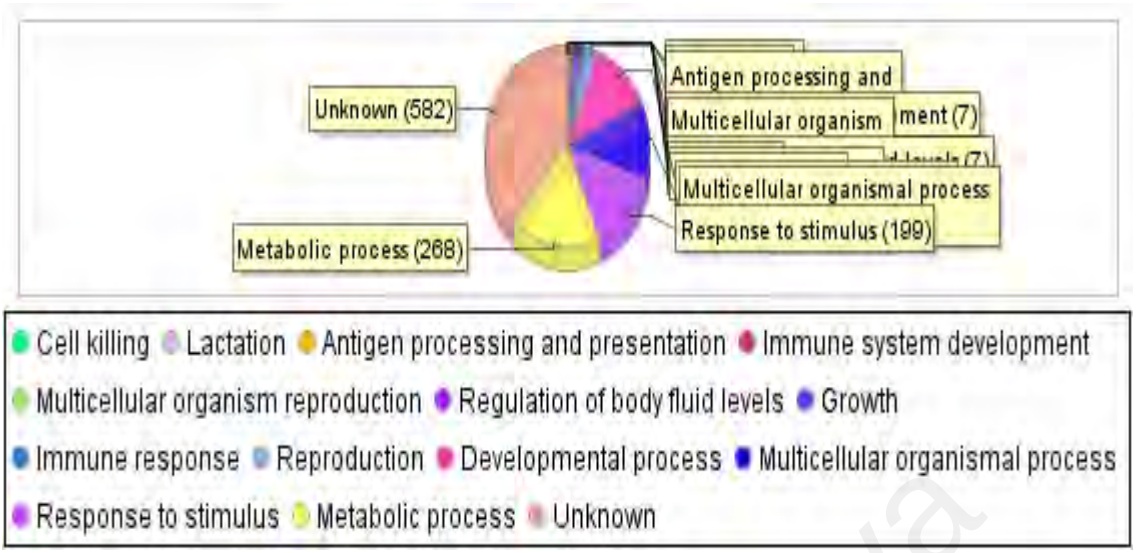


Figure 7.3: Gene ontology (GO) analysis featuring molecular functions of the identified hippocampal proteins

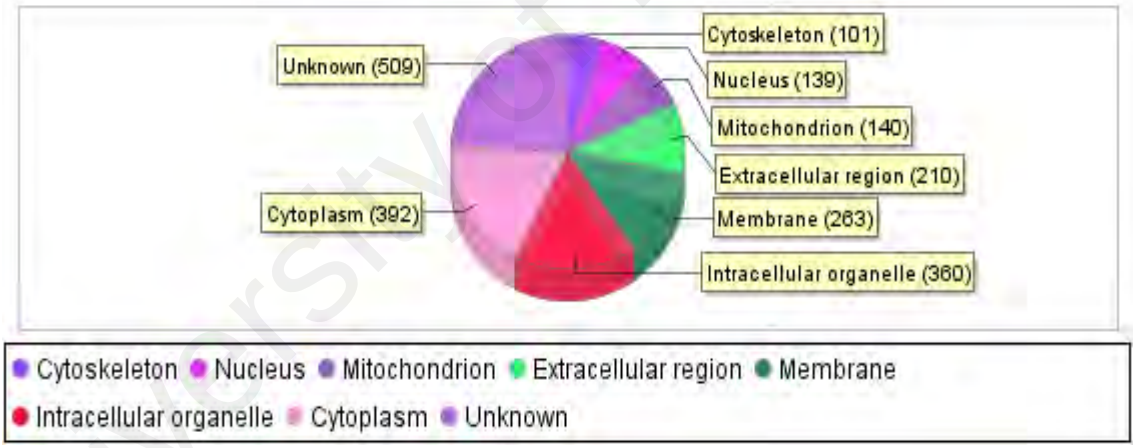


Figure 7.4: Gene ontology (GO) analysis featuring cellular localization of the identified hippocampal proteins

7.4 Discussion

7.4.1.1 Functional classification of the significantly regulated proteins

As the significantly regulated proteins are involved in diversified functions, discussions related to them have been appended below under sub-sections of their commonly known functions.

A. Proteins involved in neuronal structure and function

Differential expression of the proteins involved in neurotransmission, synaptic plasticity, neurogenesis, memory and learning related proteins such as neurochondrin, synaptophysin, synapsin-1, synapsin-2, synaptogyrin 3 (Syngr3), 4-aminobutyrate aminotransferase and 14-3-3 protein gamma were observed in the different rat groups. Associative learning and long-term memory related proteins glutathione-S-transferase 3 and tenascin R were up regulated. Synaptic plasticity promoting Ras related protein Rab 5a and nerve growth factor (NGF) signaling Rap-1A were also among the significantly up-regulated group. Similar was the case for the heat shock proteins (HSP) involved in the regulation of neuronal migration (HSP 90-alpha) and apoptosis (HSp 60). Up-regulation was also observed for the proteins involved in post-synaptic excitatory potential (serine/threonine-protein phosphatase, syntaxin 1B), pre- and post-synaptic density (isoform 2 of clathrin coat assembly protein AP180) and tyrosine phosphorylation (hemopexin) in the AD compared to the mushroom-treated (AE) group.

Proteins involved in synaptic organization (neurofascin) and synaptic vesicle budding (ADP-ribosylation factor 1), vesicle mediated transport (syntaxin 1A), neuronal differentiation and development (Dihydropyrimidinase-related protein 1 and 2), axonogenesis (2', 3'-cyclic-nucleotide 3'-phosphodiesterase), axonal choice point recognition (neuromodulin) and axonal transport (neurofilament light polypeptide) were also differentially expressed in the hippocampus of the three rat groups. Beta-soluble

NSF attachment protein (SNAP-β) involved in the regulation of glutamatergic synaptic transmission, disassemble of SNARE complex and synaptic vesicle priming was also up regulated. In addition to these proteins, differentially up regulated expression of glial fibrillary acidic protein (GFAP) was also observed. GFAP is involved in long-term synaptic potentiation, neurotransmitter uptake, neurogenesis, glial and Schwan cell proliferation.

In the present study, down-regulated expression of memory and learning related proteins in the AD group was observed when compared with both C and AE group. However, there was inter-group variation in the extent of fold change in case of the down-regulated proteins. In the AD versus control group, down regulated expression of the proteins involved in dopamine decarboxylation, clusterin (stimulator of Aβ and NFT), neuromodulin, neurofascin and NCAM 1 was observed. In the AD versus AE groups, α-synuclein, synaptogyrin 1 and transgelin-3 were among the most important down regulated proteins. In addition to these, neuromodulin, excitatory amino acid transporter and park 7 were the mostly up regulated proteins in the AE versus C groups.

Following are the AD related proteins differentially expressed in the present study

Syntaxin-1A: Syntaxin-1A regulates vesicular trafficking during exocytosis and trans-membranal protein insertion (Sudhof, 2004). Decreased expression of syntaxin-1 A in the AD rats might have affected synaptic functions (Shevchenko *et al.,* 2012). Mushroom treatment might have synaptic function improving effect as up-regulated expression of this protein has been observed in the mushroom-treated group.

Synaptogyrin-1: Synaptogyrin-1 is involved in maintaining short- and long-term synaptic plasticity. Level of hippocampal syntaxin-1 A and synaptogyrin-1 had been found to be reduced in line with AD progression (Saetre *et al.,* 2011). Its lowered

expression in the AD and increased level in the AE rats reveals the ameliorating effect of *G. lucidum*.

Neuromodulin (GAP-3): Neuromodulin is a neuronal growth and neurite forming protein whose level decreases in AD brains. As an CSF biomarker, its lowered level has been found in other studies also (Babić *et al.,* 2014; Blennow, 2004; Bogdanovic *et al.,* 2000). However, in the present study, mushroom treatment (AE) has been found to increase the abundancy of this protein as is evidenced by the up-regulated expression.

Neural cell adhesion molecule (NCAM): NCAM plays important role in brain development and increased level of NCAM 1 in transgenic AD mouse model (Tg2576) and of NCAM 2 in human AD patients have been reported (Shevchenko *et al.,* 2012; Todaro *et al.,* 2004). Current findings were compatible with the previous ones as *G. lucidum* treatment helped increase the abundancy of NCAM.

Endophilin A1: Endophilin A1 is a membrane bending protein involved in CNS development, apoptosis, signal transduction and microtubule based movement. AD rats' hippocampi showed decreased expression while the control and AE rats experienced increased expression of endophilin A1 in the present study. In the temporal neocortex of the AD patients, decreased level of endophilin A1 has been observed (Musunuri *et al.,* 2014).

Clathrin: Clathrin group of proteins are involved in neuronal secretory functions and synaptic maintenance (Cao *et al.,* 2010). AD pathogenesis involves altered clathrin-associated membrane trafficking resulting in neurodegeneration (Cao *et al.,* 2010). Between the light and the heavy chains of clathrin, impaired distribution of the former has been linked with the AD pathogenesis (Cao *et al.,* 2010; Nakamura *et al.,* 1994).

Similar pattern was observed for the AD rats in the current experiment and an increasing trend following *G. lucidum* treatment (AE).

Septin: Septins are GTP-binding proteins found to be co-localized with the NFT in the AD brains (Kinoshita *et al.,* 1998). Differential expression of septins have been observed in the present study. Contrary to the findings of Shin *et al.* (2004) and Musunury *et al.* (2014), down-regulated expression of septin-2 had been observed in the AD versus AE group of the present study. As septin-2 is involved in synaptic plasticity, its down-regulation in the AD versus AE group bears supports to the synaptic dysfunction associated with the AD pathogenesis. However, its down-regulated expression in the CE versus AE group is of intriguing. Interestingly, the isoform-2 of septin-5 had also been found down-regulated in both the AD versus AE and AE versus C groups which is compatible with the findings of Musunury *et al.* (2014), who found similar expression status in the temporal brain neo-cortex of the AD patients. Thus, differential expression even of the different isoforms of the same protein might be implicated in the AD pathogenesis and corresponding modulation demands differential therapeutic strategy. Current observation of the *G. lucidum* HWE upon differential expression of different isoforms of septin is a unique finding that demands further studies.

UCH L1: Ubiquitin carboxyl-terminal hydrolase L1 (UCH L1) is an important enzyme for maintenance of cognitive and synaptic functions (Gong *et al.,* 2006). Conflicting information regarding its expression has been documented in different AD cases. Though most of the researchers have noticed decreased and oxidatively modified form of UCH L1 in AD subjects, Sultana *et al* (2007), reported its 1.31-fold increase in the AD brain hippocampi (Castegana *et al.,* 2002; Choi *et al.,* 2004, Minjarez *et al.,*

2013). Increased expression of UCH L1 was observed in the *G. lucidum*-treated (AE) group.

Soluble NSF-attachment protein beta (SNAP-β): N-ethylmaleimide sensitive fusion proteins (NSF) are the part of APP and overexpressed in AD (Cottrell *et al.,* 2005). Soluble NSF-attachment proteins are involved in intracellular membrane fusion and vesicular trafficking. Among α-, β- and γ- SNAPs, α - and γ- SNAPs are expressed in different tissues while the β-SNAP is brain specific. In AD brain, differential expression and oxidized form of SNAP-β had been detected through redox proteomics (Butterfield *et al.,* 2006b).

Neuropolypeptide h3: Neuropolypeptide h3 is a cholinergic neuro-stimulating peptide that falls in the phosphatidyloethanolamine binding protein group and is also known as Raf-kinase inhibitor protein (RKIP) and/or hippocampal cholinergic neurostimulating peptide (HCNP) (Castegna *et al.,* 2003). Our finding of down-regulated neuropolypeptide h3 is in agreement with those of Butterfield (2004). Oxidatively modified loss of function of neuropolypeptide h3 impairs phospholipid asymmetry that might be involved in extrusion of phosphatidyl serine to the outer membrane of neuron and signal for apoptosis and cause neuronal death (Daleke & Lyles, 2000). Also, neuropolypeptide h3 mediated stimulation of acetylcholine esterase (AchE) becomes compromised and this effect is heightened when HNE interacts with AChE in presence of Aβ (1-42) in synaptosome (Butterfield & Lauderback, 2002; Morris, 2002). Thus, in AD brains, neuropolypeptide h3 is linked with cholinergic abnormalities and altered lipid metabolism that are the early events in AD pathogenesis (Butterfield, 2006).

Annexin: AD rats showed increased expression of annexin in the hippocampi. Previous studies have linked increased plasma annexin5 with increased AD risk (Sohma

et al., 2014). Transgenic AD mice (Tg2576) also expresses increased annexin in the brain cortex (Yamaguchi *et al.,* 2010).

Glycogen synthase kinase 3 β (GSK3β): Glycogen synthase kinase 3 β (GSK3β) is a serine/threonine kinase having diversified regulatory functions ranging from glycogen metabolism to gene transcription. Overactivity of GSK-3β has been linked with elevated Aβ production, tau hyperphosphorylation and impaired memory and learning activities (Kremer *et al.,* 2011). Memory affecting mechanism of GSK-3β involves interruption of intra-neuronal anterograde mitochondrial transportation and causation of "mitochondrial traffic jam" (Correia *et al.,* 2016; Zempel *et al.,* 2010).

Serine/Threonine protein phosphatase: Serine/Threonine protein phosphatase negatively regulate memory and learning abilities by impairing synaptic plasticity and LTP (Jouvenceau *et al.,* 2006). Up-regulation of serine/threonine protein phosphatase in the AD rats might contribute towards impaired memory and learning performance in the present study.

Serine protease inhibitors (serpins): Serine protease inhibitors (serpins) regulate proteolytic processing of proteins. Previous studies indicated their increased level in plasma and CSF of AD patients (Nielsen *et al.,* 2007). We also observed increased expression of serpins (α1-antitrypsin) in the AD rats' hippocampi. Alpha 1-antitrypsin (A1AT) has been reported to be co-localize with Aβ plaques and NFTs (Gollin *et al.,* 1992).

B. Proteins involved in metabolism

In the present study, the highest number of the identified up-regulated proteins was involved in the metabolic activities for all the three groups (C, AD and AE) (Appendix E, Table 10.1). Most of them were enzymes involved in both anabolism and catabolism

of carbohydrates, proteins and lipids (Appendix E, Table 10.1). Enzymes associated with glucose metabolism (glycolysis, gluconeogenesis and citric acid cycle) were among the most abundantly up-regulated proteins in the present study (Appendix E, Table 10.1). This may be due to the exclusive utilization of glucose by the brain cells as the energy source. Apolipo-protein E (ApoE), the carrier of lipo-proteins, cholesterol and lipo-philic vitamins, was also up-regulated. Indeed, defective ApoE is an important genetic risk factor of hyperlipoproteinemia type III that results in increased plasma TC and TG levels along with impaired lipo-protein (VLDL, LDL and chylomicron) clearance. ApoE4 isoform had been linked even up to 20 times greater risk of late-onset sporadic AD (Hauser & Ryan, 2013).

Glucose is the main source of energy for the brain cells and alteration in energy production has been linked with AD pathogenesis (Ferreira *et al.,* 2010; Parihar & Brewer, 2007). Present findings of the increased glycolytic enzymes (Hexokinase-1, Glucose-6-phosphate isomerase, Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), L-lactate dehydrogenase B chain), pyruvate metabolizing enzymes (Pyruvate dehydrogenase E1 component subunit alpha and beta), lactate metabolizing enzyme (L-lactate dehydrogenase B chain) are indicative of decreased utilization of glucose for energy production in the AD brains. Glycolytic enzyme GAPDH has other activities such as OS sensor of apoptosis and AD (Butterfield *et al.,* 2010). Minjarez *et al* (2013) reported differential expression of GAPDH, ubiquitin carboxyl-terminal hydrolase isozyme L1 (UCHL-1) and transferrin as the AD biomarker. UCHL-1 is linked with OS and transferrin with iron regulatory processes. Findings of the present study are in agreement with those of Boyd-Kimball *et al.,* (2005), David *et al.,* (2006), Perluigi *et al*. (2009), Shin *et al.,* (2004), Tilleman *et al.,* (2002) and Musunuri *et al.,* (2014). Differential expression of ATP synthase α, β and γ chain has been reported by Shin *et al.,* (2004).

Brain utilizes glucose as the main source of energy and about 20% of total glucose metabolism and 30% of inspired oxygen is utilized by the brain (Vannucci & Vannucci, 2000). Neurons are solely dependent on glucose for energy production and even minuscule impairment of glucose metabolism could disrupt neuronal activities including memory (Butterfield *et al.,* 2012). Altered expression of the aforementioned proteins, mostly enzymes involved in glucose metabolism, might have affected energy generation, ionic balance, membrane asymmetry and transporters in the current experimental subjects. Consequently, impaired neuronal connectivity, transportation, signal transduction and neurotransmission might have resulted in declined cognitive and memory functions of the AD rats (Butterfield *et al.,* 2012).

Oxidative modification of the energy metabolism related proteins such as creatine kinase, α-enolase and triosephosphate isomerase in the AD brains have been noticed by Butterfield *et al.,* (2004). Enolase, one of the regulatory enzymes of glycolysis, is among the differentially expressed proteins in the AD subjects in the present study. Besides its effect upon conversion of 2-phosphoglycerate to phosphoenolpyruvate through dehydration in glycolysis, α-enolase acts as neurotrophin, stress protein and plasminogen binding protein (Butterfield & Lange, 2009). It is among the most commonly upregulated and early oxidized proteins in the brains of both MCI and AD patients (Butterfield & Lange, 2009; Butterfield & Sultana, 2007; Takano *et al.,* 2013). Impaired glucose metabolism and ATP generation in the AD brain has been linked with the nitration of the glycolytic enzymes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and α enolase (Castegna *et al.,* 2002; Vanhanen & Soininen, 1998). Also, oxidation directed loss of GAPDH activity had been associated with increased Aβ (1-42) level in AD brain (Boyd-Kimball *et al.,* 2005).

Carbonic anhydrase II (CAH II) catalyzes the reversible hydration of CO_2 and important in glycolysis, lipid metabolism, CSF production, maintenance of pH and fluid balance (Sly & Hu, 1995). In CNS, CAH II is linked with oligo-dendrocytes and protoplasmic astrocytes (Sly & Hu, 1995). Its deficiency had been reported to cause cognitive decline, mental retardation, cerebral calcification, renal tubular dysfunction and osteoporosis (Sly & Hu, 1995). Its oxidative modification mediated impaired pH balance in the neurons had been linked with AD pathogenesis (Sultana *et al.,* 2006).

Beyond energy metabolism, decreased rate of glycolysis in the AD subjects has been linked with reduced generation of acetylcholine that impairs memory and learning abilities (Poon *et al.,* 2004).

Among the down-regulated enzymatic proteins, phosphoglycerate mutase 1 and acyl coenzyme A thioesterase 1 and 2 were most notable.

Phosphoglycerate mutase 1: Phosphoglycerate mutase 1 interconverts 3-phosphoglycerate and 2-phosphoglycerate in glycolysis and gluconeogenesis. In the AD rats, phosphoglycerate mutase 1 was found to be down-regulated. Decreased expression of this enzyme in the AD hippocampi and in neurons and astrocytes have been noticed by some previous researchers (Díez-Vives *et al.,* 2009; Sultana *et al.,* 2007).

Acyl coenzyme A thioesterase 1 and 2: Downregulation of acyl coenzyme A thioesterase 1 and upregulation of the acyl coenzyme A thioesterase 2 in the AD rats' hippocampi were observed in the present study. Similar type of observation has been reported for the SMP8 mouse model of AD (Díez-Vives *et al.,* 2009). The isoform 1 is cytoplasmic and 2 is mitochondrial though both are involved in the hydrolysis of acyl-coenzyme As to the free fatty acids (FFAs) and coenzyme A. FFAs themselves and their activated acyl co-A forms participate in numerous metabolic processes.

C. Proteins involved in mitochondrial respiratory chain and ATP generation

In the AD brains, lowered expression of five proteins involved in mitochondrial respiratory chain and ATP generation was observed. They were either structural subunits of ATP synthase or proton-gradient maintaining functional units.The most notable was the vacuolar (V) ATPases. V-ATPases sub-units are involved in regulation of cellular transduction processes and pH. V-ATPases sub-expressed in the AD rats were V0-subunit A (ATP6V0A1), V1-subunit D (ATP6V1D) and V1-subunit F (ATP6V1F). Our findings are relevant with those of Minjarez *et al.,* (2016).

Mitochondrial abnormality including dysfunctional and lowered level of mitochondrial enzymes is another important feature of AD pathogenesis (Bosetti *et al.,* 2002; Hirai *et al.,* 2001). APP and Aβ can affect mitochondrial respiratory chain through decreased activity of some enzymes and increasing ROS production (Suzanne & Tong, 2014). Proteins and enzymes involved in energy metabolism are highly oxidized and functionally altered in AD brains that correspond to the reduced glucose metabolism; mitochondrial reduced functioning and OS interplay in AD pathogenesis (Hoyer, 2004; Sultana *et al.,* 2006). Aβ induced lowered activity of the mitochondrial respiratory chain in the neurons had also been noticed (Hirai *et al.,* 2001; Lovell *et al.,* 2005). Defect in one of the respiratory chain complexes affect another and finally the entire process of energy production become hampered (Morán *et al.,* 2012). Comparatively, complex I is much prone to OS due to its content of Fe – S cluster. Redox proteomics studies had identified nitrated form of mitochondrial membrane associated voltage dependent anion channel (VDAC) and ATP synthase alpha chain in the AD brains (Sultana *et al.,* 2006c). Supposedly, ATP production and normal functioning of the neurons become affected and as a consequence the brain cells die. ATP synthase α is a part of the complex V of the mitochondrial respiratory chain and is located in the inner membrane. Its increased accumulation in the cytosol linked with

NFT had been observed in AD subjects (Sergeant *et al.,* 2003). Mitochondrial abnormality affects APP cleavage leading towards enhanced intra-neuronal Aβ accumulation (Busciglio *et al.,* 2002).

Selective defect in the cytochrome c oxidase of the mitochondrial respiratory chain had been linked with reduced energy production and AD complications (Maurer *et al.,* 2000; Ojaimi *et al.,* 1999). Decreased expression was also observerd for cytochrome oxidase that transfers electron from ferrocytochrome c to oxygen in the respiratory chain.

In the normal neurons, about 30% of the neuronal mitochondrial pool is involved in anterograde and retrograde shifting in response to different stimuli (Correia *et al.,* 2016; Misgeld *et al.,* 2007). Overrated mitochondria are transported from neuronal other parts to the pre-synaptic neuronal termini to fulfill increased energy demand. But, in the AD neurons, this mitochondrial transportation becomes affected due to intra-neuronal Ca2+dyshomeostasis and microtubular disassembly (Correia *et al.,* 2016).

D. Proteins involved in cellular stress responses

In the AD and the mushroom fed rat groups, proteins of both enzymatic and non-enzymatic types were significantly regulated and differentially expressed. The proteins were of overlapping functionalities against oxidative, nutritional and environmental stressors. Heat shock proteins (HSPs) of variable molecular weight and functions were among the mostly regulated ones.

a. Role of HSP in AD

Proteins performing the role of the molecular chaperones such as the heat shock proteins (HSPs) are involved in different stress responses. Altered expression of different HSPs in the present study demonstrate their altered levels in the AD

pathogenesis and corresponding amelioration in the respective subjects. As AD pathogenesis involves protein misfolding, increased chaperones are expressed in AD brains in response to Aβ and tau proteins (Blair *et al.,* 2013; van der Putten & Lotz, 2013). Increased expression of heat shock 70kDa protein 12A (Predicted), isoform CRA_a; heat shock cognate 71 kDa protein, HSP 90 – α, HSP 90 – β, and mitochondrial HSP 60 kDa were noted in the present study that are indicative of different mode and extent of the experimental subjects in response to different stressors. Current findings are in agreement with those of Yao *et al*. (2007). Oxidative modification and lowered expression of the heat shock cognate 71 in the AD brains have been noticed by Butterfield *et al.,* (2004). These observations indicate that excessive load of the oxidatively modified, ubiquinited, HNE-bound proteins disrupt the proteasomal degradation of the dysfunctional proteins resulting in increased accumulation of damaged proteins and neurodegeneration (Keller *et al.,* 2000).

Heat shock proteins (HSP) act as molecular chaperones and their role in inhibiting Aβ aggregation, facilitating Aβ degradation and clearance have been documented (Evans *et al.,* 2006; Peterson & Blagg, 2009). They are capable of inhibiting Aβ production and aggregation in a concentration dependent way. Binding with the Aβ in an ATP-dependent or –independent way, HSP 40/HSP 70 and HSP 90 can guard against Aβ aggregation (Evans *et al.,* 2006; Peterson & Blagg, 2009).

Although Aβ-induced expression of HSP 70 have been found to be variable, their activity has been reported to go beyond action against protein misfolding towards transportation of the misfolded proteins up to the proteasome for degradation (Lam *et al.,* 2013; Takano *et al.,* 2013).

Exogenous HSP 32, HSP 70 and HSP 90 act as the facilitators of Aβ clearance that has been supposed to be mediated through activation of the microglial phagocytosis

(Kakimura *et al.,* 2002). Their role in Aβ degradation has also been reported and the process might have involved activation of the NF-κB and p38 MAPK through the Toll-like receptor-4 (TLR4) pathway (Kakimura *et al.,* 2002). Degradation of the phosphorylated tau by the Hsp90 - carboxy terminus of Hsp70-interacting protein (CHIP) complex and thus lowered propensity towards NFT formation has also been observed (Dickey *et al.,* 2007). Inhibition of HSP 90 showed reduced activation of the kinases (GSK3β, CDK5, Akt, p35 and p25) involved in tau phosphorylation and thus lowered NFT formation and increased degradation (Luo *et al.,* 2010) . Also, decreased serum level of HSP 90 has been observed in AD patients. In line with this, administration of HSP 90 inhibitors have been found to prevent Aβ-induced neurotoxicity by activating the heat shock factor 1 and increasing HSP 70 level (Ansar *et al.,* 2007; Gezen-Ak *et al.,* 2013).

b. Role of Aldehyde dehydrogenase 2 (mitochondrial) in AD

Aldehyde dehydrogenases (ALDH1, ALDH2 and ALDH3), oxidatively detoxify aldehydes into carboxylic acids and its deficiency acts as AD promoting (Kamino *et al.,* 2000). ALDH usually metabolize 4-hydroxy-2-nonenal (HNE) and upregulation of HNE and acetaldehyde are indicative of reduced ALDH activity and increased vulnerability towards AD (Kamino *et al.,* 2000; Picklo *et al.,* 2002). Specifically, aldehyde dehydrogenase 2 (mitochondrial) detoxifies aldehydes in the mitochondria whose level increases with aging and in AD. Increased expression of aldehyde dehydrogenase 2 (mitochondrial) in the AD rats correspond towards excessive OS in the AD rat brains (Picklo *et al.,* 2001). Binding of aldehyde dehydrogenase with Aβ and Aβ binding peptide has been ascribed as an important aspect of AD pathogenesis. Thus, inhibition of this interaction seems a potent target of AD therapeutics (Borger *et al.,* 2013; Yao *et al.,* 2011).

E. Proteins involved in cholesterol metabolism and transportation

a. Apolipoproteins

In rats, apolipoprotein A-IV is the major constituent of HDL and chylomicrons and involved in various functions including cholesterol efflux, stimulation of lipoprotein lipase and cholesteryl ester transfer protein as well as anti-oxidtive and anti-inflammatory effects (Duka *et al.,* 2013). Apolipoprotein A-IV was downregulated in the AD groups in the present study. Obtained findings are in line with those of Cui *et al.,* (2011) who showed that genetic extirpation of apolipoprotein A-IV aggrandizes Aβ plaque deposition, aggravates neuronal loss and impairs spatial memory in the AD mouse models. Normally, apolipoprotein A-IV functions as the Aβ clearing agent and *in vitro* studies showed that they co-localize with Aβ plaque in the brain (Cui *et al.,* 2011). Down-regulation or loss of function of this lipo-protein impairs the Aβ clearing process that ultimately leads towards Aβ deposition (Cui *et al.,* 2011).

Down-regulated expression of apolipoprotein A-I (apo A-I) was observed in the hippocampus of the AD versus control rats. Current findings are compatible with those of Liu *et al.,* (2006) who demonstrated that AD patients' hippocampi and serum possess lowered apo A-I compared with their age matched controls. Redox proteomics has identified apo A-I as one of the most promising biomarker of neurodegenearation (Keeney *et al.,* 2013). In this regard, upregulated expression of apo A-I found in the AE versus control group of the present study is indicative of neurohealth giving effect of the *G. lucidum* HWE.

In the present study, up-regulated expression of apolipoprotein E (apo E) was noticed in the AD rat groups. Apolipoprotein E is the principal carrier of cholesterol and aids in lipid transportation and injury repairment in the brain (Liu *et al.,* 2013). Individuals with its ε4 allele are at increased (about 8 fold) risk of AD pathogenesis than those with the

ε3 allele and ε2 allele possessing people are at rather protection against AD generation (Corder *et al.,* 1993). Though, any specific allele has not been detected in the present study but the total apolipoprotein E, observed findings are compatible with some established knowledge that apolipoprotein E is involved in AD pathogenesis (Sizova *et al.,* 2007). Like that of binding with the cell surface receptors, apolipoprotein E transfers lipids to the hydrophobic amyloid-β (Aβ) peptide and aids formation of Aβ, cerebral amyloid angiopathy, generation of OS, neurotoxicity and neurodegeneration in AD (Ellis *et al.,* 1996). They also stimulate Aβ aggregation and participate in metabolism, neuro-signaling and neuroinflammation (Kim *et al.,* 2009). Recently, Choi *et al.,* (2015) had shown that brain level of Apo E and Aβ are regulated by the low-density lipoprotein receptor (LDLR) degrading E3 ubiquitin ligase, idol. Idol had been implicated as the principal regulator of LDLR degradation and an inhibitor to the clearance of brain level of both Apo E and Aβ (Choi *et al.,* 2015). Its absence in AD transgenic mice increased brain level of LDLR as well as decreased the levels of both Apo E and Aβ as well as improved Aβ-induced neuroinflammation (Choi *et al.,* 2015). Genome wide association studies (GWAS) also have linked Apo E with LDLR and CLU (clusterin) *inter alia* as the cholesterol dysregulatory factors of AD pathogenesis (Tosto & Reitz, 2013). Thus, increased expression of apolipoprotein E might have been among the most favored factors of AD pathogenesis in the current experiment. Likewise, decreased expression in the control and *G. lucidum* HWE fed rats bear testimony to the AD enhanced protection against AD pathogenesis in the respective rat groups.

b. Apolipoprotein J (Clusterin)

Apolipoprotein J (Clusterin) is a glycoprotein normally expressed in different tissue but highly in the brain and with aging and AD pathogenesis. Clusterin gene, CLU, is the

third most risk gene linked with late onset AD and it is responsible about 9% of AD risks (Bertram *et al.,* 2007). Single nucleotide polymorphism of clusterin gene (CLU) heightens AD risk (Bertram *et al.,* 2007). Hong *et al.,* (2013) reported its elevated expression in the hippocampus of the 5XFAD mice. Clusterin affects AD pathogenesis in various ways such as promoting Aβ aggregation, neuro-inflammation, apoptosis and controlling lipid metabolism and cell cycle regulation and also through epigenetic mechanism (Yu & Tan, 2012). Binding with Aβ, clusterin facilitates Aβ transportation in the blood in chaperone-like mechanism and thus clusterin has been regarded as a blood biomarker of AD (Weinstein *et al.,* 2016). In this way, clusterin also acts as the Aβ clearing agent (Yu & Tan, 2012). Thus, compelling conclusion is yet to be drawn about the effect of clusterin on AD and a state-of-the time explanation is that at low ratio of clusterin to Aβ, clusterin acts as the Aβ clearing agent but as the ratio changes, clusterin adds injury to AD pathogenesis (Yerbury *et al.,* 2007).

c. ACAT (acyl-coA : cholesterol acyltransferase)

Elevated expression of ACAT (acyl-coA - cholesterol acyltransferase), enzyme involved in cholesterol esterification, points towards altered cholesterol metabolism in the AD rats (Chang *et al.,* 2001; Puglielli *et al.,* 2001). Impaired ACAT and cholesterol metabolism have previously been linked with increased Aβ production and neurotoxicity (Puglielli *et al.,* 2001; Sizova *et al.,* 2007; Sjögren *et al.,* 2006).

d. Fibrinogen

Fibrinogen was overexpressed in the AD rats' brain of the current study. Fibrinogen is the precursor of fibrin, the primary protein involved in blood clotting. Normally, fibrinogen circulates in plasma and can not cross the blood brain barrier (BBB). But damaged vasculature of the AD subjects allows its accumulation in the extravascular

spaces that further intensifies AD pathogenesis (Cortes-Canteli *et al.,* 2012). Elevated level of fibrinogen has been linked with AD propensity. Indeed, interaction between Aβ and fibrinogen produces structurally modified fibrin oligomer that causes altered clot formation and vasculopathies (reduced cerebral blood flow, neuroinflammation and neurodegeneration) associated with AD (Ahn *et al.,* 2010; Cortes-Canteli *et al.,* 2012). Oxidative modification of the plasma isoforms of fibrinogen γ-chain precursor and α1-antitrypsin precursor had been linked with AD pathogenesis (Choi *et al.,* 2002).

e. Transthyretin

Transthyretin (TTR) co-localizes Aβ in the human AD brains and has been suggested to sequester Aβ, prevent Aβ aggregation and fibrillation and thus protective against AD (Li & Buxbaum, 2011) . Downregulation of transthyretin in the AD rats bears proof of increased Aβ plaque formation. On the contrary, *G. lucidum* treatment showed boosting up effect upon TTR expression as the extent of its down-regulation was less in the AE group.

f. Clathrin

Defected clathrin-mediated endocytosis has been reported to be associated with AD pathogenesis (Cao *et al.,* 2010; Wu & Yao, 2009). Clathrin coat consists of three heavy chains (~190 kDa) connected in a hub and each heavy chain containg an axial light chain (~25 kDa). The light chain has been mostly been implicated in AD pathogenesis (Nakamura *et al.,* 1994). They contain multiple adaptors (AP) to bind to the plasma membranes among which AP2 is clathrin-coated vesicle specific and AP 180 is synaptic vesicle specific. Increased expression of the clathrin heavy chain and isoform 2 of clathrin coat assembly protein AP180 were found in all the rat groups. Though present findings are seemingly in contrast with some others, it can not be claimed that the

clathrin-mediated endocytosis remain unaffected in the AD subjectspresent study (Cao *et al.,* 2010). May be, exogenous Aβ bound to the plasma membrane needed excessive rate of clathrin-mediated endocytosis and to fulfill this demand, expression of the clathrin heavy chain and isoform 2 of clathrin coat assembly protein AP180 had to be triggered (Wu & Yao, 2009).

F. Proteins involved in Ca^{2+}transportaion, homeostasis and signaling

Dysregulation of Ca^{2+} metabolism and signaling has been linked with neurodegeneration and AD pathogenesis (Brawek & Garaschuk, 2014). Differential expression of the proteins involved in Ca^{2+} transportation, homeostasis and signaling such as calmodulin, CamK2a and CamK2b were noticed in the present study.

a. Calmodulin

Calmodulin is a Ca^{2+}- binding protein involved in Ca^{2+}-mediated signaling and maintenance of ion channel and enzymatic (kinases and phosphatases) activities (Brawek & Garaschuk, 2014). Calmodulin is a biomarker of AD whose increased expression and its binding proteins are associated with AD pathogenesis (O'Day *et al.,* 2015). AD rats showed upregulated expression of calmodulin. Upregulated expression of calmodulin in the AD group of the present study points towards Ca^{2+} metabolism dysregulatory state of the AD rats compared with the C and the AE rats.

b. CamK2a

Ca^{2+}/calmodulin-dependent serine/threonine protein kinase (Camk2a) is highly important for maintenance of the glutametargic synaptic plasticity (Zhou *et al.,* 2010). Its role in spatial learning becomes also evident from its supporting role towards NMDAR-dependent LTP in the hippocampus (Molnár, 2011). Through Ca^{2+}/calmodulin

kinase II signaling, Camk2a also regulates neurotrophin-3 and BDNF secretion from the hippocampal post-synaptic neurons (Kolarow *et al.,* 2007). In the present study, the CamK2a showed downregulated pattern compared with their control and mushroom-treated counterparts. Current findings are in line with those of Yamasaki *et al.* (2008) and Zhou *et al.* (2010). Its deficiency has been shown to hamper neuronal development and dentate gyrus formation as well as behavioral alteration (Yamasaki *et al.,* 2008). On the other hand, up-regulated expression of CamK2a has been linked with improved cognitive performance of the SMP8 mice. Its stimulatory role towards the ubiquitin-proteasome system has also been documented (Djakovic *et al.,* 2009).

c. Annexins (Annexin-1, -5 and -6)

Annexins (1,5 and 6) have been found to be up-regulated in the AD rats. Observed increased annexin expression in the AD rats' hippocampi is compatible with those of Yamaguchi *et al.,* (2010), Sohma *et al.,* (2014) and Hondius *et al.,* (2016). Annexins are intracellular Ca^{2+}-respondents capable of binding with membrane phospholipids and participate in membrane trafficking, endo- and exo-cytosis (Hondius *et al.,* 2016; Lizarbe *et al.,* 2013).

Aβ-induced Ca^{2+} dysregulation (increased intracellular level) hyperactivates c-Jun N-terminal kinase (JNK), cyclin-dependent kinase 5 (CDK5), tau phosphorylation and disrupts microtubule network (Correia *et al.,* 2016; Zempel *et al.,* 2010). It leads towards mitochondrial trafficking defects that impair the normal movement of mitochondria across the microtubules and thus cause "mitochondrial traffic jam" in the AD neurons and affects neuronal functions (Correia *et al.,* 2016). Thus, intracellular Ca^{2+}dyshomeostasis might be among different mechanisms involved in disrupted neuronal activity and corresponding impaired memory of the AD rats in the present study.

G. Proteins involved in signal transduction

a. 14-3-3 Proteins (Ywhag)

In the current proteomics experiment, up-regulated expression of the 14-3-3 proteins (ζ/δ, θ, η, γ, β/α) were found in the AD rat brains. 14-3-3 proteins constitute about 1% of total soluble proteins of the normal brain. They participate in signaling through binding with the phospho-serine containing proteins and can regulate the activities of the kinases, phosphatases and trans-membrane proteins (Foote & Zhou, 2012). Thus, they mediate diversified activities involving neuronal plasticity, neurotransmission, neurite outgrowth generation and neurogenesis (Fu *et al.,* 2000). In AD brains, they have been found to be closely associated with tau and aid in the formation of the NFTs (Foote & Zhou, 2012). Differential expression of 14-3-3 proteins β/α, ζ/δ and ε had been observed in the temporal neocortex and other parts of the brains of the AD patients (Andreev *et al.,* 2012; Musunuri *et al.,* 2014). In the AD hippocampi, both intra- and extra-cellular expression of the 14-3-3 proteins have been detected. Among different isoforms, the highest immunoreactivity towards NFT has been observed for the 14-3-3ζ (Thomas *et al.,* 2005). 14-3-3ζ – mediated tau phosphorylation involves protein kinases such as glycogen synthase kinase-3 beta (GSK3β) (Gao *et al.,* 2014). GSK-3β hyperactivity impairs mitochondrial intra-neuronal anterograde movement and causes "mitochondrial traffic jam" and disrupts neuronal activities (Correia *et al.,* 2016; Zempel *et al.,* 2010). 14-3-3ζ also binds with δ-catenin and disrupts the formation of the adherens junction complex that compromises the neural structure and cognitive performance (Matter *et al.,* 2009). Also, interaction of δ-catenin with pre-senilin 1 is an important stimulator of the wnt signaling and thus of AD pathogenesis and neuronal under-development (Zhou *et al.,* 1997). Protective effect of the 14-3-3 proteins towards α-synuclein driven dopaminergic neurotoxicity has been found to be mediated through

Lrrk2 (leucine-rich repeat protein kinase 2) and Syna (Abbott & Nelson, 2000; Li *et al.,* 2011).

Besides signaling, the 14-3-3 proteins participate in the regulation of stress response, redox mechanisms, cell cycle, apoptosis and cytoskeletal maintenance (Thomas *et al.,* 2005). In addition to the different brain regions, their increased expression has been observed in the CSF also (Jayaratnam *et al.,* 2008).

b. VDAC (Voltage-dependent anion selective channel 1)

Voltage-dependent anion selective channels (VDAC1 and VDAC 2) are mitochondrial porins involved in transportation of ATP and Ca2+and in apoptotic signaling (Reddy, 2013). Its altered expression has been noticed in AD and other NDs and in mitochondrial dysfunctions. However, conflicting expression status of VDAC 1 has been reported for different AD patients and model animals (Cuadrado-Tejedor *et al.,* 2011; Manczak & Reddy, 2012; Yoo *et al.,* 2001).

c. SLC12A5

SLC12A5 are neuronal K^+/Cl^- symporter involved in maintaining intra-neuronal low Cl^- concentration (Cherubini *et al.,* 2012). It mediates neuronal excitotoxicity and synaptic inhibition (Gauvain *et al.,* 2011). Its increased expression is linked with the glutamate transporter Slc17a7 that actively participates in GABAergic neurotransmission (Gauvain *et al.,* 2011).

Oxidative modification and lowered expression of the excitotoxicity-linked proteins such as glutamine synthetase, glutamate transporter – excitatory amino acid transporter 2 (EAAT2) in the AD brains have been noticed by Butterfield *et al.,* (2004). Aβ (1-42) induces increased 4-hydroxy-2-trans-nonenal (HNE) generation through lipid

peroxidation and HNE oxidatively modify glutamate transporter EAAT2 and impair transporter function (Lauderback *et al.,* 2001; Mark *et al.,* 1997; Masliah *et al.,* 1996). Its coupled inactivity with glutamate synthetase result in accumulation of excessive glutamate outside of the neuron that stimulates NMDA receptor driven increased Ca2+influx and compromised LTP and neurodegeneration (Kim *et al.,* 2011).

H. Proteins involved in apoptosis

Apoptosis is an important feature of neuronal and synaptic cell losses. Decreased levels of the apoptosis regulatory enzymes found in the present study are distinct hallmarks of AD pathogenesis (Chou *et al.,* 2011).

a. Peptidyl prolyl cis-trans isomerase (Pin1)

In the AD versus AE groups, significantly down regulated pattern of the peptidyl prolyl cis-trans isomerase (Pin1) was observed. Targeted proteomic search for oxidized protein in the AD hippocampus had reported the protein peptidyl-prolyl isomerase, Pin1 to be oxidized and significantly down-regulated (Sultana *et al.,* 2006). Normally, Pin1 is involved in dephosphorylation of tau protein, cell cycle regulation and in protection against neurodegeneration (Liou *et al.,* 2003; Lu *et al.,* 1999). Pin1 acts as a chaperone and isomerizes the pSer/Thr-Pro peptide bond and thus participates in protein folding, assembly, cellular transportation and apoptosis (Butterfield *et al.,* 2006a).

Beyond glycolysis, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is also involved in neuronal apoptosis (Chuang *et al.,* 2005; Cumming & Schubert, 2005). In the cortical neurons, Aβ-mediated increased aggregation of GAPDH leading towards neuronal death emanated from the chronic Aβ exposure have been documented

(Cumming & Schubert, 2005). Non-native GAPDH is considered as a component of the amyloid and its binding to the GAPDH reduces the enzyme's activity and to complement the reduced functioning and to fulfill energy demand, AD brains might have triggered the increased expression of GAPDH (Mazzola, J. L. & Sirover, 2001; Naletova *et al.,* 2008; Rhein *et al.,* 2009).

b. Ppp2ca (protein phosphatase) Protein

Protein phosphatase (Ppp2ca) is involved in dephosphorylation of tau, whose hyperphosphorylation causes NFT formation (Qian *et al.,* 2010). Thus a balance occurs between protein kinase and phosphatase in the normal state (Martin *et al.,* 2013). Up-regulation of Ppp2ca is indicative of increased protein phosphatase 2A activity and thus ameliorating effect towards AD pathogenesis (Manavalan *et al.,* 2013).

I. Proteins involved in neuronal cytoskeleton maintenance

Derangement of neuronal cytoskeleton through microtubule disassembly is an important feature of neurodegeneration (McMurray, 2000). STRING analysis revealed strong networks among microtubule assembling the cytoskeletal proteins such as tubulin, β-actin and keratin.

a. Tubulin

AD neurons suffer from disrupted microtubule structure and functioning (Butler *et al.,* 2007). Tubulin is the main component of microtubule and consists of dimers imparted by the alpha and beta chains. Differential expression of tubulin α -1c, -4a, β -2a, -2B, -3 and -5 chains were observed in the present study. Molecular function based sub-network analysis showed different tubulin chains to be clustered together and deranged in AD. Both animal and human studies have linked decreased level of α and β

tubulin with human AD (Pollack *et al.,* 2003; Poon *et al.,* 2006) . In AD brain, β tubulin becomes abnormally hyperphosphorylated and modified tubulin fails to assemble microtubules. Consequently, microtubule disassemblage leads towards cytoskeletal vulnerability (Vijayan *et al.,* 2001). Recently, microtubular disassembly has been implicated in causing "mitochondrial traffic jam" in the AD neurons as mitochondrial shifting across the "rail-road of microtubule" becomes impeded in the AD brain (Correia *et al.,* 2016)

b. β-Actin

Normally, β-actin is involved in maintenance of cytoskeleton, internal cell motility, neuronal network integrity and aids in memory and learning performances. Its altered expression and oxidized form had been linked with AD pathogenesis (Castegna *et al.,* 2003). Impaired expression of actin is in agreement with the synaptic dysregulation associated with AD and age-related altered cytoskeletal structure, axonal dystrophy, reduced dendritic spines and impaired transport across membranes (Penzes & VanLeeuwen, 2011). Enhanced accumulation of actin enhances tau-governed neurotoxicity (Fulga *et al.,* 2007; Takano *et al.,* 2013).

c. Dihydropyrimidinase-related protein 2 (DRP-2):

Dihydropyrimidinase related protein 2 (DRP2) is involved in regulation of axonal outgrowth and becomes hyperphosphorylated in NFT and its increased level is observed in AD model animals (Sizova *et al.,* 2007). Compared to the normal neurons, AD neurons possess shortened dendrites which is a characteristic of their lowered communication with neighboring neurons (Coleman & Flood, 1987). DRP2 is expressed highly in the developing brains and altered in AD (Castegna *et al.,* 2002; Lubec *et al.,* 1999). It regulates the activity of collapsin that elongates the dendrites,

increases their communicability and repairs the damaged neurons (Castegna *et al.,* 2002; Lubec *et al.,* 1999). Oxidative modification of DRP2 might cause reduced length of the dendrites and communication leading to lowered cognitive performance (Butterfield *et al.,* 2006b).

d. Glial fibrillary acidic protein (GFAP)

Glial fibrillary acidic protein (GFAP) provides structural support to the astrocytes and its elevated level in AD model animals and in human subjects (Sizova *et al.,* 2007; Sultana *et al.,* 2010b). Its differential and reduced expression has been reported in AD animal studies (Korolainen *et al.,* 2005; Shiozaki *et al.,* 2004).

e. RhoA proteins

Ras homolog gene family, member A (RhoA) proteins are involved in cytoskeleton regulatory processes such as dendrite development, axonal extension and protrusion (Fujita & Yamashita, 2014). They also stabilize the Aβ-disrupted microtubules (Fujita & Yamashita, 2014). Aβ increases RhoA-GTPases and decreases neuronal spine production and neural connection both in the cell lines and also in the brains of the transgenic AD models (Minjarez *et al.,* 2016; Petratos *et al.,* 2008). Inhibition of the RhoA/ROCK signaling pathway seems ameliorating towards axonal growth and cognitive decline (Fujita & Yamashita, 2014).

f. Septin

Septins are microtubule associated, filament-forming and GTP-binding proteins that participate in dendritic spine formation and in neurotransmitter release (Ito *et al.,* 2009; Spiliotis, 2010). Like that of Musunury *et al.,* (2014), increased expression of septin -2 and -3 were noted in the AD rats' hippocampi. Its increased expression in the AD rats

might be involved in disrupting microtubular filament formation and associated cytoskeletal derangement in the AD rats (Hanai *et al.,* 2004; Marttinen *et al.,* 2015).

g. Cofilin

Brain cofilin activity reduces with age and in the AD subjects, it goes down aberrantly (Barone *et al.,* 2014). Transgenic mice studies also showed its lowered expression (Tilleman *et al.,* 2002). However, unlike other cytoskeletal proteins, its levels have not been found to be up-regulated in AD ameliorating cases (Barone *et al.,* 2014; Hwang *et al.,* 2015). As cofilin is a regulator of actin, increased cofilin expression points towards increased actin turnover and increased depolymerization of actin filament (Hwang *et al.,* 2015).

h. Dynamin

Dynamin is a neuronal GTPase capable of free entry into and release from the synaptic vesicles (Fulga *et al.,* 2007). Current finding of its decreased expression in the AD rats is consistent with those of Cao *et al.,* (2010), Watanabe *et al.* (2010) and Zhou *et al.* (2010) and Kelly *et al.,* (2005). Aβ-induced depleted dynamin1 level has been found to impair memory in the AD model rats (Watanabe *et al.,* 2010). Although some other findings found its variable level, altered dynamin induced affected neurotransmitter release has commonly been correlated with AD pathogenesis (Kelly *et al.,* 2005; Takano *et al.,* 2013).

i. Gelsolin

Gelsolin is a member of the actin-binding proteins having antioxidative, Aβ binding and fibrillation inhibitory potentiality (Chauhan *et al.,* 2008; Ji *et al.,* 2015). Its overproduction and/or administration showed Aβ lowering effect and thus, gelsolin has

been regarded as an AD therapeutic agent (Carro, 2010). More importantly, administration or overexpression of gelsolin results in significant reduction of amyloid load and decrease of Aβ level in AD transgenic mice (Carro, 2010, Chauhan *et al.,* 2008). Down-regulated expression of gelsolin was noted in the AD rats' hippocampus that is in par with those of Manavalan *et al.* (2013). However, some studies have reported confounding results regarding the expression of gelsolin in AD brains (Güntert *et al.,* 2010; Ji *et al.,* 2009).

Oxidative modification and reduced expression of the neuronal cytoskeletal proteins such as dihydropyrimidinase-related protein 2 (DPYSL 2), β-actinin the AD brains have been noticed by Butterfield *et al.,* (2004).

j. Proteins involved in anti-oxidative defense

Anti-oxidative proteins up-regulated in the mushroom fed rats were mitochondrial superoxide dismutase [Mn], superoxide dismutase [Cu-Zn], glutathione S-transferase pi and mu, glutathione peroxidase 3; peroxiredoxin-1, -2, -5, -6; catalase, isoform 2 of haptoglobin and mitochondrial stress -70 protein.

Mitochondrial superoxide dismutase coverts superoxide anion (O_2^-) into H_2O_2 and thus reduces the deleterious effect of O_2^- (Flynn & Melov, 2013). In the AD patients, its overexpression at the lymphocytic mRNA level has been detected (De Leo *et al.,* 1998). Studies upon AD mouse model has shown that deficiency of this enzyme stimulates Aβ formation, plaque deposition and tau phosphorylation whereas its increased level demolishes Aβ plaque deposition through diminishing the Aβ-42/Aβ-40 (De Leo *et al.,* 1998). Thus, to thwart the OS and to maintain synaptic plasticity, upregulation of superoxide dismutase [Cu-Zn] (Sod1), and mitochondrial superoxide

dismutase [Mn] might have been achieved through feedback mechanism (Manavalan *et al.,* 2013).

Glutathione-S-transferases pi and mu (GSTP1 and GSTM1) are involved in glutathione conjugation and phase II detoxification. The stress regulator Nrf2 regulates these enzymes in response to OS and other stressors. Through protein-protein interaction, GSTP1 lowers the activity of the stress kinase JNK and Cdk5 (Sun *et al.,* 2011).

Catalase decomposes H_2O_2 into H_2O and molecular oxygen and can also inhibit Aβ aggregation (Luo *et al.,* 2014) . On the other side, Aβ-42 can thwart anti-oxidative and other functions of catalase by forming "catalase- Aβ-42" complex and generate ROS. Thus, inhibitors of the "catalase- Aβ-42" complex have been recommended for diminishing the OS generated by this interaction (Habib *et al.,* 2010).

Haptoglobin is another anti-oxidant protein that is either oxidized and/or downregulated in the AD patients (Cocciolo *et al.,* 2012). It also binds with the Apo-A1 of HDL and is involved in reverse cholesterol transportation (Alayash, 2011). Its direct influence on ApoE and Aβ cross-talk has also been reported (Spagnuolo *et al.,* 2014).

In the current experiment, increased levels of peroxiredoxin-1, -2, -5 and -6 in the AD brains were observed that are indicative of increased OS in the AD model rats (Minjarez *et al.,* 2016; Poynton & Hampton, 2014). Peroxiredoxins are thioredoxin peroxidases capable of eliminating hydroperoxides through thioredoxin/thioredoxin reductase. Their oxidative modification has been linked with AD and other NDs (Minjarez *et al.,* 2013).

Thus, increased expression of both enzymatic and non-enzymatic anti-oxidant protein levels in the *G. lucidum* fed rats indicate the OS attenuating capacity associated with AD pathogenesis (Sultana *et al.,* 2007; Yao, Jun *et al.,* 2007).

7.4.1.2 Protein-protein interaction (PPI) findings

In addition to functional, modular and pathway-related insights, PPI maps provide disease specific information. As identification of the target protein in any disease pathogenesis is an important aspect, PPI analyses shed light towards understanding the complex connectivity and identifying the protein of interest for further evaluation and management (Koh *et al.,* 2012).

Based on the analyses of the PPI networks and pathways of the differentially expressed proteins, it is obvious that AD causes a disturbed protein expression affecting the global protein-protein interactive networks and the relevant biological pathways. They have been categorized into functional framework of metabolic process, intracellular signaling cascade, signal transduction, oxidation reduction, cell communication, molecular transport, regulation of biological processes, regulation of cellular processes and apoptosis.

1. PPI among the up-regulated proteins

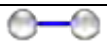
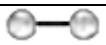
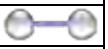
A. PPI among up-regulated proteins of AD vs control group

a. PPI network of the upregulated protein clusters in the AD vs control group

PPI interaction among the upregulated proteins in the AD versus control group can be divided into several network clusters (Figure 7.5). Among them, the first two consists mainly of the cytoskeletal proteins such as 14-3-3 proteins along with tubulin, actin, Ras-related proteins, cofilin, sirtuin, actin, myosin and the Ras-like proteins. The heat shock proteins (HSP) of different molecular weight and function formed another

important network cluster (Figure 7.5). Calmodulin, Ca^{2+}/calmodulin-dependent protein kinases, serine/threonine-protein phosphatase and synapsin-1 formed another functional interaction (Figure 7.5). Enzymatic proteins involved in metabolism and energy generation formed functional intearactions among themselves (Figure 7.5). There was strong interaction among the proteins (at high confidence score of 0.700). As some of the proteins had been involved in different functions, functional overlap of the proteins led them towards extended integration and interaction beyond any single class (Figure 7.5). The following figure demonstrates the identification keys used in interpretation of PPI analysis.

Node	**Node size**		**Node color**	
	 **Small**	 **Large**	 **Colored**	 **White**
Network nodes represent proteins	Protein of unknown 3D structure	Some 3D structure is known or predicted	Query proteins and first shell of interactors	Second shell of interactors

Edges	**Known interactions**		**Predicted interactions**			**Others**		
								
Edges represent protein-protein associations	From curated data bases	Experimentally determined	Gene neighborhood	Gene fusions	Gene co-occurrence	Text mining	Co-expression	Protein homology

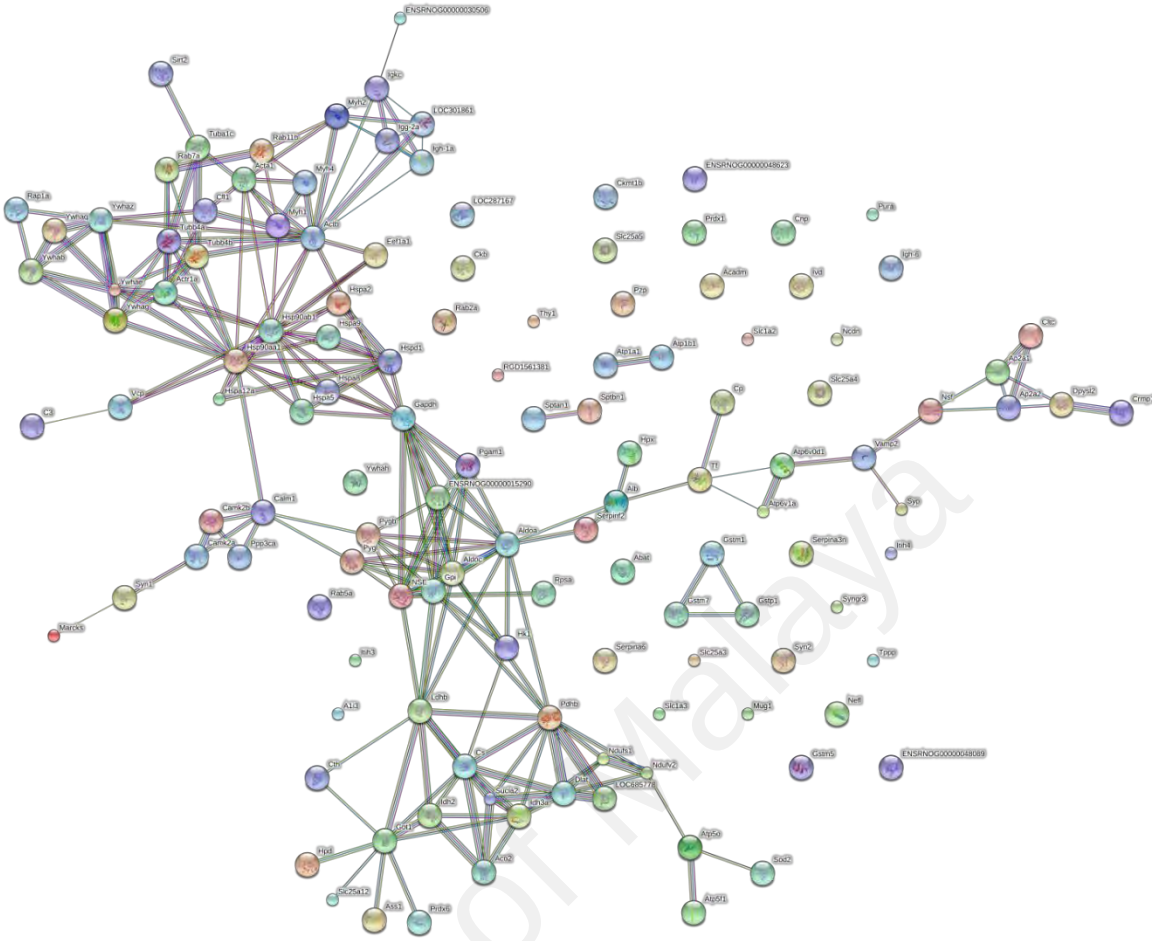


Figure 7.5: PPI network of the up-regulated proteins of the AD vs Control group

b. Pathway analysis of the upregulated proteins in the AD vs control group

Through KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway analysis, 65 pathways were found to be significantly enriched (P<0.05) in the AD versus control group. Among them the most notable were the AD pathway (pathway ID 05010) involving the genes *Camk2a, Camk2b, Ndufs1, Ndufv2* and *Atp5o* (FDR 0.0176); the LTP pathway (pathway ID 04720) involving the genes *Calm1, Camk2a, Camk2b* and *Rap1a* (FDR 0.0037) and the neurotrophin signaling pathway (pathway ID 04722, FDR 0.0246) involving the genes *Calm1, Camk2b, Rap1a* and *Ywhae*. The P13K-Akt signaling pathway (pathway ID 04151, FDR 0.0161) involving the genes *Hsp90aa1, Ywhab, Ywhae, Ywhag, Ywhah* and *Ywhaq*; Ca^{2+}signaling pathway (ID 04020, FDR

0.0176) with the genes *Calm1, Camk2a, Camk2b, Ppp3ca, Slc25a4* and *Slc25a5; cGMP-PKG* signaling pathway (ID 04022, FDR 0.0032) involving the genes *Camk2a, Camk2b, Atp1a1, Atp1b1* and *Slc25a5* were also among the interacted signaling pathways.

PFAM and INTERPRO protein domain analyses identified 14-3-3 (PF0024) as the most significantly enriched protein in the AD versus control group.

B. PPI among upregulated proteins in AD vs AE group

a. PPI network of the upregulated protein clusters in the AD vs AE group

In the AD versus AE group, the most notable protein networks included those involved in the biological processes such as redox mechanisms, neurogenesis, neurotransmission, neuronal development and metabolism (Figure 7.6). Interacted protein networks involved in molecular functions such as SNARE binding, syntaxin binding, syntaxin-1 binding were also enriched (Figure 7.6).

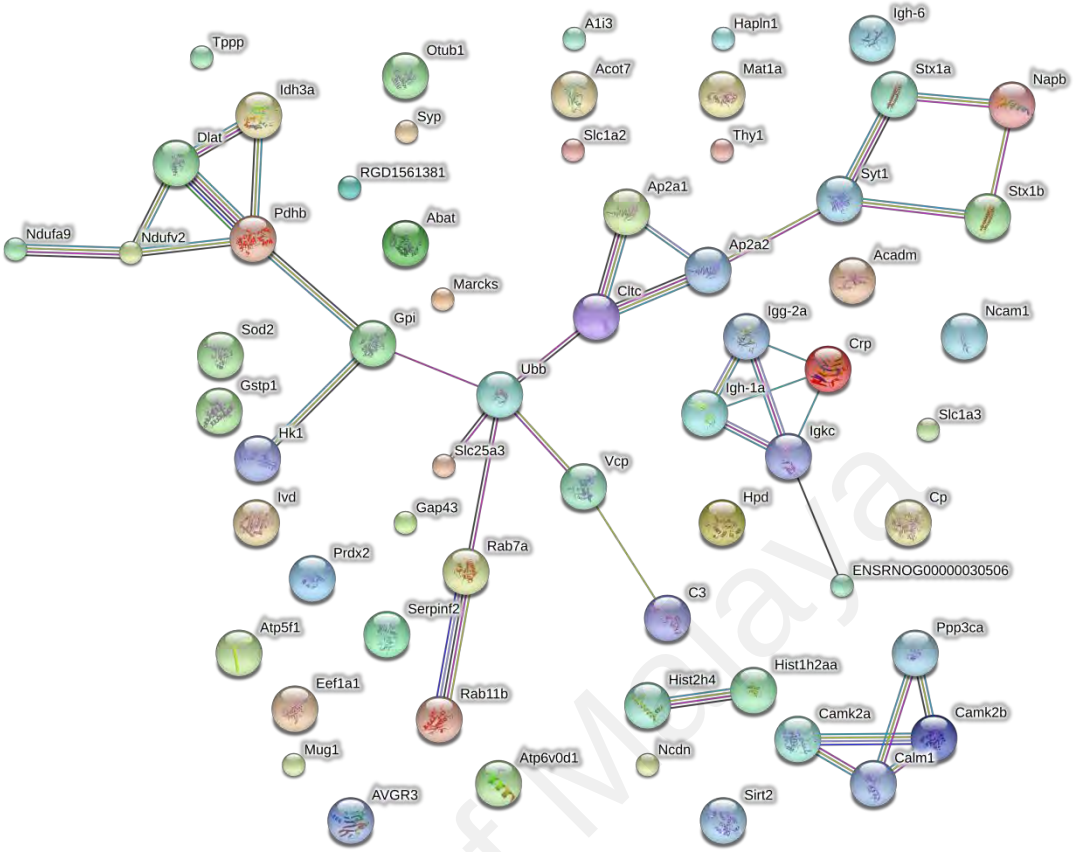


Figure 7.6: PPI network of the up-regulated proteins in the AD vs AE group.

The main network of the upregulated proteins can be divided into several subsets with respect to their gene content such as sub-set 1 consisting of synaptogamin-1, syntaxin 1A, syntaxin 1B, beta soluble NSF attachment protein *(Syt1, Stx1a, Stx1b* and *Napb),* sub-set 2 involving AP2 forming proteins and clathrin heavy chain *(Ap2a2, Ap2a1, Cltc),* sub-set 3 consisting of metabolic enzymes NADH dehydrogenase, dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex, pyruvate dehydrogenase E1 component subunit beta and isocitrate dehydrogenase *(Ndufv2, Ndufa9, Dlat, Pdhb and Idh3a)* and those involved in signal transduction, membrane trafficking and cytoskeletal maintenance *(Ubb, Vcp, C3, Rab7a, Rab11b, Gpi* and *HK1)* (Figure 7.6). Besides the main network, two other protein-protein interaction networks involved immune-regulatory proteins *(Igg-2a, Igh-*

1a, IgkC and *Crp)* and proteins associated with Ca^{2+}-mediated signaling and metabolism *(Calm1, Camk2a, Camk2b* and *Ppp3ca)* (Figure 7.6).

b. Pathway analysis of the upregulated proteins in the AD vs AE group

Among the 21 enriched KEGG pathways, the highly notable were the synaptic vesicle cycle, LTP, AD, HIF-1 signaling, SNARE interaction in vesicular transport and those associated with glutametargic synapse.

PFAM protein domain analysis identified alpha adaptin AP2, C-terminal (pathway ID PF02296) and INTERPRO analysis showed clathrin adaptor, alpha adaptin AP2, appendage, C-terminal as the mostly enriched domain.

A. PPI among upregulated proteins in the AE vs C group

a. PPI network of the upregulated protein clusters in the AE vs control group

The upregulated proteins constitute the main interaction network that can be splitted into several network sub-sets as depicted through the STRING analysis (Figure 7.7). The first sub-set consists of the genes related with microtubule assembly and cytoskeletal structure and function such as sirtuin 2 and tubulin (Figure 7.7). The proteins involved in this network are sirtuin2 and tubulin with the corresponding genes *Sirt2, Tuba1b, Tuba1c, Tubb2a, Tubb3, Tubb2b, Tuba4a, Tubb4a, Tubb4b, Tubb5* and *Tubb6* (Figure 7.7). The second network sub-set consists of the proteins linked with anti-oxidative defense (peroxidredoxins, SOD), metabolism (malate dehydrogenase, phosphoglutarate mutase, glucose 6 phosphate isomerase, pyruvate dehydrogenase) and molecular chaperone activity (polyubiquitin, T complex protein and HSP 10 KDa) (Figure 7.7). The interacted genes in this network are *Prdx2, Sod1, Mdh1, Pgm1, Gpi, Pdhb, Ubb, Cct3* and *Hspe1* (Figure 7.7).

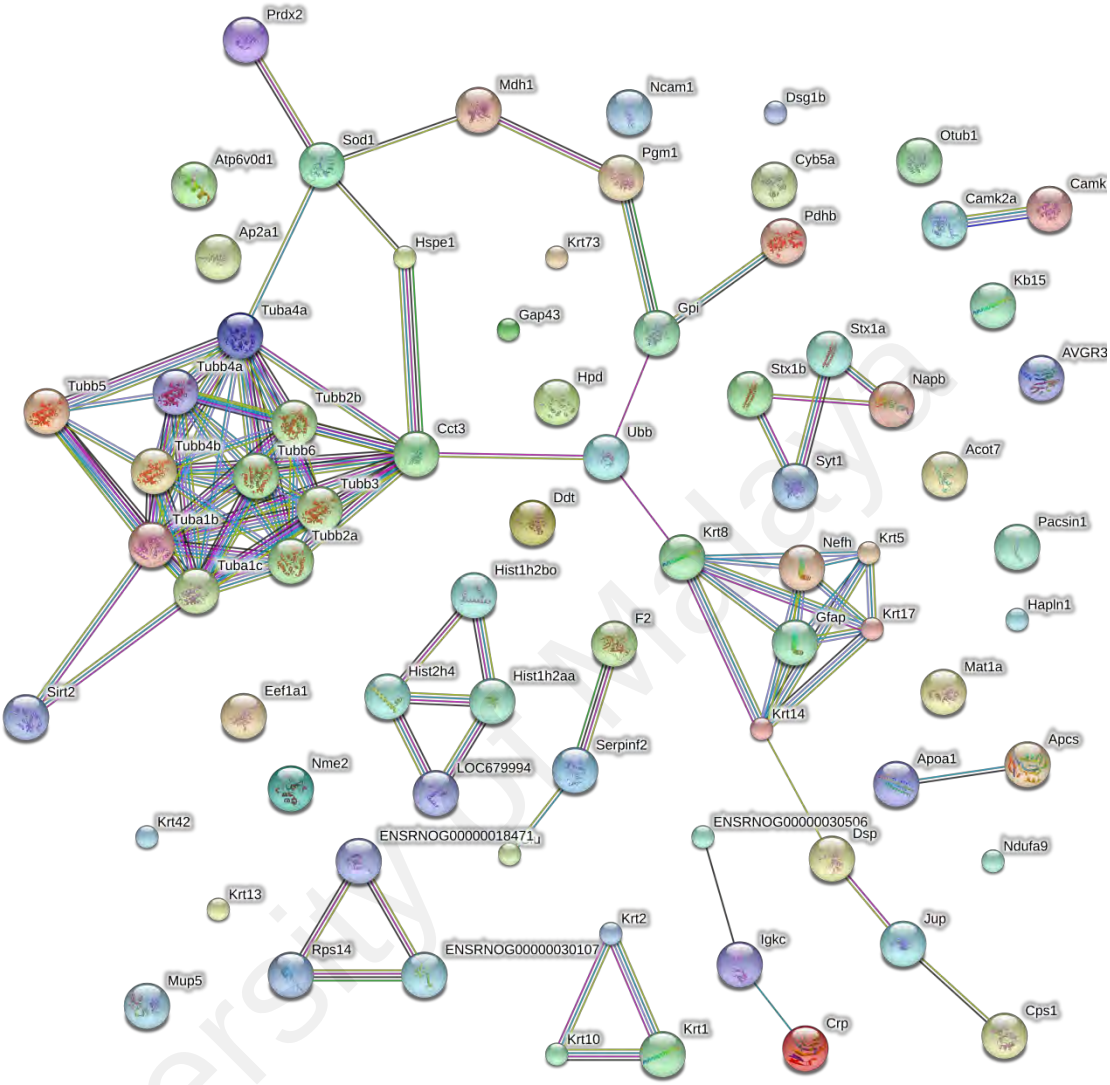


Figure 7.7: PPI network of the up-regulated proteins in the C vs AE group.

The third sub-set consists of the cytoskeletal proteins including keratin, neurofilament heavy polypeptide, GFAP, Dsp, junction plakoglobin and carbamoyl phosphate synthase 1 (Figure 7.7). Interacted genes in this category are *Krt8, Nefh, Krt5, Krt17, Gfap, Krt14, Dsp, Jup* and *Cps1* (Figure 7.7).

Besides the main network, there were some small networks of which the first one consisted of the genes *Stx1a, Stx1b, Napb* and *Syt1* that are correspondent of the

proteins syntaxin 1a, syntax 1b, periplasmic nitrate reductase and synaptotagmin 1, respectively (Figure 7.7). The second small network incorporated histones *(Hist1h2aa, Hist1h2bo* and *Hist2h4)* and unknown protein LOC679994 (Figure 7.7).

b. Pathway analysis of the upregulated proteins in the AE vs control group

KEGG pathway anlaysis showed gap junction, phagosome, synaptic vesicle cycle and carbon metabolism pathways as the enriched.

PFAM and INTERPRO protein domain analyses identified tubulin, tubulin/Ftsz family – C terminal domain and GTPase domains to be significantly ($P<0.05$) enriched.

2. PPI among the downregulated proteins

A. PPI among the down-regulated proteins of the AD vs control group

Among the downregulated AD versus control group, the most notable interaction had been observed among the cytoskeletal proteins such as keratin isoforms *(Krt1, Krt2, Neflh, Krt5, Krt8, Krt10, Krt13, Krt15, Krt14, GFAP, Krt17, Krt42, Krt73)*, junction plakoglobin (Jup), neurofilament heavy chain (Neflh) and glial fibrillary acidic protein (GFAP) (Figure 7.8). Downregulated anti-oxidant proteins peroxidredoxin 5, superoxide dismutase 1, protein disulfide isomerases *(Prdx5, Sod1, P4hb, Hspe1* and *Cct3)*, oxoglutarate/malate carrier protein and alcohol dehydrogenase also formed a sub-network (Figure 7.8). Besides, metabolic and ATP *(Atp5c1, Atp6v1d* and *Atp6v1e1)* generating proteins were also interacted with each other (Figure 7.8).

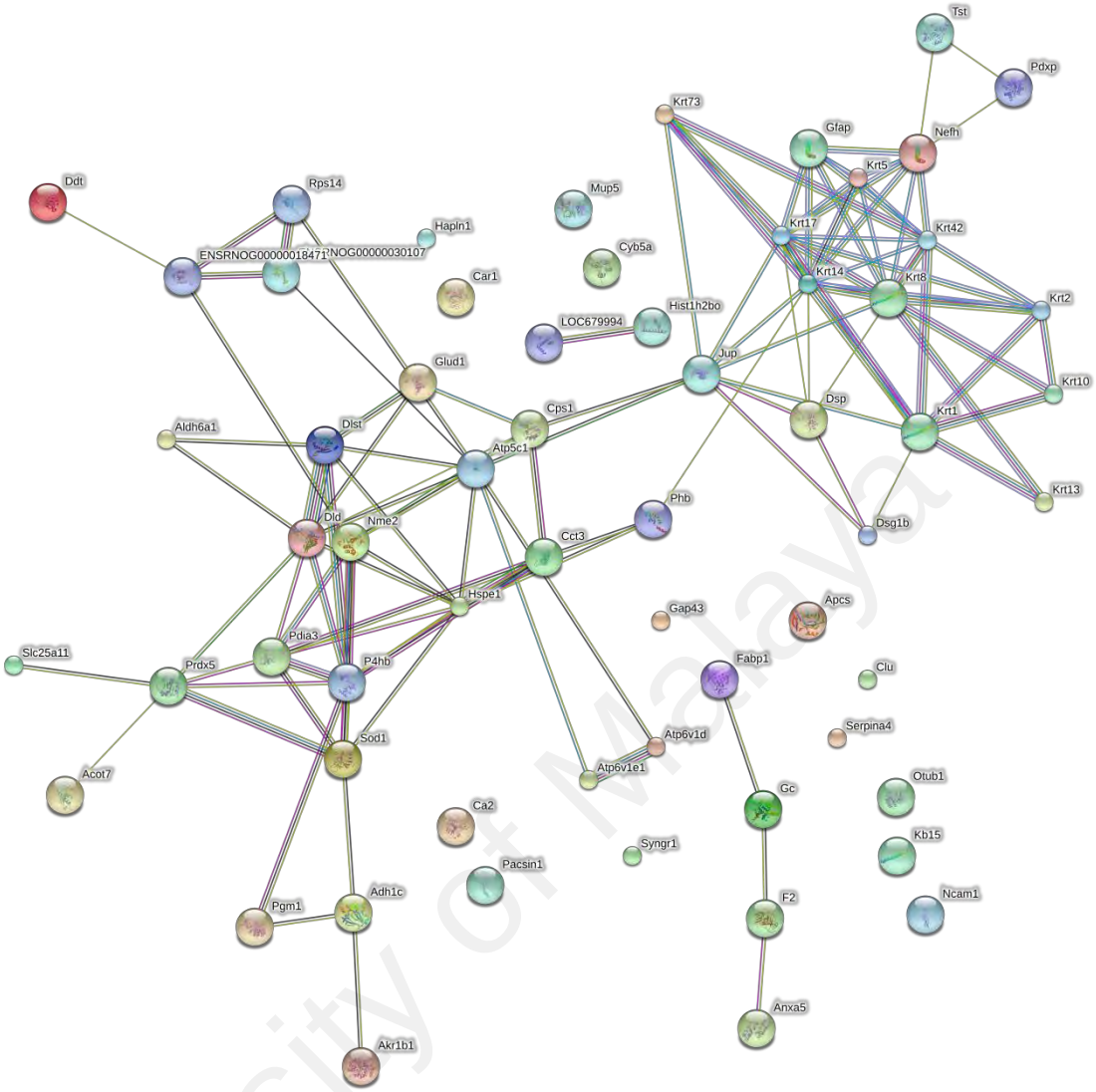


Figure 7.8: PPI network among the down-regulated proteins of the AD vs C group.

B. PPI among down-regulated proteins of the AD vs AE group

In the AD versus AE group, the most notable interaction was among the downregulated proteins of anti-oxidant (SOD, GPX, peroxiredoxin, glutathione-S-transferases) activities (Figure 7.9). Besides, several sub-networks were also noted among the proteins involved in metabolism, energy generation and ribosomal structure and function related proteins *(Rpsa, Rps7, Rps2, Rpl13, Rplp0* and *Rpl14)* (Figure 7.9).

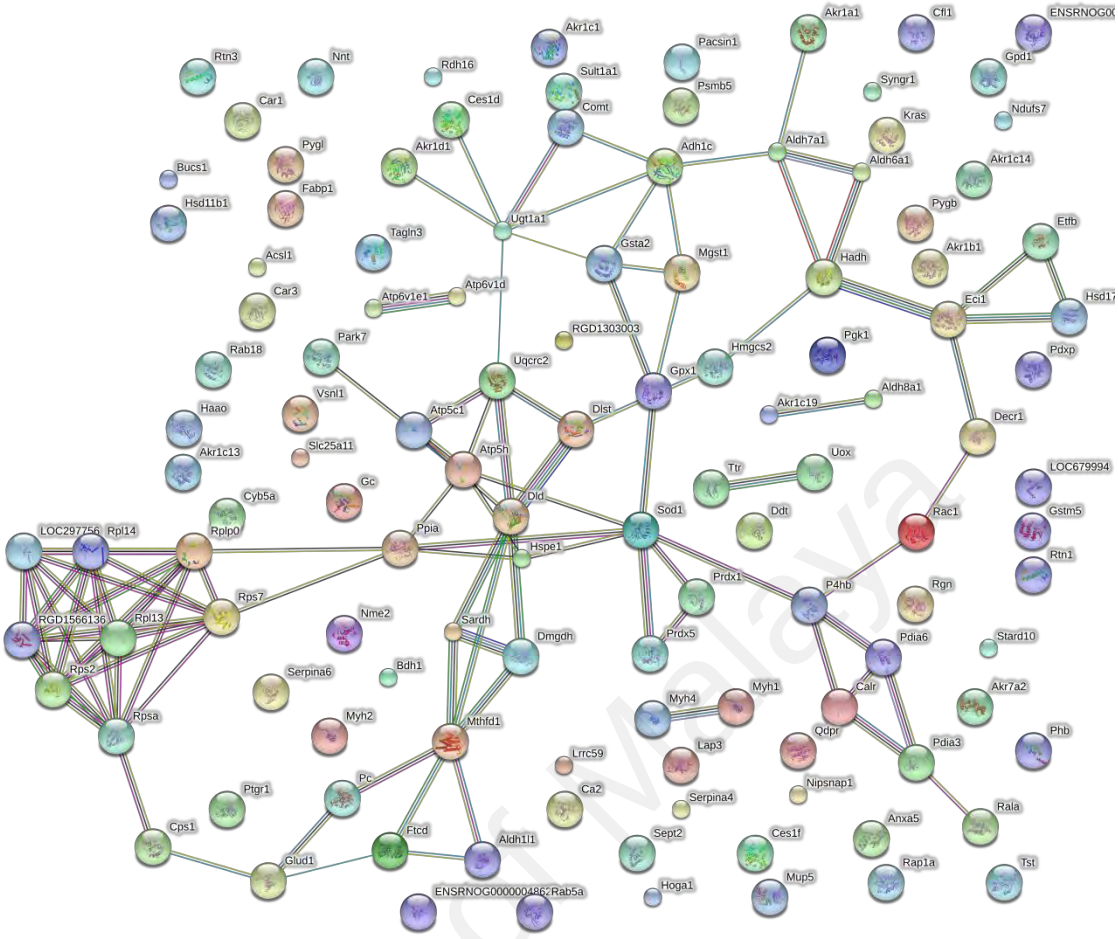


Figure 7.9: PPI among the down-regulated proteins of the AD vs AE group.

Ribosome is the protein synthesis machinery and abnormality in its structure and protein synthesis activity highly affects AD pathomechanisms (Ferreira *et al.,* 2016). Thus, downregulation of the relevant proteins might be involved in impaired memory and learning activities of the AD rats (Ferreira *et al.,* 2016).

From the entire PPI analyses of the present study, it has been clearly understood that AD pathogenesis mostly affected the protein networks linked with neuronal cytoskeleton. Other affected networks include those associated with metabolism, Ca^{2+}dyshomeostasis, mitochondrial structure and function, signal transduction and anti-oxidant defense activities. Our findings are compatible with those of Minjarez *et al*. (2013), Zahid *et al*. (2014) and Hondius *et al*. (2016). Actually, derangement of the neurocytoskeleton is an important hallmark of AD pathogenesis and demands

considerable attraction in identifying AD therapeutics (Hwang *et al.,* 2015; Pollak *et al.,* 2003; Vickers *et al.,* 2016).

7.4.1.3 Identification of functional pathway interaction through IPA

Based on ingenuity pathway analysis Knowledge Base (IPAKB), genes are transformed into relevant networks. In the network, relationships among the genes are expressed as the "edges" and genes become connected with each other only if there is any path among them in the global network. In this case, molecules from the dataset that are uploaded are called the "focus molecules". Core analysis of IPA was performed to interpret the datasets in the form of their functional networks. In the IPAKB, corresponding objects were mapped with the protein identifiers (James *et al.,* 2012) . Depending on the physical interaction (direct relationship) among the eligible proteins, IPAKB generated the networks and the score (probability value) of the networks (James *et al.,* 2012). Higher the network connectivity greater is the representation of significant biological functions of the relevant genes (James *et al.,* 2012). Statistical justification of the network connectivity is performed through measuring "p scores (-log10 p value, Fisher's exact test)" and "network score". Network score is also measured through Fisher's exact test that is based on the focus protein and biological functions and thus shows the relevancy of the analysis.

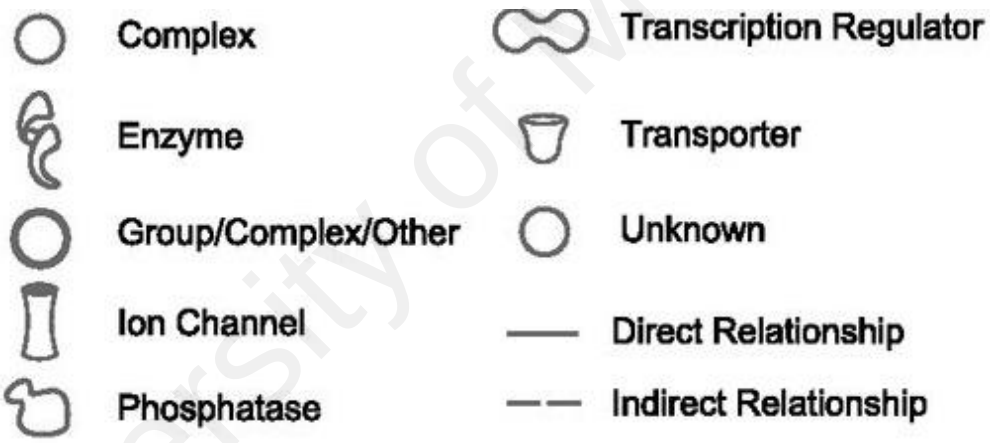
A. Functional networks in AD vs C

IPA of the AD vs C identified 18 networks of which the top-most one had score of 90 having 93 focus molecules among 140 total selected molecules (Figure 7.10; Summary in Appendix F, Table F 11.1). The top-most network has been associated with cell death and survival, neurological diseases and psychological disorders.

The second network had score of 54 and focus molecules 64 among 140 total selected molecules (Figure 7.11). The relevant physiological system development and functions included molecular transport, cell-cell signaling and interaction, nervous system development and functions. Nervous system development and functions involved 40 molecules (p value 4.99E-02 - 3.48E-05). The other networks had score of 1 and focus molecule 1 and most were irelated to metabolism.

Legends of the nodes and edges used in IPA

Red color is indicator of up-regulation and green color of down-regulation. Nodes and edges are indicators as follows:



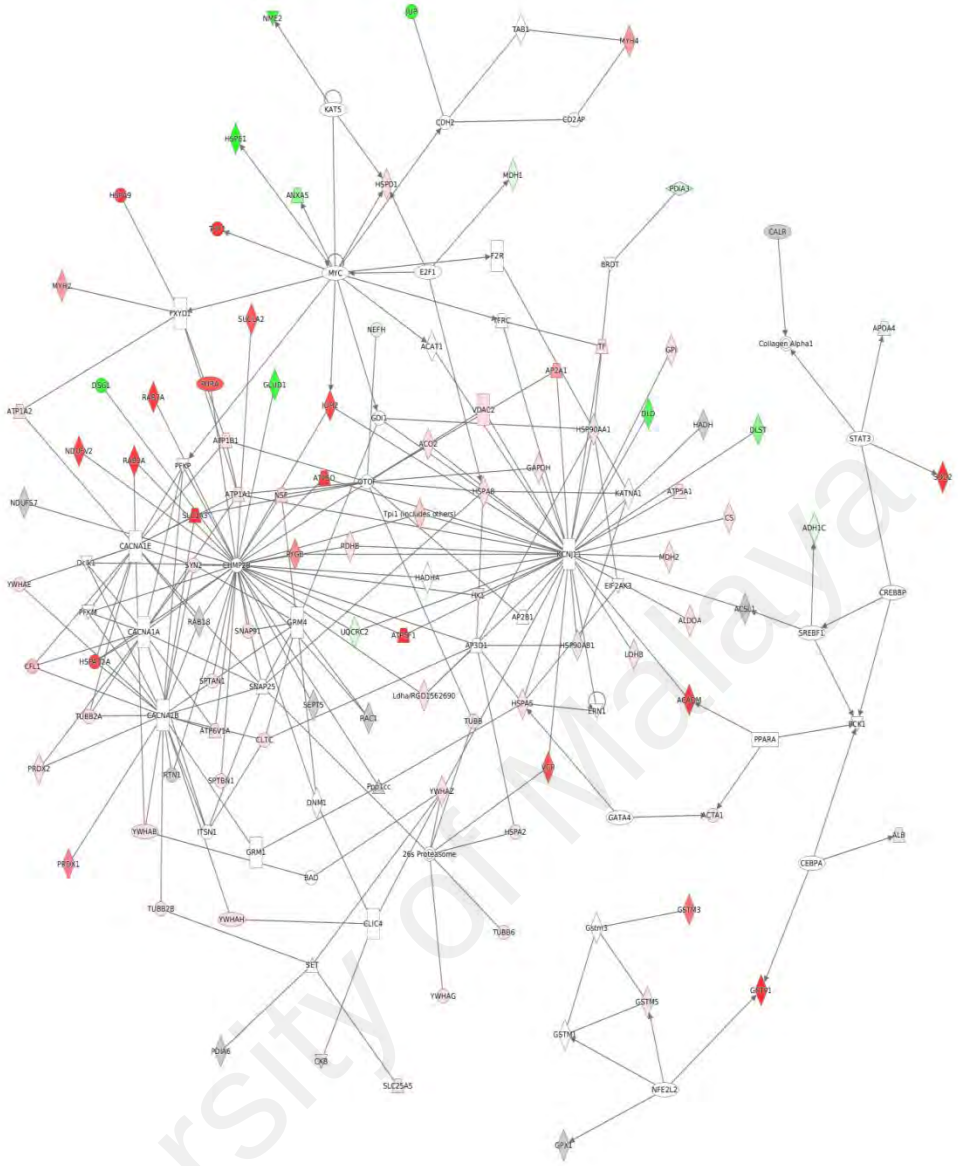


Figure 7.10: Top-scoring gene networks differentially expressed between AD and C group (top-most network 1).

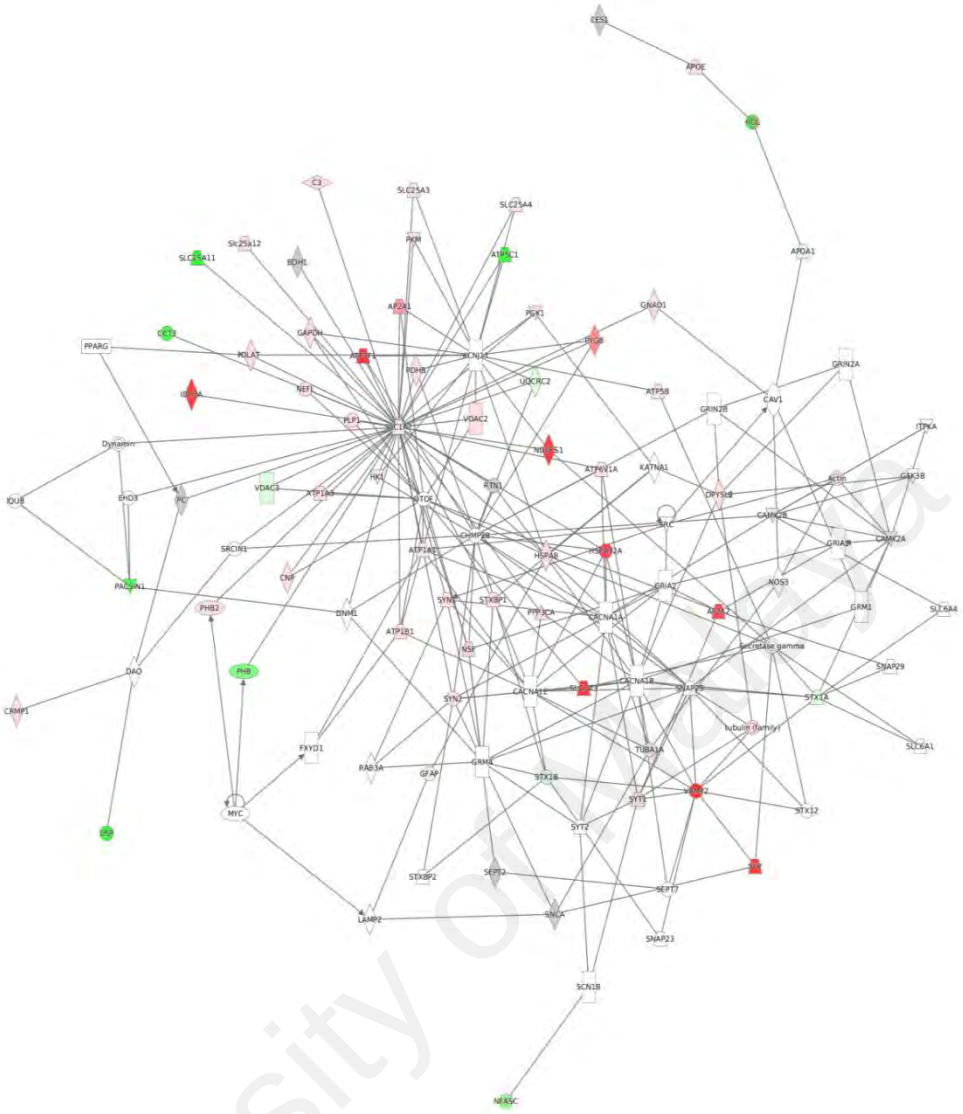


Figure 7.11: Top-scoring gene networks differentially expressed between AD and C group (top-most network 2)

The top upstream regulators with corresponding p value of overlapping were PPARG (6.50E-05), NFE2L2 (3.34E-04), STAT3 (1.36E-03), CEBPA (5.25E-03), GATA4 (5.25E-03). Peroxisome proliferator activated receptors (PPARs) are the groups of nuclear hormone receptors that regulate lipid metabolism, energy production, metabolic balance between lipid and carbohydrates by acting as the lipid sensors (Kummer & Heneka, 2008). AD ameliorating effect of the non-steroidal anti-inflammatory drugs

(NSAIDs) have been associated with PPAR stimulating effect (Camacho *et al.,* 2004; Weggen *et al.,* 2001).

Neurological diseases were among the top diseases and bio-functions with 14 molecules and p value of 4.74E-02 - 5.26E-04. Among the top toxicity lists were OS (22.2%, 7.95E-11), mitochondrial dysfunction (12.7%, p 2.30E-12), LXR/RXR activation (14.4%, 5.73E-11), positive acute phase response proteins (34.6%, 2.43E-10) and FXR/RXR activation (13.0%, 9.72E-10).

The top-most up-regulated molecules with their log ratios were (THY1 18.403), ATP5F1 (18.175), GSTP1 (17.998), VAMP2 (17.922), Igh-6 (17.867), SLC1A3 (17.808), SOD2 (17.784), HSPA9 (17.354), TPPP (17.347) and RAB11B (17.254).

The top-most down-regulated molecules with their log ratios were DDT (-18.544), LOC259246 (-18.299), CYB5A (-18.203), SOD1 (-17.609), HIST1H3E (-17.581), DSG1 (-17.409), FABP1 (-17.253), HSPE1 (-17.230), LOC100911847 (-16.892) and JUP (-16.852).

Top canonical pathways with their corresponding overlap and p values were phagosome maturation (21.5%, 2.98E-19), 14-3-3-mediated signaling (16.1%, 2.37E-14), remodeling of epithelial adherens junctions (24.6%, 6.49E-14), epithelial adherens junction signaling (14.0%, 1.55E-12) and mitochondrial dysfunction (12.9%, 1.80E-12). 14-3-3-mediated signaling and the network associated affected proteins have been depicted in Figure 7.12. Besides, pathways associated with glucose metabolism (glycolysis and gluconeogenesis), TCA cycle, oxidative phosphorylation, unfolded protein response, HIPPO signaling and xenobiotic metabolism signaling were also among the notable functionally interacted ones. The HIPPO signaling is entails the protein kinase “Hippo (HPO)” and is evolutionarily conserved for developing the

mammalian nervous system. Its emerging role in AD pathogenesis has intrigued the current AD research field (Godoy *et al.,* 2014)

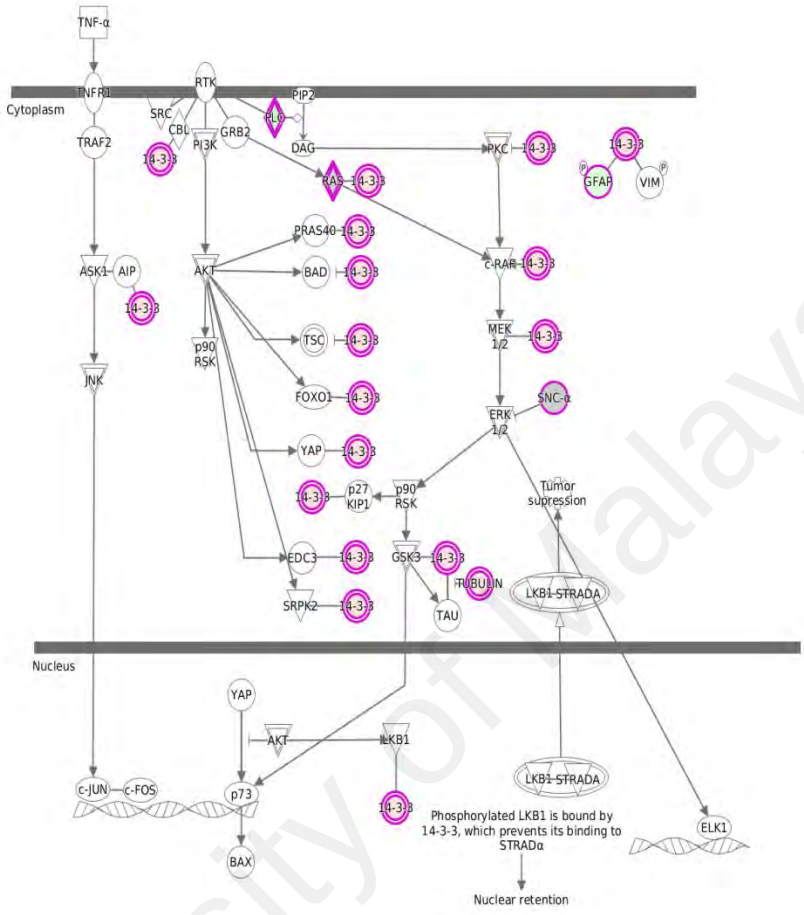


Figure 7.12: 14-3-3 protein-mediated signaling and associated proteins in the AD vs C group.

Consistent with our quantitative proteomics findings and interaction analysis by STRING, the proteins altered in their expression are also functionally related with each other. The most prominent proteins in this regard are those involved in nervous system development and function (YWHAE; NCAM1,NEFH,SIRT2,VSNL1; NCAM1, NEFH, SIRT2, VSNL1; NME2; UGT1A1; SEPT1; CAMK2A; 2,SEPT5,SNCA,SOD1; KNG1, TUB1A1). Proteins involved in anti-oxidative defense through free-radical scavenging (GPX1, GPX3, PRDX1, PRDX2, PRDX6, SNCA, SOD1 and SOD2) also

were functionally related. Similar trend was observed for the proteins involved in synaptic functions, cytoskeletal arrangements, microtubule-associated proteins, cellular stress response (especially the HSPs) and calcium binding. Our findings closely resemble those of Hondius *et al.,* (2016).

Besides, proteins involved in mitochondrial structure and functions were functionally closed. In the previous section under differential expression related description, we focused on the deranged expression pattern of the metabolism, energy generation and mitochondria-associated proteins. The IPA analysis tally with those and indicate that the AD mitochondria suffer from disrupted expression and functionality of the proteins involved in metabolism, energy generation, OS regulation and Ca^{2+} homeostasis (James *et al.,* 2012). Another important aspect of neuronal mitochondria is maintenance of cellular dynamics so that mitochondrial "traffic jam" is overcome through proper translocation of mitochondria through the neuronal cytoskeleton (Correia *et al.,* 2016). Present findings of the affected proteins linked together in the AD subjects are "red signal" across the neuronal cytoskeletal "cross-roads". In line with the mitochondrial derangement, the functional interaction among the affected cytoskeleton and microtubule-associated proteins (especially tubulins) reinforce the observed jumble of the AD hippocampal proteins.

Proteins involved in metabolism of almost all the biomolecules have been affected in the AD groups. The most notable alteration was those metabolizing glucose and lipids (APOA1, HSD11B1, PHB, SERPINA1, SIRT2; Akr1c14, COMT, HSD11B1, Sult1a1; ACSL1, ALB, FABP1; ABAT, ACSL1, ALB, APOA4, APOE, CS, DLAT, DLD, F2, FABP1, KNG1, MDH1, MDH2, PDHB, RAC1, RGN, STX1A, SUCLA2). Top-ranking proteins involved in protein metabolism and interacted together were ACADM, ALDOC, ANXA5, CRP, EEF1A1, Gnmt/LOC100911564, HBA1/HBA2, HBB,

HSD17B10, IVD, JUP, MAT1A, NEFL, NME2, SOD2, VCP and YWHAB. Metabolism-related proteins were linked in the peripheral nodes of the IPA.

B. Functional network in AD vs AE

IPA of the AD vs AE identified 20 networks of which the top-most one had score of 75 and focus molecules 83 among 140 total selected molecules (Figure 7.13, Summary in Appendix F, Table F 11.2). The top-most network functions include cell death and survival, cell morphology and organ morphology. The second one had score of 56 and focus molecules 73 among 140 total selected molecules (Figure 7.14). The second-top network related functions include molecular transport, cell signaling, nervous system development and function categories. The third network had score of 8 and 25 focus molecules among 135 total selected molecules involved in CVD and functions, cellular and organismal development (Figure 7.15).

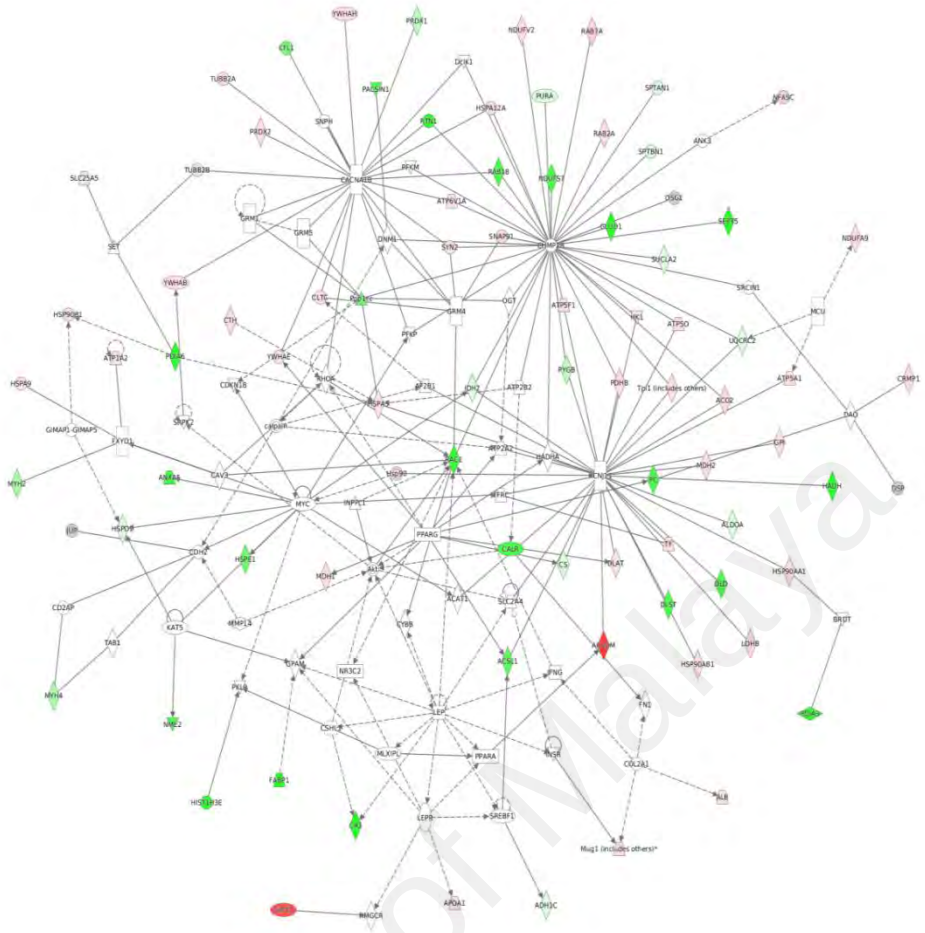


Figure 7.13: Top-scoring gene networks differentially expressed between AD ane AE group (top-most network 1).

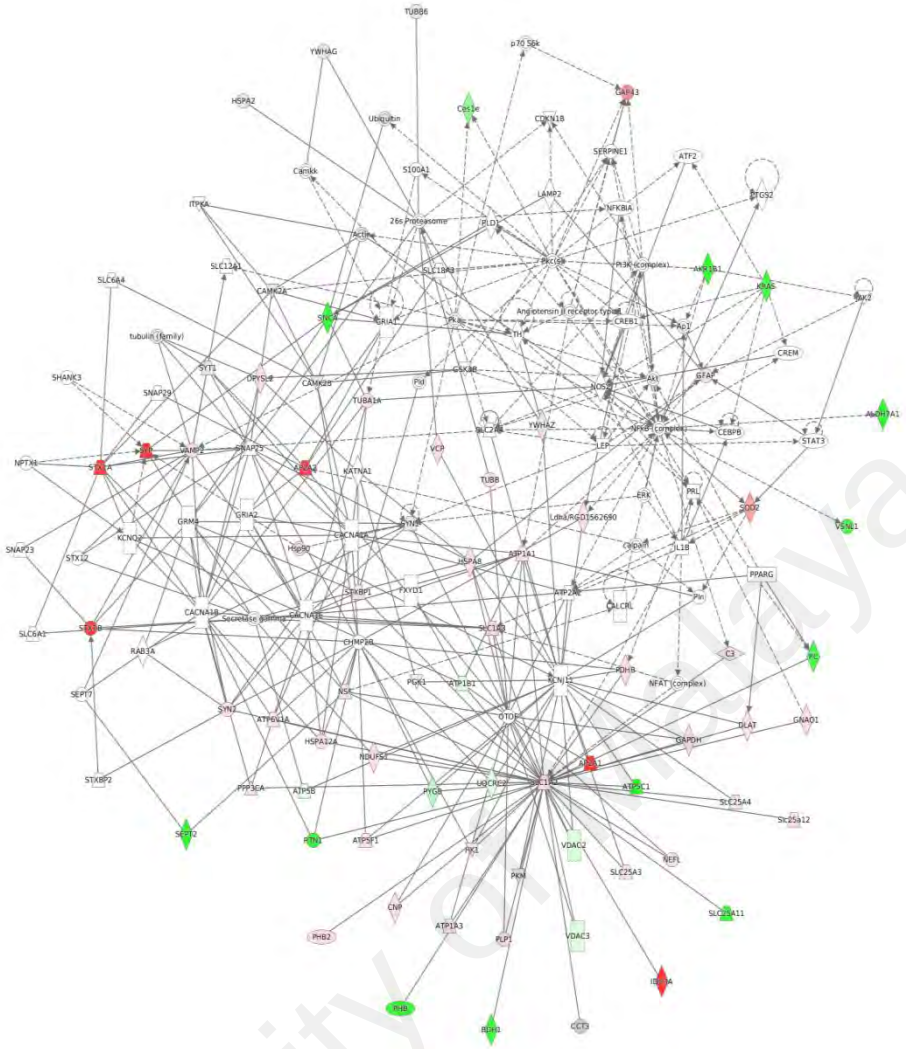


Figure 7.14: Top-scoring gene networks differentially expressed between AD and AE group (top-most network 2).

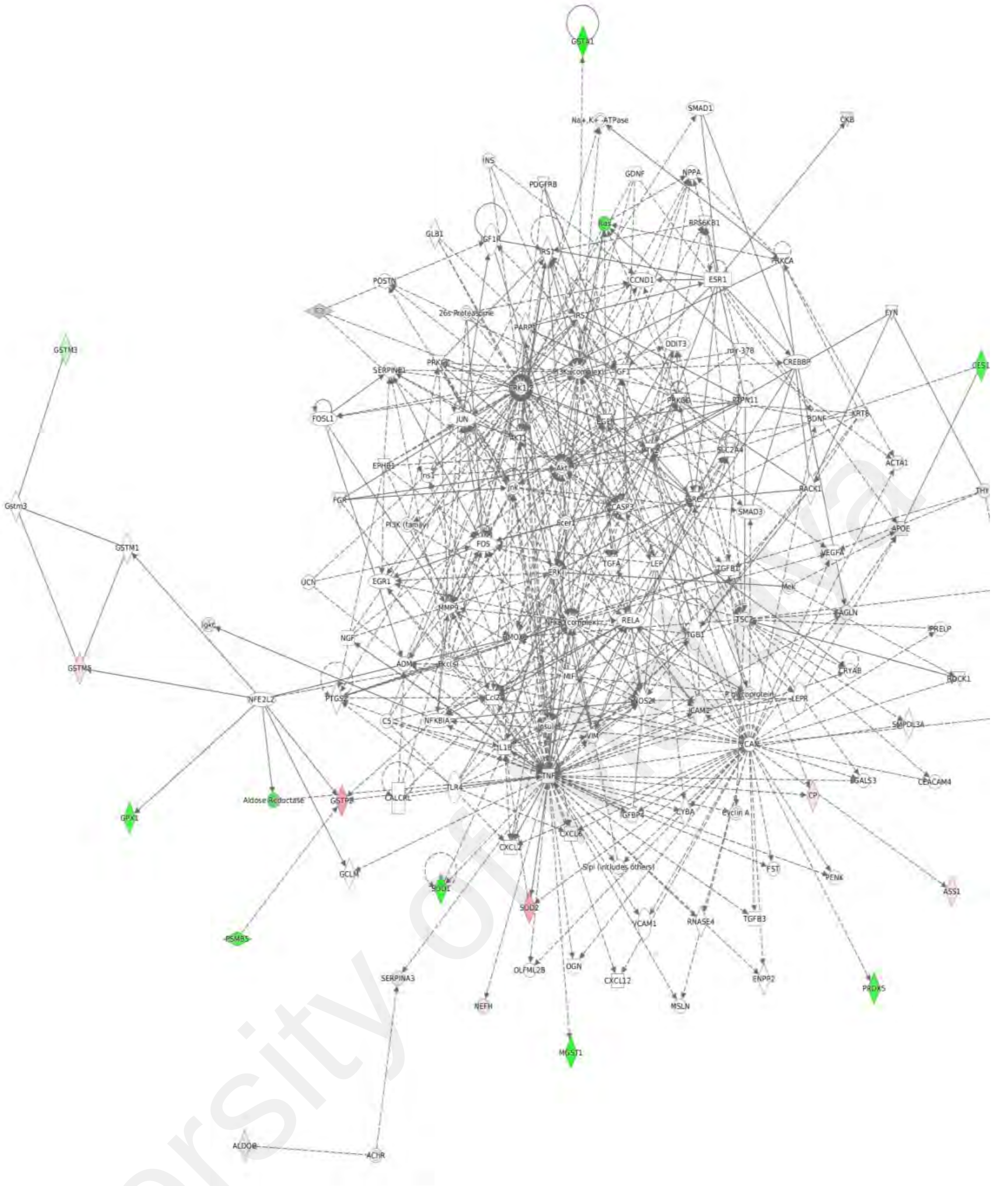


Figure 7.15: Top-scoring gene networks differentially expressed between AD and AE group (top-most network 3).

The top physiological system development and functions with their corresponding p value range and involved molecules were nervous system development and function (4.99E-02 - 3.48E-05, 40), tissue morphology (3.35E-02 - 5.29E-05, 22), tissue development (4.99E-02 - 1.62E-04, 31), organ morphology (3.35E-02 - 2.52E-04, 6) and organismal development (3.35E-02 - 2.52E-04, 18).

The top upstream regulators with corresponding p value of overlapping were MCU (1.69E-05), PPARG (6.50E-05), PDIA6 (2.64E-04), GIMAP1-GIMAP5 (2.64E-04) and NFE2L2 (3.34E-04). Metabolic (p value 1.69E-02 - 1.68E-06, molecules involved 16) and neurological (p value 4.74E-02 - 5.26E-04, molecules involved 14) diseases were among the top physiological disorders affected.

The top-most up-regulated molecules with their log ratios were AP2A1 (17.321), TNR (17.234), UPB1 (17.067), HPD (16.998), IVD (16.946), SYP (16.918) and ATP6V0D1 (16.827).

The top-most down-regulated molecules with their log ratios were ACSM1 (-18.169), ALDH6A1 (-17.880), GSTA1 (-17.673), TST (-17.527), FABP1 (-17.339), GLUD1 (-17.265), CA3 (-17.190), ALDH8A1 (-17.183), RGN (-17.177) and SEPT5 (-17.092).

Proteins involved in cellular communication and signaling (CALR, F2, KNG1, KRAS; ARF1, GSTP1, HSPA8, RAP1A, RGN, SOD1, THY1; ABAT, CLU, NCAM1; RAC1, SNCA; CKB, NME2) were highly affected in the AD vs AE group and interacted together.

Another important aspect was the functional networks of the kinases (CAMK2A, CAMK2B, GSK3β and CDK5). These kinases are members of the post-synaptic phosphoproteome and their associated network points towards the disrupted phosphoproteome of the AD subjects (Zhou *et al.,* 2010).

Top-most canonical pathways were like those of AD vs C. Exceptional was the eNOS and melatonin signaling pathways. The eNOS signaling has been reported to be beneficial for AD as eNOS forms a feedback loop with pin1 and Aβ that prevents deposition and stimulates clearance of Aβ in the cerebral microvessels (Wang *et al.,*

2014). But, the process is disrupted in AD brains due especially to the aberrantly increased level of Aβ when eNOS fails to perform its feed-back loop formation (Wang *et al.,* 2014).

Melatonin (N-acetyl-5-methoxytryptamine) is a pineal gland hormone whose level decreases in the aged and AD subjects (Lin *et al.,* 2013). Its neuroprotective role involves anti-oxidative, anti-Aβ producing and anti- Aβ fibrillation strategies (Lin *et al.,* 2013; Poeggeler *et al.,* 2001). Their regulatory roles on the protein kinases and phosphatases and protective effect upon cholinergic system have also been regarded (Lin *et al.,* 2013). The melatonin signaling network and the associated proteins in the present study highlights another therapeutic target of the damaged AD proteome (Cecon & Markus, 2011).

C.Functional networks in AE vs C

IPA of the C vs AE identified 20 networks of which the top-most one had score of 73 and focus molecules 82 among 140 total selected molecules (Figure 7.16, Summary in Appendix F, Table F 11.3). The top-most network functions include cell death and survival, cell morphology and organ morphology. The second one had score of 56 and focus molecules 71 among 140 total selected molecules (Figure 7.17). The second-top network related functions include molecular transport, cell signaling, nervous system development and function categories. The third network had score of 8 and 25 focus molecules among 135 total selected molecules involved in CVD and functions, cellular and organismal development (Figure 7.18).

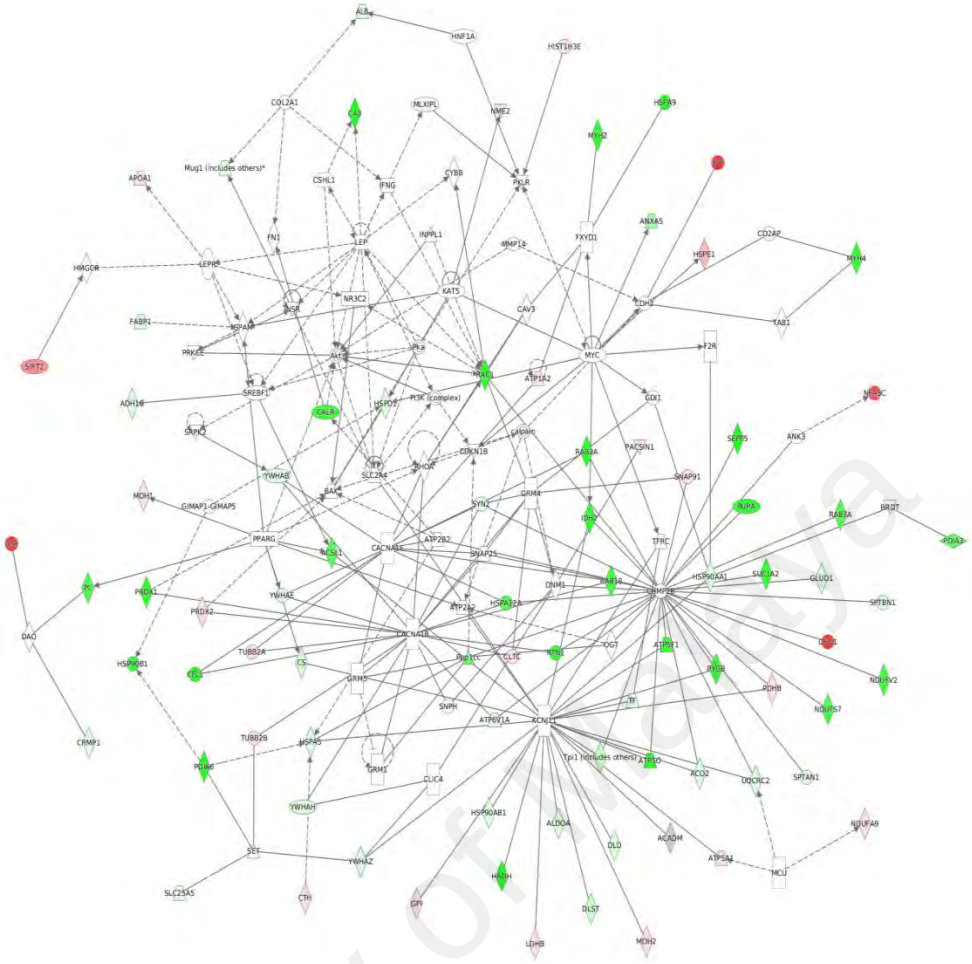


Figure 7.16: Top-scoring gene networks of the differentially expressed between C and AE group (top-most network 1).

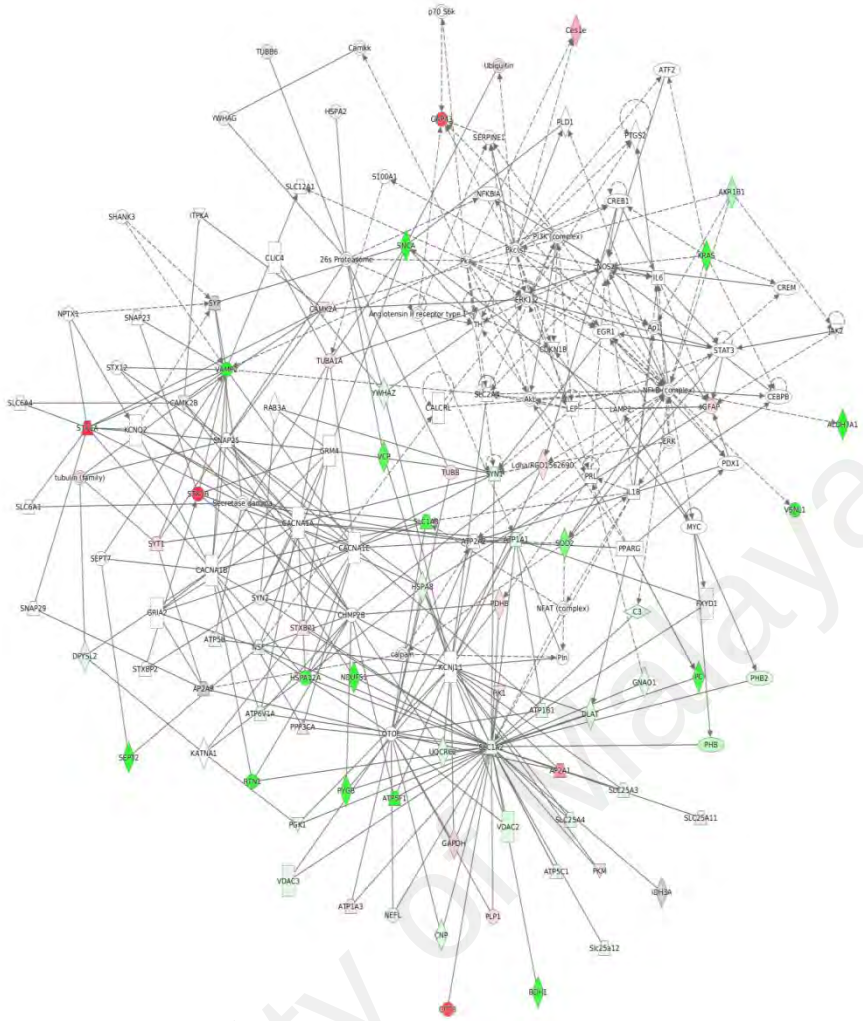


Figure 7.17: Top-scoring gene networks of the differentially expressed between C vs AE group (top-most network 2).

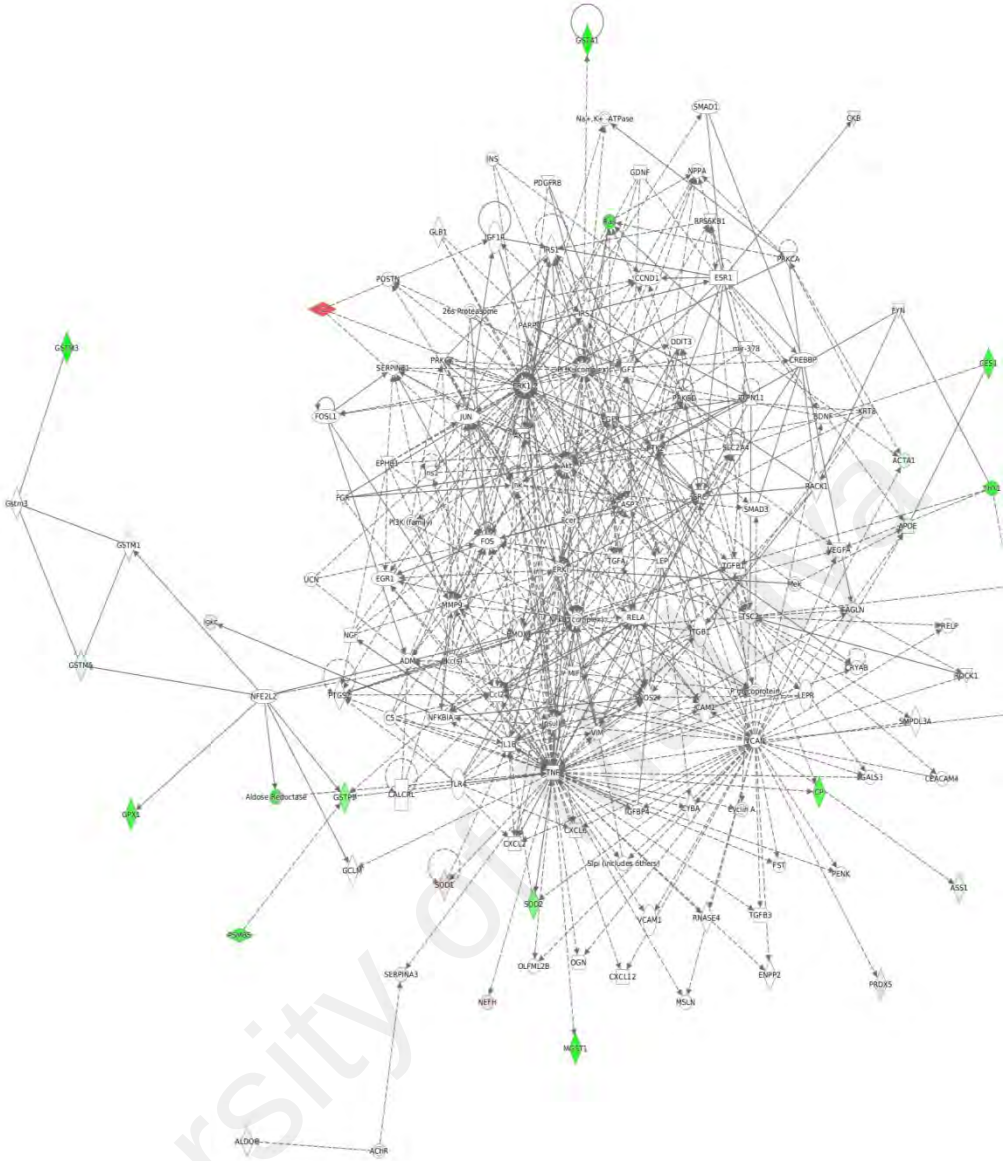


Figure 7.18: Top-scoring gene networks differentially expressed between C and AE group (top-most network 3).

Nervous system development and function was the top-most feature of the physiological system development and function with 40 molecules and p values in the range of 4.99E-02 - 3.48E-05.

The top-most up-regulated molecules with their log ratios were DSG1 (17.409), LOC100911847 (16.892), JUP (16.852), MAT1A (16.707), STX1B (16.560), DSP (16.474) and STX1A (16.313).

The top-most down-regulated molecules with their log ratios were ACSM1 (-18.169), GSTA1 (-17.673), GSTM3 (-17.204), CA3 (-17.190), ALDH8A1 (-17.183), PDHA2 (-17.179), RGN (-17.177), HSPA9 (-17.175), ARF1 (-17.101) and SEPT5 (-17.092).

Mitochondrial Ca^{2+} uniporter (MCU) was the top-most up-stream regulator. Its involvement in OS-mediated cellular damage has been confirmed recently (Liao *et al.,* 2015). Exceptionally noted canonical pathways in the C vs AE included actin cytoskeleton signaling, ROS and NOS production, NRF2 mediated OS response, RhoA signaling, Rho family GTPases, protein kinase A signaling, EIF2 signaling, ILK signaling and paxillin signaling pathways. These findings are consistent with those of STRING analysis in the sense that maintenance of neuronal cytoskeleton, mitochondrial function, metabolism proper and defense against free-radical induced damage are the top-notch concern of AD pathogenesis. Impairment in any one and/or combined system(s) poses threat towards AD vulnerability.

7.5 Conclusion

Up to present day, about 96 proteins have been reported to be linked with AD (Musunuri *et al.,* 2014). Most of the differentially expressed proteins identified in the present study fall within that category. The reason for not detecting direct expression of APP or Aβ in the AD rats of the present study might be that Aβ was infused intra-ventricularly and this doesnot fall under the category of expression or repression. But its effect is revealed through up- and/or – down regulation of multiple proteins in the AD subjects. Provided the same amount of protein being processed for quantification, variability in the output may be considered due to reduced accessibility of the AD proteins towards trypsin digestion as the proteins in AD subjects are much aggregated and less soluble compared to the controls. Importantly, inability to detect proteins of

target does not imply their absence in the sample; rather it is an indication of their differential expression. As the AD rats have been found to experience impaired memory and learning activities in the previous chapter and altered expression and networks (both interacting and functional pathways, as revealed by the STRING and IPA analysis, respectively) of the memory and learning related proteins in the present chapter, differential expression of the proteins might be attributed to the infused Aβ in the respective model animals. On the other hand, proteins up- and down-regulated in the control and mushroom treated animals might have been involved in improving those derangements and thus ameliorating the AD consequences as observed through memory related tests in the previous chapter. Another important feature of the present study is that some of the proteins differentially expressed in the AD model rats are related with cholesterol metabolism (such as apolipoprotein A-I, A-IV, E) and OS management (such as SOD, GPX, peroxiredoxin, glutathione -S-transferase). They were also highly interacted with each other and formed pathway-based functional networks. This inter-relationship reinforces the OS and hypercholesterolemia links of AD pathogenesis. Thus, current findings suggests towards management of OS and hypercholesterolemia in addition with AD therapeutics *per se.* And incorporation of *G. lucidum* as an AD ameliorating agent seems pertinent. Differential expression of the proteins in the mushroom-fed AD rats might either be an adaptive response or protective strategy against Aβ-mediated stresses. Admittedly, the regulated proteins, identified in the current experiment but not previously been studied, warrants extensive exploration for much conclusive remarks. In this endeavor, western blot analysis of the mostly regulated proteins for validation of the current findings is the immediate future aspect of the present study.

CHAPTER 8: GENERAL DISCUSSION

The present study was aimed at elucidating the anti-oxidative and cholesterol lowering capacities of the selected edible-medicinal mushrooms in AD amelioration. The selected edible mushrooms were *H. erinaceus, L. edodes* and *F. velutipes* while the medicinal representative was the *G. lucidum.* Though mushrooms have been regarded highly for their immense nutritive and medicinal values since ancient times, identification and utilization of those bio-components in human ailments have been practised recently. Contents of different bio-components enable mushrooms to provide nutritional and medicinal support in human health and diseases (Guillamón *et al.,* 2010; Paterson, 2006; Rahman *et al.,* 2015 and Wasser, 2011). Among various mushroom bio-components, phenolics, polysaccharides and proteins have been ascribed for their health giving properties such as immunostimulating, anti-microbial, anti-cancer and anti-CVD (Guillamón *et al.,* 2010; Paterson, 2006; Rahman *et al.,* 2015 and Wasser, 2011). Although different aspects of different mushrooms have been explored worldwide, studies encompassing combined anti-oxidative, cholesterol lowering and AD amelioarating effect of edible-medicinal mushrooms are scarce. Been intrigued by this, the current comparative anti-oxidative studies of the aforementioned edible-medicinal mushrooms have been conducted followed by the identification of the bio-active components in the potent mushroom extracts and fractions as well as evaluated the effect of the most potent fraction upon cholesterol lowering, AD ameliorating and proteomics status. As activity of different bio-components varies depending on the extracting media, hydro-philic (aqueous, hot water and methanol), lipo-philic (DCM, hexane) and mixed (methanol:DCM) type of solvents were used in the present study to extract the corresponding solvent-solvent partitioned fractions and extracts (Mazzola *et al.,* 2008).

Utilizing the mushroom fractions and extracts, both *in vitro* and *in vivo* anti-oxidative experiments have been performed in the present study. *In vitro* anti-oxidative tests included DPPH free radical scavenging, total phenolic assay, lipid peroxidation and LDL oxidation assays. Although, performance of the selected edible-medicinal mushrooms varied in different tests, *G. lucidum* showed the most potent *in vitro* anti-oxidative potentiality. Notably, the hydro-philic HWE of *G. lucidim* excelled in DPPH free radical scavenging and in Folin-Ciocalteu assay (chapter 3, section 3.3.1 and 3.3.2). Folin-Ciocalteu assay measured the reducing capacities of the mushroom fractions and extracts and compared to others, the observed reducing capacities were of higher values (Abdullah *et al.,* 2011; Heleno *et al.,* 2015; Kim *et al.,* 2008; Mau *et al.,* 2002; Rawat *et al.,* 2013; Reis *et al.,* 2012; Wong *et al.,* 2009). On the contrary, the lipo-philic hexane fraction showed better inhibition towards lipid peroxidation and LDL oxidation (chapter 3, section 3.3.3 and 3.3.4). Subsequent identification of the bio-components in the respective extracts and fractions revealed the presence of different bio-components. Among the most notable hydrophilic bio-components were phenolics such as protocatechuic acid, p-coumaric acid, ellagic acid, cinnamic acid and triterpenoids such as ganoderic acids of different types (chapter 4, section 4.3.5, Table 4.5). Among the lipo-philic bio-components were ergosterol and fatty acids. In the present study, 11-octadecanoic acid (vaccenic acid) was identified as a novel fatty acid present in the *G. lucidum* (chapter 4, section 4.3.1, Table 4.1). Current findings support the notion that solvents of higher polarity extract the phenolic compounds much than those of the non-polar (Roby *et al.,* 2013). Lipophilic bio-components present in the non-polar hexane might have imparted lipid peroxidation and LDL oxidation inhibition either becoming oxidized themselves or through quenching the free radicals (Turchi *et al.,* 2009). Another important finding was the content of as much as 44.15% β-D-glucan in the HWE of *G. lucidum* (chapter 4, section 4.3.6). As the physiological properties of β-D-

glucan such as molecular size and structure as well as source and extracting condition affect the anti-oxidative performance, the hot water extraction process might have conferred favorable anti-oxidative arsenal to the β-D-glucan and the cumulative effect has been demonstrated as the enhanced anti-oxidative potency of the HWE of *G. lucidum* (Kofuji *et al.,* 2012).

As the HWE of *G. lucidum* showed better *in vitro* anti-oxidative potential, it was chosen for *in vivo* anti-oxidative and hypocholesterolemic tests. At 200 mg/kbw, this mushroom extract improved anti-oxidative defenses of the experimental animals as revealed by the increased levels of plasma catalase, glutathione peroxidase and superoxide dismutase (chapter 5, section 5.3.1,Tables 5.1 and 5.2). Present findings are in line with those of Hasnat *et al.* (2013), Jang *et al.* (2014) and Zhu *et al.* (2016). In hypocholesterolemic study, the same mushroom extract at the same dosage could reduce body weight gain and lower plasma TC, TG and LDL-C levels and increase HDL-C level (chapter 5, section 5.3.2, Figure 5.1 and Table 5.3). Similar observations have been noticed by Amin *et al.* (2011) and Chu *et al.* (2012). Importantly, feeding of the HWE of *G. lucidum* has not evoked any adverse health effects to the rats as evidenced by the organ function tests (chapter 5, section 5.3.3, Table 5.4).

For evaluating the AD ameliorating effect of the HWE of *G. lucidum,* the above mentioned dosage (200 mg/kbw) was applied to the intraventricularly $A\beta_{1-42}$-infuesed AD rat models and compared with the controls. AD specific tests included memory and learning related behavior tests (8-armed radial maze and novel object recognition tests) antibody and ELISA based tests and proteomics (Table 6.2). As described in the chapter 6 (section 6.3), improved memory and learning abilities have been observed in the *G. lucidum* HWE fed rats (Figures 6.4, 6.5, 6.6 and 6.7). As memory impairment is the main symptom affecting the AD subjects, its improvement is the prime target of AD

therapeutics (Ross, 2009; Davis & Couch, 2014). Thus, the observed memory enhancing effects seemed promising in proceeding with other aspects of AD in the present study. To substantiate the memory enhancing effect of the HWE of *G. lucidum,* neuro-biochemical tests had been conducted. Enhanced level of the synaptic and memory related proteins (BDNF, SNAP 25, PSD 95 and VAchT) and decreased anti-$A\beta_{(1-42)}$ oligomer and TNFα levels substantiated the AD ameliorating effect of the *G. lucidum* HWE (Table 6.2). Another set of evidence emanates from the TEM studies of the hippocampi of the rats. TEM studies showed that the neurons of the *G. lucidum* HWE fed rats' hippocampi possessed prolonged dendrites whereas those of the AD that possessed degenerative neurons and diffused Aβ plaque like deposition (Figure 6.9).

In addition to direct abnormality with Aβ, altered expression of various proteins has been implicated in AD pathogenesis (Butterfield & Dalle, 2014). Current study also focused on that arena through proteomics of the selected rat groups (controlled, AD and the *G. lucidum* HWE fed AD rats). A total of 822 proteins were identified with minimum peptide 2, threshold 95.0% and peptide threshold at 0.1% FDR. Among them, 329 were differentially expressed ($P<0.05$) and 289 had log fold change of 1.5 and above (chapter 7, section 7.4). Number of proteins linked with AD, OS and hypercholesterolemia was 59, 20 and 12, respectively. Most of the down-regulated proteins in the AD rats were involved in neurotransmission, synaptic functions, stress response, mitochondrial oxidative phosphorylation and neuronal cytoskeleton maintenance (chapter 7, section 7.4). As a whole, number of the proteins differentially expressed in the AD rats was those involved in metabolism. PPI analyses of the significantly expressed proteins with log fold change value of 1.5 and above were showed different interacting networks among the proteins (chapter 7, section 7.4.1.2). Last of all, IPA analyses of the same proteins were performed that revealed the connectivity strength among different networks and identified the functional pathway

interactions among them (chapter 7, section 7.4.1.3). The top-most network has been associated with cell death and survival, neurological diseases and psychological disorders. The second top-most network involved physiological system development and functions included molecular transport, cell-cell signaling and interaction, nervous system development and functions. Findings of the present study closely resemble those of Musunury *et al.* (2014) and Hondius *et al.* (2016). Thus, the present study interprets the anti-oxidative and hypocholesterolemic effects of edible-medicinal mushrooms in AD amelioration. The *G. lucidum* HWE has been found as the interpretor and thus it can be regarded as an AD therapeutic agent.

CHAPTER 9: CONCLUSION

Based on the ever increasing global AD burden and need of functional food-based therapeutic approach, the present study had been designed to judge the suitability of *G. lucidum* in ameliorating AD through anti-oxidative and hypocholesterolemic approaches. Perhaps, this is the first in type subjected to the combined anti-oxidative, cholesterol lowering and AD ameliorating effects of this and the other selected (*H. erinaceus, L. edodes* and *F. velutipes)* mushrooms.

Among the selected edible-medicinal mushrooms, the best one was screened through *in vitro* anti-oxidative tests (DPPH free radical scavenging, Folin-Ciocalteu assay, lipid peroxidation inhibition and LDL oxidation inhibition). Based on their *in vitro* anti-oxidative performances, the selected mushrooms could be ranked as *G. lucidum* > *H. erinaceus* > *L. edodes* > *F. velutipes*. Among the *in vitro* anti-oxidative outcomes, one important aspect was the identification of the higher reducing power of the mushroom fractions and extracts expressed as the gallic acid equivalents. Besides, lengthening of the lag time of CD formation by them was a unique approach in the chosen field and also for the selected mushrooms. Identification of 11-octadecanoic acid (vaccenic acid) in the *G. lucidum* was a novel finding. According to the β-D-glucan detecting kit information, presence of 44.15% β-D-glucan in the HWE of *G. lucidum* in the present study represents very close conformity to the highest amount of β-D-glucan ever detected.

In vivo studies showed that the hot water extract of *G. lucidum* at 200 mg/kbw could boost up the levels of plasma anti-oxidant enzymatic levels (catalase, glutathione peroxidase and superoxide dismutase), lower plasma and liver cholesterol levels without any adverse effect to the other organs. Beyond anti-oxidative studies, this outcome would be helpful for the hyperlipidemia and toxicity studies. Thus, the objectives of the

present study concerned with the anti-oxidative and cholesterol lowering performances have been fulfilled.

As oxidative stress and hypercholesterolemia have been implicated as the common causative factors of AD, the anti-oxidative and hypocholesterolemic outcomes of the *G. lucidum* HWE turned out promising in the amelioration of AD. Improved memory and learning related behavioral performances of the *G. lucidum* HWE-fed rats are the ground-breaking findings in respect of disrupted memory functioning of AD. Compared with the AD and age-matched controls, the elevated levels of the memory and learning related proteins in the *G. lucidum* HWE-fed rats substantiates the improved cognitive accomplishments highly. TEM outcomes of regular arrangement, prolonged neuronal dendrites and absence of Aβ plaque-like diffused neurons in the mushroom-treated rats bear evidence to the neuroprotective feat.

Mushroom-treated AD rats' brain proteome has not been explored yet. Proteomics outputs support the already mentioned findings of the present study. The most noteworthy are the disrupted anti-oxidative proteomic defense, mitochondrial oxidative phosphorylation, metabolic enzymes, stress response and neuronal cytoskeleton. Network analyses of the significantly altered proteins also identified unique structural and functional interactions among them. A limitation of the present study was that the proteomics outcomes could not be verified through Western blot studies. This remains one of the future aspects of the current study.

Thus, through *in vitro* anti-oxidative tests, the current study screened the HWE of *G. lucidum* among the four edible-medicinal musrhooms (*H. erinaceus, L. edodes, F. velutipes* and *G. lucidum*) and undertook *in vivo* anti-oxidative, cholesterol lowering and AD specific tests including proteomics. Observed results suggest the AD ameliorating effect of the hot water extract of *G. lucidum* that would be of immense importance in

formulating functional food-based AD therapeutics. In this regard, studies entailing specific bio-component such as β-D glucan, ganoderic acid of any specific type or ergosterol could be recommended. At the same time, both acute and chronic toxicity tests of the experimental subjects are suggested.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

Rahman, M. A., Abdullah, N., & Aminudin, N. (2014). Inhibitory effect on in vitro LDL oxidation and HMG Co-a reductase activity of the liquid-liquid partitioned fractions of *Hericium erinaceus* (Bull.) persoon (Lion's Mane Mushroom). *BioMed Research International*, 2014.

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Research Article

Inhibitory Effect on *In Vitro* LDL Oxidation and HMG Co-A Reductase Activity of the Liquid-Liquid Partitioned Fractions of *Hericium erinaceus* (Bull.) Persoon (Lion's Mane Mushroom)

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Oxidation of low-density lipoprotein (LDL) has been strongly suggested as the key factor in the pathogenesis of atherosclerosis. Mushrooms have been implicated in having preventive effects against chronic diseases due especially to their antioxidant properties. In this study, *in vitro* inhibitory effect of *Hericium erinaceus* on LDL oxidation and the activity of the cholesterol biosynthetic key enzyme, 3-hydroxy-3-methyl glutaryl coenzyme A (HMG Co-A) reductase, was evaluated using five liquid-liquid solvent fractions consisting of methanol : dichloromethane (M : DCM), hexane (HEX), dichloromethane (DCM), ethyl acetate (EA), and aqueous residue (AQ). The hexane fraction showed the highest inhibition of oxidation of human LDL as reflected by the increased lag time (100 mins) for the formation of conjugated diene (CD) at 1 μg/mL and decreased production (68.28%, IC_{50} 0.73 mg/mL) of thiobarbituric acid reactive substances (TBARS) at 1 mg/mL. It also mostly inhibited (59.91%) the activity of the HMG Co-A reductase at 10 mg/mL. The GC-MS profiling of the hexane fraction identified the presence of myconutrients: *inter alia*, ergosterol and linoleic acid. Thus, hexane fraction of *Hericium erinaceus* was found to be the most potent *in vitro* inhibitor of both LDL oxidation and HMG Co-A reductase activity having therapeutic potential for the prevention of oxidative stress-mediated vascular diseases.