

CHAPTER ONE

1.0 INTRODUCTION

Western society constantly searches for new natural substances that can improve and enhance the biological functioning of humans, and has recently turned to foods as a potential source of these. These products are known by a variety of names: dietary supplements, functional foods, nutraceuticals, nutriceuticals, phytochemicals, biochemopreventatives and designer foods (Zeisel, 1999).

Mushrooms are highly nutritive as they contain good quality proteins, vitamins and minerals (Khanna and Garcha, 1984 and Flegg and Maw, 1976). Mushrooms are low calorie food with very little fat and are highly suitable for obese persons. With no starch and very low sugars, they are the 'delight of the diabetics' (Bano, 1976).

Mushrooms being rich in amino acids on dry basis are suitable for the supplementation, especially to cereals and millets. The Food and Agricultural Organization recognized mushrooms as food contributing to the protein nutrient of the countries which depend largely on cereals because of their high protein quantity and quality. Besides, it enhances aesthetic, sensory and medical quality as well (Chang, 1993).

Several types of mushroom products are available on the market today:

- ❖ Dried naturally growing mushroom fruiting bodies in the form of capsules or tablets.
- ❖ Artificially cultivated fruiting body powders, hot water or alcohol extracts of these, or the same extract concentrates and their mixtures.
- ❖ Dried and pulverized preparations of the combined substrate, mycelium, and mushroom primordial after inoculation of edible semisolid medium (usually grains).

- ❖ Biomass or extracts from mycelium harvested from submerged liquid cultures grown in a fermented tank.

In this aspect, mushroom-based dietary supplement have some advantages over herbal preparations. These advantages are based on the following facts:

- ❖ The overwhelming majority of mushrooms used for production of dietary supplement are cultivated commercially (and not gathered in the wild). This guarantees proper identification, pure and unadulterated products. In many cases it also means genetically uniformity.
- ❖ Mushrooms are easily propagated vegetatively, and thus safeguard clonality. The mycelium can be stored for a very long time, and the genetic and biochemical consistency might be tested after considerable time.
- ❖ Many edible and medicinal mushrooms are capable of growing in the form of mycelial biomass in submerged cultures.

The problem is that mushroom-based dietary supplements are so diverse and that there are currently no standard protocols for guaranteeing their product quality and critical testing. There is a serious need for improved quality and legal control, which are essential both to increase and maintain consumer confidence, and to meet current and future standards set by the regulatory authorities (Chang and Buswell, 1999). However, there is still a criticism of dietary supplement safety, and most of it is concerned with standardization, i.e. producing mushrooms in such a way that there is consistency in chemical composition and effects across batches. This depends on numerous factors including cultivation conditions, purity and quality of ingredients and composition of substrates, stability of active compounds and storage, etc. (Wasser *et al.*, 2000).

Mushrooms are promising resource of physiologically functional food and as well as materials for the development of harmless medicines, pharmaceutical products such as new drugs, dietary supplements, healthy beverages and some cosmetic products

(Mizuno *et al.*, 1992; Hobbs, 1995). Mushrooms have been long appreciated for their flavour and texture. Now they are recognized as a nutritious food as well as an important source of biologically active compounds of medicinal value (Breene, 1990). Mushrooms accumulate a variety of secondary metabolites, including phenolic compounds, polyketides, terpenes and steroid.

Many pharmaceutical substances with potent and unique valuable properties have been isolated recently from mushrooms and distributed worldwide. Most mushroom-derived preparations and substances find their use not as pharmaceuticals (“real” medicine), but rather as a novel class of dietary supplements (“nutriceuticals”).

Biological response modifiers deserve special attention, as many mushroom preparation are described as such modifiers, or immunopotentiators (Hobbs, 1995; Wasser and Weis, 1999). Biological response modifiers are defined as agents that modify the host’s biological response by stimulation of the immune system. This may result in various therapeutic effects, according to the following criteria:

- ❖ They should cause no harm and place no additional stress on the body.
- ❖ They should help the body to adapt to various environmental and biological stresses.
- ❖ They must have a nonspecific action on the body, supporting some or all of the major systems, including nervous, hormonal, and immune system, as well as regulatory functions.

Another shortcoming of the term “biological response modifiers” is that many of the mushroom preparations possess particular physiological effects, such as lowering of blood cholesterol, or hepatoprotective activity (Hobbs, 1995; Chang and Buswell, 1999, Wasser and Weis, 1999).

Fungi of the *Pleurotus* genus have an important place among the commercially employed basidiomycetes because they have gastronomic, nutritional and medicinal

properties and can be easily cultivated on a large range of substrates. Besides the studies in solid culture aiming for the production of fruit bodies, the submerged culture of the genus *Pleurotus* has also been studied by several authors with the most varied objectives including the production of liquid inoculum (Rosado *et al.*, 2002), extracellular enzymes (Garzillo *et al.*, 1994), flavoring agents (Martin, 1992), β -glucosidases (Morais *et al.*, 2002), antimicrobials (Wisbeck *et al.*, 2002) and vitamins (Solomko and Eliseeva, 1988). Biomass and intra and extracellular polysaccharides are also the aim of several studies. Jung *et al.* (2003) investigated the effect of thinned fruits, apple, pear and peach on the mycelial growth of *Pleurotus* finding superior yields of biomass than those found in control medium. Wang *et al.* (2005) used a response surface methodology to determine the optimal conditions for production of water soluble polysaccharides of the culture broth of *Pleurotus citrinopileatus*. Sarangi *et al.* (2006) purified proteoglycan fractions from *P. ostreatus* mycelia that could be used as immunomodulators and anticancer agents.

The polysaccharides represent the major constituent that determines the rigidity and morphological properties of the fungal cell wall and, depending on the culture conditions they can also be excreted to the culture medium. Among the polysaccharides produced by *Pleurotus* spp., β -1,3 glucans play an important role as biological response modifiers (Bohn and BeMiller, 1995) which stimulate the immune system of the host and exert an extensive range of immunopharmacological activities, in particular an antitumor effect and the inhibition of metastasis, as well as the stimulation of hematopoiesis (Mizuno and Zhuang, 1995, Gunde-Cimerman, 1999 and Wang *et al.*, 2005).

The edible mushrooms of the genus *Pleurotus* are biologically versatile fungi which could therefore serve as the basis for various biotechnological and environmental

applications. Some of these applications are traditional and practiced throughout the world and some need further research and development (Cohen *et al.*, 2002).

Pleurotus species are temperate mushrooms that require cool temperatures of 20°C to 24°C to produce fruitbodies but it is now being successfully domesticated to adaptation to tropical climate in Malaysia. To our knowledge, there is no information available on the biological activities of the mycelial of *Pleurotus* species that is locally grown. Therefore, the objectives of the study were to investigate and determine the medicinal activities of mycelial extracts of *Pleurotus* species grown in Malaysia.

Previous findings have showed that *Pleurotus citrinopileatus* possesses anti-diabetic properties and *Pleurotus sajor-caju* with antioxidant properties. Hence, in this proposed research, these medicinal properties in nine *Pleurotus* species of Malaysian strain including *Pleurotus sajor-caju* and *Pleurotus citrinopileatus* will be determined. Besides that, additional findings including the determination of insulin levels in plasma and biomarker response in liver enzymes will be conducted to support the theory.

1.1 OBJECTIVES OF STUDY

The main objectives of this study were to:

- a) To produce mushroom mycelia in liquid culture.
- b) To determine antioxidant levels in extracts from mycelia of *Pleurotus* species using FRAP, DPPH and TPC methods.
- c) To evaluate anti-diabetic activities of extracts from *Pleurotus citrinopileatus* mycelia by measuring glucose and plasma insulin levels in diabetic rats and analyze the kidney tissues of diabetic rats and non-diabetic rats.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 MEDICINAL AND THERAPEUTIC PROPERTIES OF MUSHROOMS

Mushrooms have been used in folk medicine since ancient times (Wasser and Weis 1999). Of approximately 10000 known species, about 2000 are safe, while about 300 possess significant pharmacological properties (Hobbs, 1995; Miles and Chang, 1997). Most of the traditional knowledge about medicinal properties of mushrooms comes from the Far East (China), Japan, Korea and Russia), where such mushrooms as reishi [*Ganoderma lucidum* (Curt.: Fr.) P. Karst.], shiitake [*Lentinula edodes* (Berk.) Sing.], chaga [*Inonotus obliquus* (Pers.: Fr.) Bond. *et. Sing.*] and others have been collected, cultivated, and used for millennia.

Mushrooms represent a major and as yet largest untapped source of powerful new pharmaceutical products. Many pharmaceutical substances with potent and unique properties were isolated recently from mushrooms and distributed worldwide. They are also known as nutraceuticals, phytochemicals, mycochemicals and biochemopreventatives (Wasser and Weis, 1999). Some of the recently isolated and identified substances of the mushrooms express promising antitumor, immune modulating, cardiovascular and antihypercholesterolemia, antiviral, antibacterial, antiparasitic, hepatoprotective and diabetic effects (Wasser and Weis, 1999).

Fresh and dried edible mushrooms such as Maitake, Shiitake and *Pleurotus sajor-caju* are used as materials for Japanese, Western-style, and Chinese cooking, because of their excellent flavour, texture and condiment value. Nutritionally it is considered to be rich in protein, fiber, carbohydrates along with vitamin C, B and a good source of minerals (Chang, 1999).

Wasser and Weis (1999) reviewed the recently isolated and identified substances of higher basidiomycete mushroom origin that show promise for medicinal use. In particular, mushrooms are useful against cancers of the stomach, esophagus, lungs, etc. are known both in China, Japan, Korea and Russia and in the United States and Canada. Both cellular components and secondary metabolites of a large number of mushrooms have been shown to affect the immune system of the host and therefore could be used to treat a variety of diseases (Wasser and Weis, 1999).

At present, the shiitake mushroom (*Lentinus edodes*) is valued for its properties as an immunomodulator (it increases the action of natural killer cells and amount of interferon) and an antiviral, antibacterial, antiparasitic and liver – fortifying agent. Shiitake produces a high molecular weight cell-wall polysaccharide called lentinan, which has been extensively used as an injectable anticancer drug in Asia (Mizuno *et al.*, 1999a). This species has also been known for its cholesterol lowering, cardiovascular and hepatoprotective properties (Irinoda *et al.*, 1992; Hobbs, 1995 and Wasser and Weis, 1997b). Shiitake is rich in several antioxidants such as selenium, uric acid and vitamin A, E, C and D.

Ganoderma species are not normally eaten as a food item but are used as traditional medicine in China and Japan (Quimio, 1986). These mushrooms belong to the order Aphyllophorales and are generally called polypores. The fruit bodies are usually tough and leathery, reddish in colour and grow as shell-like forms on tree trunks, logs and stumps. They are called Ling-Zhi in China and mannentake in Japan. The fruit bodies have traditionally been used for medicinal purposes. Quimio has pointed out that both the mycelia and fruit bodies can be used as medicine and had briefly described how they are cultivated in Japan and China (Quimio, 1986).

Reishi has been called an “immune potentiators” and can increase the production of Interleukin-1 and 2. It has been reported that Reishi extracts “exerted an inhibition

effect on tumor growth” (Nanba *et al.*, 1987). Recent studies have also indicated that Reishi can have a number of other effects such as analgesic, anti-inflammatory, antioxidant, antiviral through inducing interferon production, lowers blood pressure, cardiotonic action through lowering serum cholesterol, expectorant and antitussive, liver (hepatitis)-protecting and detoxifying. Reishi contains calcium, iron and phosphorus as well as vitamins C, D and B-including pantothenic acid, which is essential to nerve function and the adrenal glands.

Red Ling-Zhi is used in China to treat neurasthenia, insomnia, coronary heart disease, hypercholesterolemia, chronic hepatitis, bronchial asthma, stomach ulcers and indigestion, chronic inflammation of the liver, kidney and bone joints and high blood pressure (Lindequist *et al.*, 2005).

Grifola frondosa or maitake has been extensively studied (Zhuang and Mizuno, 1999), and has been proven to be a more powerful anticancer and immune-enhancing agent than any other medicinal mushrooms tested so far. It is recommended as a supplement for people with cancer, AIDS and other immune-system disorders as well as those with chronic fatigue syndrome, chronic hepatitis and environmental illnesses that may represent toxic overloads.

Hericium erinaceus contains a number of biologically active substances. Most important, it is a high molecular weight fraction that possesses immune-enhancing and anticancer activities; alcohol extracts promote nerve growth factor synthesis and lectin from its fruiting bodies inhibits the agglutination of blood. It is also rich in dietary fibers (Mizuno *et al.*, 1999b). Besides that, it is also known for its antioxidant property (Wong, *et al.*, 2009).

Tremella fuciformis or silver ear has been used in China since ancient times for strengthening health and curing several kinds of diseases; furthermore, it contains skin nutrients and can improve personal appearance (Yang and Jong, 1989). *T. fuciformis* is

a member of the so-called jelly mushrooms. Many of these mushrooms possess similar medicinal properties owing to the presence of the heteropolysaccharide glucuronoxylomannan in their fruiting bodies. According to modern investigations, *Tremella* mushrooms possess pharmacological activities such as stimulation of the immune system (Ma and Lin, 1992), antitumor (Ukai *et al.*, 1972), hypolycemic (Kiho *et al.*, 2000) and decreases the cholesterol level in blood serum (Yui *et al.*, 1995). They are effective for treating cough, phlegm and asthma and also for chronic bronchitis (Yang and Jong, 1989; Hobbs; 1995).

In vitro evaluation of antioxidant activities of *Auricularia auricular* has shown significant inhibition of lipid peroxidation and potent hydroxyl radical scavenging activity when compared with the drug catechin.(Acharya *et al.*, 2004 and Kho *et al.*, 2009). *Auricularia auricula* had very high dietary fiber content (Cheung, 1997) which may have potential hypocholesterolemic effect similar to other high-fiber foods and considerable attention has been drawn to the immunomodulating activities, such as anti-tumor activities, exhibited by their non-starch polysaccharide components, especially β -glucans (Misaki *et al.*, 1993).

Several bioactive molecules have been identified in various edible mushroom species showing strong anti-cancer activities: polysaccharides, mainly β -D-glucans of heterogeneous molecular weights, proteoglycans or β -D-glucans bound to peptides, lectins, fibres, terpenoids, steroids, nucleic acids and others (Wang *et al.*, 2000).

Lectin was isolated from mycelia of *P. cornucopiae* grown on solid medium (Oguri *et al.*, 1996). The appearance of this lectin was associated with fruiting body formation; and its hemagglutinating activity was studied. A reductase inhibitor, the anti-cholesterol compound lovastatin, has been found in *Pleurotus* species, including *P. eryngii* (Gunde-Cimerman and Cimerman, 1995).

In humans 50 % or more of the cholesterol is derived from *de novo* synthesis (Rosenfeld, 1989). Clinical intervention studies have demonstrated the therapeutic importance of correcting hypercholesterolemia. The initial step in lowering cholesterol is a special diet low in fat and saturated fatty acids and rich in crude fibers. *Auricularia auricula-judae* has shown the following effects and activities in studies on mice and rat; anticoagulant, lowered total cholesterol, triglyceride and lipid levels (Sheng and Chen, 1989); antiaggregatory activity on blood platelets, which might make it beneficial for coronary heart disease (Agarwal *et al.*, 1982). This mushroom is used traditionally as an immune tonic.

2.2 PLEUROTUS SPECIES AND ITS BIOLOGICAL ACTIVITIES

The genus *Pleurotus* (Jacq.: Fr.) Kumm. (Pleurotaceae, higher Basidiomycetes) is a cosmopolitan group of mushrooms with therapeutic properties and various environmental and biotechnological applications. This species are characterized by a white spore print, attached to the current gills, often with an eccentric stipe. The common name “oyster mushroom” stems from the white, shell – like appearance of the fruiting body. It is a white rot fungus that is capable of degrading both lignin and cellulose. *Pleurotus* species are primary agents of decomposition. They have the ability to directly break down cellulose and lignin bearing materials without chemical or biological preparation or composting. According to Singer (1978), the genus *Pleurotus* has a total of 39 species distributed into 4 sections. They have mostly lignicolous, saprophytic habit, sometimes occurring as a parasite.

Pleurotus species are among the easiest mushrooms to cultivate (Hilber, 1997). In nature, they grow on wood, usually on dead, standing trees or on fallen logs. Various substances that contain lignin and cellulose can be used for *Pleurotus* cultivation, such

as wood chips, corn wheat, rice straw, cotton stalks, waste hulls and other agricultural wastes, of which can be recycled and upgraded for use as animal feed or in the preparation of other products. Utilization of these materials is clearly dependent on *Pleurotus* ability to secrete a range of enzymes, including peroxidases, laccases, cellulases, hemicellulases and xylanases.

The genus *Pleurotus* is one of the most diverse groups of cultivated mushrooms and are flavourful edible mushrooms with high nutritional value found throughout temperate, tropical and subtropical climate zones of the world. This mushroom represents a valuable source of protein for rural populations and its production has increased significantly in recent years (Chang, 1999).

In this genus, more species have been reported as cultivated than in any other genus, of agaric. Zadrazil (1978), Raper (1978a) and Martinez – Carrera *et al.*, (1991) have listed the following species as cultivated ones.

- i. *Pleurotus ostreatus* (Jacq.: Fr.) P. Kummer
- ii. *Pleurotus sp. florida* (Mont.) Singer
- iii. *Pleurotus eryngii* (DC.: Fr.) Quél.
- iv. *Pleurotus cornucopiae* (Pers.) Rolland
- v. *Pleurotus sajor-caju* (Fr.) Singer
- vi. *Pleurotus flabellatus* (Berk. & Broome) Sacc.
- vii. *Pleurotus citrinopileatus* (Singer)
- viii. *Pleurotus sapidus* (Schulz.) Sacc.
- ix. *Pleurotus smithii* (Guzmán)
- x. *Pleurotus cystidiosus* (O. K. Mill)

Pleurotus spp. can also be cultivated in submerged – liquid cultures or on solid media for the production of fungal protein, or as a source of spawn or flavouring agents. Hadar and Cohen – Arazi (1986) studied the chemical compositions of *P. ostreatus*

mycelia produced on cotton straw. A comparison of the fruiting bodies and mycelial pellets (total protein, amino acids and fatty acids) revealed highly similar chemical compositions.

Pleurotus ostreatus is considered a good dietary option because of its nutritional value – it is rich in protein, fibre, carbohydrates, vitamins and minerals and its low content of calories, fats and sodium. *P. ostreatus* contains B-D-Glucan substance that gives antibacterial, immunomodulating, antitumor and antioxidant effects (Bobek and Galbavy, 2001). This species contains ubiquitin-like protein that acts as antiviral property (Wang *et al.*, 2000). Substances derived from this species also possess anticholesterol effect (Bobek *et al.*, 1995,).

Among the wild species of the genus *Pleurotus*, the economic importance of *Pleurotus eryngii* is well established (Stamets, 1995; Hilber, 1997). The fruiting bodies of *P. eryngii* are comparable to those of the cultivated oyster mushroom (*P. ostreatus*), but are consistently better (cap and stem) with a more pleasant aroma and better culinary qualities (Zadrazil 1978).

Pleurotus eryngii and *P. cornucopiae* had an active compound known as lovastatin that enhances the anticholesterol property (Gunde-Cimerman and Cimerman, 1995). Besides that, *P. cornucopiae* also possesses lectin that acts on hemagglutination (Oguri *et al.*, 1996). Mau *et al.*, (1998) studied the flavour compounds in *P. eryngii* and found them to be mostly volatiles and taste components.

A novel lectin was isolated from mycelia of the basidiomycete *P. cornucopiae* grown on solid medium (Oguri *et al.*, 1996). The appearance of this lectin was associated with fruiting body formation; and its hemagglutinating activity was studied. A reductase inhibitor, the anticholesterol compound lovastatin, has been found in *Pleurotus* spp, including *P. eryngii* (Gunde – Cimerman and Cimerman 1995).

Krasnopolskaya *et al.*, (1998) reported the extraction of appreciable amounts of lovastatin from *P. cornucopiae*.

Generally, *Pleurotus* spp. possessed a wide range of medical effects. Mycelia and polysaccharide of *Pleurotus* spp. acts as an antibiotic (Bianco 1981; Wang and Wang, 1997). It also functions as an antibacterial and antiviral, antitumor and anti-inflammatory product (Hidaka and Ikegawa, 1998; Suzuki and Ikegawa, 1998).

Polysaccharide from *Pleurotus citrinopileatus* acts as an antiviral medicine in the era of new research (Zhang, *et al.*, 1994a).

2.3 OXIDATIVE STRESS

Oxygen is one of the most abundant element in our world, consisting 21% of the air we breath (Cantin, 1999). It is essential for the oxidation of organic compounds, which is the process by which mammalian cells generate the energy needed to sustain life. Most intracellular oxidations results in transfer of two electrons to appropriate acceptors such as NAD^+ or FAD, which are then oxidized by the electron transport chain. The terminal step in this chain is catalyzed by cytochrome c oxidase, which tightly binds O^2 to the binuclear center where stepwise reduction of O_2 occurs without release of intermediates in the oxidation process.

The electronic structure of O_2 , however favours its reduction by addition of one electron at a time, leading to the generation of oxygen radicals that can cause cellular damage. A radical is a molecule with a highly reactive unpaired electron in an outer orbital, which can initiate chain reactions by removal of an electron from another molecule to complete its own orbital. The stepwise transfer of electrons to O_2 results in formation of superoxidase anions (O_2^-), then hydrogen peroxidase (H_2O_2), and finally hydroxyl free radicals (OH^\cdot) (Halliwell and Gutteridge, 1990) as shown in Figure 2.1.

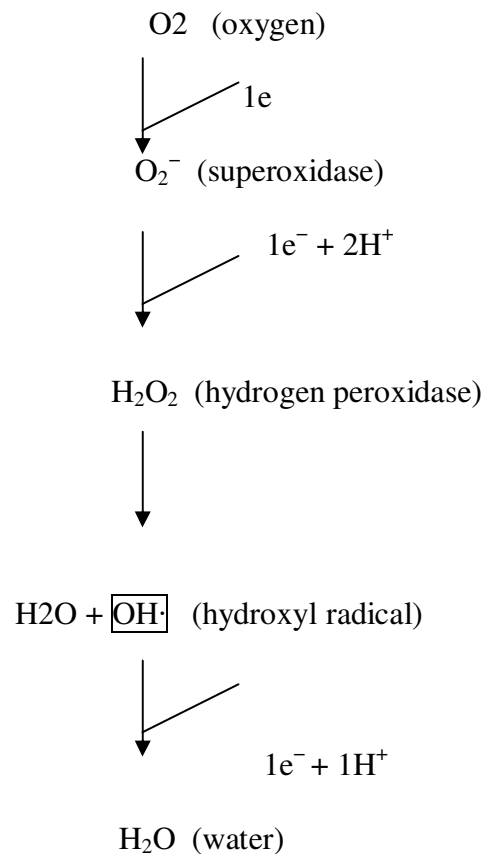


Figure 2.1: One – electron steps in reduction of oxygen, leading to formation of reactive oxygen species superoxidase, hydrogen peroxidase and hydroxyl radical.

The hydroxyl radical is undoubtedly the most dangerous free radical because it is involved in reactions such as lipid peroxidation and generation of other toxic radicals. Hydrogen peroxide itself is not a free radical but is converted by Fenton or Haber – Weiss reactions (Figure 2.2) to the hydroxyl radical in the presence of Fe^{2+} or Cu^+ , which are prevalent in cells.

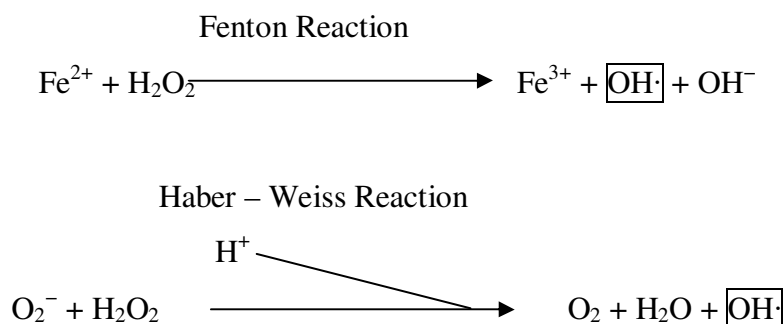


Figure 2.2: The Fenton and Haber – Weiss reaction for formation of toxic hydroxyl radical.

While oxidative processes in cells generally result in transfer of electrons to O_2 to form water without release of intermediates, a small number of oxygen radicals are inevitably formed due to leakage in electron transfer reactions. Under normal physiological conditions, the majority of reactive oxygen species (ROS) is produced in the mitochondrial electron transport chain since 90% of the oxygen consumption by the body is reduced to water in the mitochondria (Ames, 1995).

Mitochondrial electron transport chain is the major intracellular source of oxygen radicals where superoxidase is produced by transfer of one electron to O_2 from the stable semiquinone produced during reduction of ubiquinone by complexes I and II as shown in Figure 2.3. Superoxidase can also be produced by transfer of an electron from a flavin such as FMN.

Oxygen radicals are also produced in certain cells during inflammation due to bacterial infection. To combat microbial infections, phagocytes produce toxic oxygen radicals in a process known as the respiratory burst. The phagocytes then kill the engulfed bacteria. In an acute infection, production of oxygen radicals and killing of bacteria are efficient processes; however, in prolonged infection, phagocytes tend to die, releasing toxic oxygen radicals that affect surrounding cells.

Cosmic radiation, ingestion of chemicals and drugs, as well as smog can lead to formation of reactive oxygen species. Damage from ROS often occurs during perfusion of tissues with solutions containing high concentration of O_2 levels as happens in patients who have suffered an ischemic episode in which localized O_2 levels are lowered due to blockage of an artery and have then undergone thrombolytic or other procedures to remove the blockage.

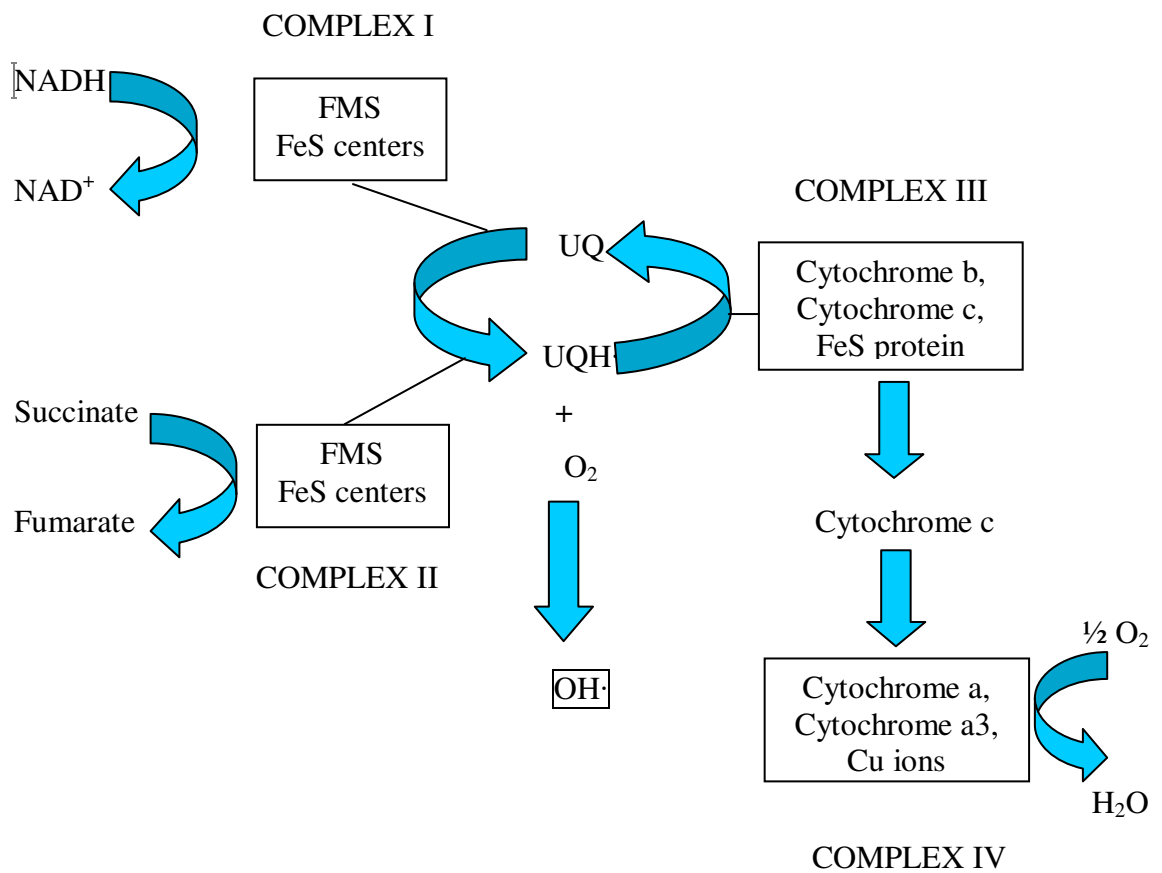


Figure 2.3: Generation of superoxidase anions by mitochondrial electron transfer chain.

The semiquinone formed during the two electron reduction of ubiquinone by the iron – sulphur centers of both complexes I and II can transfer an electron to oxygen to form the superoxidase anion. By contrast, the binuclear center of cytochrome c oxidase prevents release of intermediates in the reduction of oxygen.

2.4 DAMAGE CAUSED BY REACTIVE OXYGEN SPECIES.

Recently, it was suggested that the reactive oxygen species are the most probable cause of aging – related diseases, such as Parkinson’s disease and cancer (Fukae *et al.*, 2005) in which spontaneous mutations induced by reactive oxygen species might be accumulated during the aging process. Besides that, the effect of lipid peroxidation in humans is exemplified by the brown spots commonly observed on hands of the elderly. These “age” spots contain the pigment lipofuscin, which is probably a mixture of linked lipids and products of lipid peroxidation, which accumulate over the course of a lifetime.

Oxidative stress is a major determinant in conditions such as infection, inflammation, and aging, and there are multiple studies showing an association among oxidative damage, inflammation, cancer, and age-related disorders (Muller *et al.*, 2007). ROS cause damage to all major classes of macromolecules in cells. The ROS can damage intracellular components such as lipids, proteins and DNA (Imlay, 2003). The phospholipids of plasma and organelle membranes are subject to lipid peroxidation, a free radical chain reaction initiated by removal of hydrogen from a polyunsaturated fatty acid by hydroxyl radical. The resulting lipid radicals then react with O₂ to form lipid peroxy radicals and lipid peroxidase along with malondialdehyde, which is water – soluble and can be detected in blood. Proline, histidine, arginine, cysteine and methionine are susceptible to attack by hydroxyl radicals with subsequent fragmentation of proteins, cross – linking and aggregation. Proteins damaged by oxygen radicals may be targeted for digestion by intracellular proteases.

The most important consequence of oxygen radicals is damaged by mitochondrial and nuclear DNA, resulting in mutations. The non-specific binding of ferrous ions (Fe²⁺) to DNA may result in localized formation of hydroxyl radicals that

attack individual bases and cause strand breaks. Mitochondrial DNA is more susceptible to damage, since the electron transport chain is a major source of toxic oxygen radicals. Nuclear DNA is protected from permanent damage by a protective coat of histones as well as by active and efficient mechanisms for DNA repair. Damage to mtDNA generally results in mutations that affect energy production (Barja, and Herrero, 2000). The symptoms in affected individual are manifest in energy requiring processes such as muscle contraction. Besides that, accumulation of excessive amounts of Ca^{2+} in mitochondria may trigger apoptosis.

Damage caused by lipid peroxidation is highly detrimental to the functioning of the cell and its survival. It has been implicated in the pathogenesis of a number of diseases and clinical conditions. These include adult respiratory distress syndrome, Alzheimer's disease, Parkinson's disease, ischaemia – reperfusion injury of various organs, chemical and radiation – induced injury. Experimental and clinical evidence suggest that aldehyde products of lipid peroxidation can also act as bioactive molecules in physiological and pathological conditions (Devasagayam, *et al.*, 2003). It is now generally accepted that lipid peroxidation and its products play an important role in liver, kidney and brain toxicity (Poli, *et al.*, 1987 and Usyal, *et al.*, 2005).

Oxygen – mediated mechanism of damage will be the inhaling of ozone and nitric oxide which may induce toxic processes that impair lung function (Cantin, 1999). Besides that, another damage will be the inflammation, during which leucocytes, macrophages, and mast cells release mediators that may cause bronchoconstriction and edema as observed during an asthmatic reaction (Calhoun, 1992). Lung tissue can also be destroyed during reperfusion after an ischemic period such as that produced by surgery (Rahman, *et al.*, 2001.).

The elevated levels of blood glucose in diabetes produce oxygen – free radicals (OFR), which cause membrane damage due to peroxidation of membrane lipids and

protein glycation (Sato *et al.*, 1979). Oxidative damage due to hyperglycemia contributes to the microvascular pathology of diabetes that occurs particularly in the retina, renal glomerulus and peripheral nerves, causing blindness, renal failure and peripheral neuropathy (Rosen, *et al.*, 2001; West, 2000; Brownlee, 2001 and Vincent, *et al.*, 2002).

2.5 ANTIOXIDANTS

An antioxidant is a molecule capable of inhibiting the oxidation of other molecules. Oxidation is a chemical reaction that transfers electrons from a substance to an oxidizing agent. Oxidation reactions can produce free radicals. In turn, these radicals can start chain reactions that damage cells. Antioxidants terminate these chain reactions by removing free radical intermediates, and inhibit other oxidation reactions. They do this by being oxidized themselves, so antioxidants are often reducing agents such as thiols, ascorbic acid or polyphenols (Sies, 1997).

Common antioxidants include the vitamins A, C, and E, glutathione and the enzymes superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase. Other antioxidants include α -lipoic acid, mixed carotenoids, coenzyme Q₁₀, several bioflavonoids, antioxidant minerals (copper, zinc, manganese and selenium) and the cofactors (folic acid, vitamins B₁, B₂, B₆, B₁₂). Vitamin E suppresses the propagation of lipid peroxidation; vitamin C, with vitamin E, inhibits hydroperoxide formation; metal complexing agents, such as penicillamine, bind transition metals involved in some reactions in lipid peroxidation (Feher, *et al.*, 1987) and inhibit Fenton and Haber-Weiss-type reaction; vitamins A and E scavenge free radicals (Halliwell, 1990).

Antioxidants are widely used as ingredients in dietary supplements in the hope of maintaining health and preventing diseases such as cancer, coronary heart disease and even altitude sickness.

2.6 ANTIOXIDANT ACTIVITY OF MUSHROOMS

Recent evidence indicates that mushrooms contain a large number of biologically active components that offer protection against degenerative diseases. Since the role of free radicals has been implicated in a large number of diseases, the antioxidant activity of mushrooms is of significant importance in exploiting their therapeutic potential. A number of medicinal mushrooms have recently been reported to possess significant antioxidant activity (Ajith and Janardhanan 2001; Mau, *et al.*, 2001; Ekanem and Ubengama, 2002 and Jose, *et al.*, 2002).

Phellinus rimosus is a wood – inhibiting polypore – macrofungus often found growing on jackfruit tree trunks in Kerala. Earlier investigations showed that ethyl acetate extracts of *P. rimosus* possessed antioxidant activity (Ajith and Janardhanan 2001).

Ganoderma lucidum, commonly known as Reishi, is considered as a panacea in Chinese medicine because of its effectiveness in the treatment of a large number of diseases. It is known to possess DNA protective properties (Kim, *et al.*, 1994). Both aqueous and lipophilic extracts of Lingzhi have shown antioxidant properties in *in vitro* systems (Lee *et al.*, 2000).

Flammulina velutipes and *Lentinula edodes* were better in antioxidant activity, reducing power and scavenging abilities and higher in total phenol contents as shown in recent study (Yang, *et al.*, 2002). Mau *et al.*, 2002 proved that *Dictyophora indusiata*, *Grifola frondosa*, *Hericium erinaceus* and *Tricholoma giganteum* possessed the total

polyphenols of major naturally occurring antioxidant components by methanolic extracts. Methanolic extracts that were prepared from the mycelia of *Morchella esculenta* and *Termitomyces albuminosus* showed a good antioxidant effect (Mau, *et al.*, 2003).

2.7 DIABETES

Diabetes mellitus is a non-communicable disease, which is considered one of the five leading causes of death in the world. About 100 million people around the world have been diagnosed with diabetes and by the year 2010, it is projected that 215 million people will have the disease (Zimmet 1999). Diabetes mellitus is a generic disorder in which superimposed environmental factors bring out the phenotypic expression of the disease. It is a disorder of metabolism of carbohydrate, protein and fat associated with a relative or absolute insufficiency of insulin secretion and with various degrees of insulin resistance. It is characterized by fasting hyperglycemia, and in most patients with long-standing disease by microangiopathic and atherosclerotic macrovascular disease and by neuropathy. Diabetes, therefore, is not a single disease but a syndrome (Fagan and Deedwania, 1998).

The present classification includes three clinical classes:

- 1) Diabetes mellitus is characterized either by fasting hyperglycemia or levels of plasma glucose above defined limits during a glucose tolerance test.
- 2) Impaired glucose tolerance is characterized by plasma glucose levels during a glucose tolerance test that exceed normal but are below those defined as diabetes. The levels of plasma glucose in the fasting state or during a glucose tolerance test that are defined as normal, impaired, or diabetic are also compromises and not subscribed to as ideal by all investigators.

- 3) The third clinical class is gestational diabetes. Patients with gestational diabetes (GDM) have detection or onset of glucose intolerance during pregnancy. A known diabetic who becomes pregnant is not classified as having GDM. Gestational diabetes occurs in approximately 2% of all pregnant women and is associated with increased perinatal morbidity and increased frequency of loss of viable fetuses. Gestational diabetes usually returns to a state of normal glucose tolerance after parturition; even so, 60% of such women develop diabetes within 15 years after parturition.

2.8 OXIDATIVE STRESS AND DIABETES

The human body is exposed to free radicals from outside the body (exogenous) and inside the body (endogenous). Some of the factors that lead to free radicals are smog, cigarette smoke, radiation, consumption of excessive amounts of alcohol, and even sunlight. Yet, some factors that led to free radicals come from within the body. The cells necessitate oxygen to produce the energy they need to work properly. In the process known as mitochondrial respiration, the cells take in oxygen, burn it, and release energy. During the process, free radicals are produced.

Oxidative stress occurs when free radical production exceeds the body's ability to neutralize them. This imbalance happens when the antioxidant production is decreased or when the free radicals are produced in excess. Oxidative stress functions on both sides, meaning that it help the progression and the development of diabetes and its complications (Ha and Lee, 2000). In the study, it was shown that oxidative stress is one of the important mediators of vascular complications in diabetes including nephropathy (Ha and Lee, 2000). High glucose produces ROS as a result of glucose

auto-oxidation, metabolism, and the development of advanced glycosylation end products.

Hyperglycemia is a connector between diabetes and diabetic complications (Rolo and Palmeira, 2006). Hyperglycemia-induced overproduction of superoxide is the causal link between high glucose and the pathways responsible for hyperglycemic damage. In fact, diabetes is typically associated with increased generation of free radicals and/or impaired antioxidant defense qualifications, representing a central contribution for ROS in the onset, progression, and pathological consequences of diabetes. Besides oxidative stress, some evidence has demonstrated a link between various disturbances in mitochondrial functioning and type 2 diabetes. Mutations in mitochondrial DNA and decreases in mitochondrial DNA copy number have been connected to the pathogenesis of type 2 diabetes (Rolo and Palmeira, 2006). In addition, in the research of Brownlee, 2001, it was shown that hyperglycemia-induced process of overproduction of superoxide by the mitochondrial electron-transport chain by the four main molecular mechanisms and has been implicated in glucose-mediated vascular damage.

Oxidative stress is increased in diabetes and is more definite in women and this leads to cardiovascular disease (Marra *et al.*, 2002). In the study of Marra *et al.*, it was examined whether type 1 diabetic patients with short duration of disease and without complications have an altered oxidative status and whether there are differences between men and women. Compared with the control subjects the women with type 1 diabetes had lower plasma antioxidant capacity and higher lipid hydroperoxide levels. These findings indicate that reduced antioxidant activity and increased oxidative stress occur early after the diagnosis of type 1 diabetes, especially in women, and this might explain the increased susceptibility of diabetic women to cardiovascular complications (Marra *et al.*, 2002).

Indicative parameters of lipoperoxidation, protein oxidation, and changes in antioxidant defense system status were measured in blood samples and results showed erythrocyte glutathione peroxidase activity, glutathione content, and plasma beta-carotene to be significantly lower in diabetic patients compared with control subjects. Antioxidant enzyme superoxide dismutase activity was significantly higher in the erythrocytes of diabetic patients independently of the presence of microvascular complications. (Martin-Gallan *et al.*, 2003). In the survey of Ceriello *et al.*, in type 2 diabetes, free radical production has been reported to be increased in diabetic patients and to be implicated in the development of diabetic complications. A meal was given to patients and after the meal, plasma malondialdehyde and vitamin C increased, while protein SH groups, uric acid, vitamin E, and total plasma radical-trapping parameter decreased more considerably in the diabetic subjects than in control subjects. This result demonstrates that in the absorptive phase, free radicals are produced in diabetic patients. Since plasma glucose arose extensively more in diabetic subjects than in control subjects, hyperglycemia may play a significant role in the generation of postprandial oxidative stress in diabetic patients (Ceriello *et al.*, 1998).

2.9 CAUSES OF DIABETES

The presence of inflammatory cells in the islet of Langerhans was termed “insulitis” (Von Meyenburg, 1940). The significance of this insulitis at the clinical onset of insulin-dependent diabetes was first studied in detail by Gepts and LaCompte (1981). During this time antibodies and T cells reactive with beta-cell antigens can be detected, and the immunologic attack on the beta cells is occurring.

At least 80 to 90% of the functional capacity of the beta cells must be lost before hyperglycemia occurs. Because even the most sensitive tests of beta-cell function

remain normal until islet insulin content is very low (McCulloch, 1988), the immune markers of type I diabetes usually antedate evidence of beta-cell deficiency by a long period of time. Until very recently it was believed that beta-cell destruction was the primary mechanism responsible for the loss of insulin secretory capacity during the preclinical period of IDDM.

It is likely that in insulin-dependent diabetes there are one or more immune response genes in linkage disequilibrium with HLA antigens that may impart increased susceptibility to beta-cell damage by permitting interaction of an environmental factor with specific cell membrane antigens. In the most common type of IDDM, it has been postulated that environmental (acquired) factors, such as certain viral infection, and possibly nutritional or chemical agents, superimposed on genetic factors, may lead to cell-mediated autoimmune destruction of beta cells. Thus, genetically determined abnormal immune responses (linked to HLA associations), characterized by cell-mediated and humoral autoimmunity, are thought to play a pathogenetic role after evocation by an environmental factor. (In a very small subset of patients, an overwhelming viral infection may lead to destruction of beta-cells without a genetic predisposition.)

This immune injury is indicated by the presence of islet cell antibodies and insulin autoantibodies, which appear several years before the diagnosis of type I diabetes mellitus (Palmer, 1990). In addition, reduced insulin responses to intravenous glucose also appear months to years prior to severe hyperglycemia (Srikanta, 1986).

Immune-related damage of islet beta-cells has been shown to be related to islet beta-cells insulin-secretory activity at the time of exposure to IL-1 (Nerup, *et. al.*, 1988). Thus, increased secretion of insulin seems to be followed by an enhanced ability of IL-1 to impair beta cell function. Because increased beta cell secretion of insulin has been found during physical stress and infection, it has been postulated that the beta cells

would be more sensitive to injury at that time. This increase in insulin level is usually associated with insulin resistance. Glucocorticoids, growth hormone, and the catecholamines all causes insulin resistance, and it has been hypothesized that they are the cause of insulin resistance.

The second type of diabetes, type II or noninsulin – dependent diabetes mellitus (NIDDM), present in approximately 90% of diabetics in the world, also has a genetic basis that is commonly expressed by a more frequent familial pattern of occurrence than is seen in IDDM. Patients with type II diabetes may have a body weight that ranges from normal to excessive; indeed, NIDDM has been subclassified according to association with obesity. The intake of excessive calories leading to weight gain and obesity and resulting in or exacerbating insulin resistance is an important factor in the pathogenesis of NIDDM.

Obesity and pathologic insulin resistance are by no means essential in the evaluation of NIDDM. In NIDDM patients who are not overweight, even small increases in body weight (including normal growth in childhood and adolescence) can exacerbate glucose intolerance and precipitate fasting hyperglycemia (Fagan and Deedwania, 1998).

In Japanese NIDDM, Kuzuya and Matsuda (1982) also found that patients with definite obesity in the past had a lower frequency of a family history of diabetes and a lower prevalence of diabetes in their parents than did patients without obesity, supporting the concept that the presence or absence of obesity may mark heterogeneous groups of diabetics within the category of type II diabetes.

There is a persistent and important impairment of islet sensitivity to glucose in NIDDM that reduces the insulin response to all stimuli, but the hyperglycemia compensates for a reduced response to the nonglucose inputs (Halter, *et al.*, 1984). Thus, any stimulus that tends to impair insulin action or accelerate hepatic glucose

production will be more effective in elevating glucose in NIDDM patients because it would require a greater degree of hyperglycemia for the islet-cell adaptation to occur. In addition, it has now become apparent that islet beta cells are probably more sensitive to the inhibitory actions of epinephrine in NIDDM because the ability of epinephrine to impair islet function is also glucose-sensitive (Halter, *et al.*, 1984).

2.10 MECHANISMS OF DIABETES

After an overnight fast, glucose is produced solely in the liver by glycogen breakdown and gluconeogenesis, and the rate of production is dependent on the availability of hepatic glycogen and gluconeogenic precursors. About 80% of this glucose released by the liver is metabolized independent of insulin by the brain and other insulin-insensitive tissues, such as the gut and red blood cells. Insulin-sensitive tissues, such as muscle and fat, use only small quantities. A number of neural and hormonal influences regulate hepatic glucose production, and in the presence of adequate amounts of insulin, the glucose level itself can regulate hepatic glucose release (Liljenquist, *et al.*, 1979). Short-term hormonal regulators of physiologic importance include insulin, glucagons and the catecholamines; a more long-term influence on hepatic glucose production is provided by growth hormone, thyroid hormone and glucocorticoids.

The liver is exquisitely sensitive to changes in insulin and glucagon levels, which, due to the fact that these hormones drain directly into the liver, are ideally suited to regulate moment-to-moment changes in hepatic glucose output. A reduction in insulin concentration removes the inhibitory effect in insulin on the liver and permits a slow rise in hepatic glucose production and the development of hyperglycemia (Ward, *et al.*, 1984). On the other hand, a decrease in the glucagon level reduces glucose

production by the liver and is associated with a concomitant fall in plasma glucose level (Liljenquist, *et al.*, 1979).

Restoration of the original plasma glucose level will occur if the feedback loop is intact due to the effect of the glucose level to in turn regulate pancreatic insulin and glucagon secretion. In situations where peripheral insulin sensitivity changes, this will also be reflected by a change in plasma glucose level. For example, if peripheral glucose use decreases, a rise in the fasting plasma glucose level will occur, to which the pancreatic islet will appropriately modify its secretion by reducing glucagon output by the alpha-cell and increasing insulin secretion by the beta-cell. These secretory changes will reduce the rate of hepatic glucose output so that the glucose level will tend to be restored to near normal.

In instances where peripheral glucose use rises, the opposite will occur, so that hepatic glucose production will increase and glucose level will once again return toward normal. It is important to realize that complete islet adaptation cannot occur; otherwise no stimulus for the changes in insulin and glucagon secretion would be present. Thus, when tissue insulin sensitivity changes, a new steady-state glucose level results at a value somewhere between that expected for the change in insulin action and that expected for the change in pancreatic hormone secretion, with the exact level depending on the islet alpha and beta-cells' responsiveness and sensitiveness and sensitivity of glucose.

Following food ingestion, plasma glucose excursions are minimized by the islet. This is accomplished by a reduction in hepatic glucose production and an increase in peripheral glucose uptake. These changes in glucose metabolism arise as a result of alterations in insulin and glucagon secretion, which are regulated on a minute-to-minute basis by an interaction between glucose, amino acids and the gut hormones. Glucose is the key regulator of the islet in this system since it not only regulates insulin and

glucagon secretion directly but also modulates responses to the other substrates as well as gut hormones and neural factors released during nutrient ingestion. A basic model of normal regulation of plasma glucose level is shown in Figure 2.4.

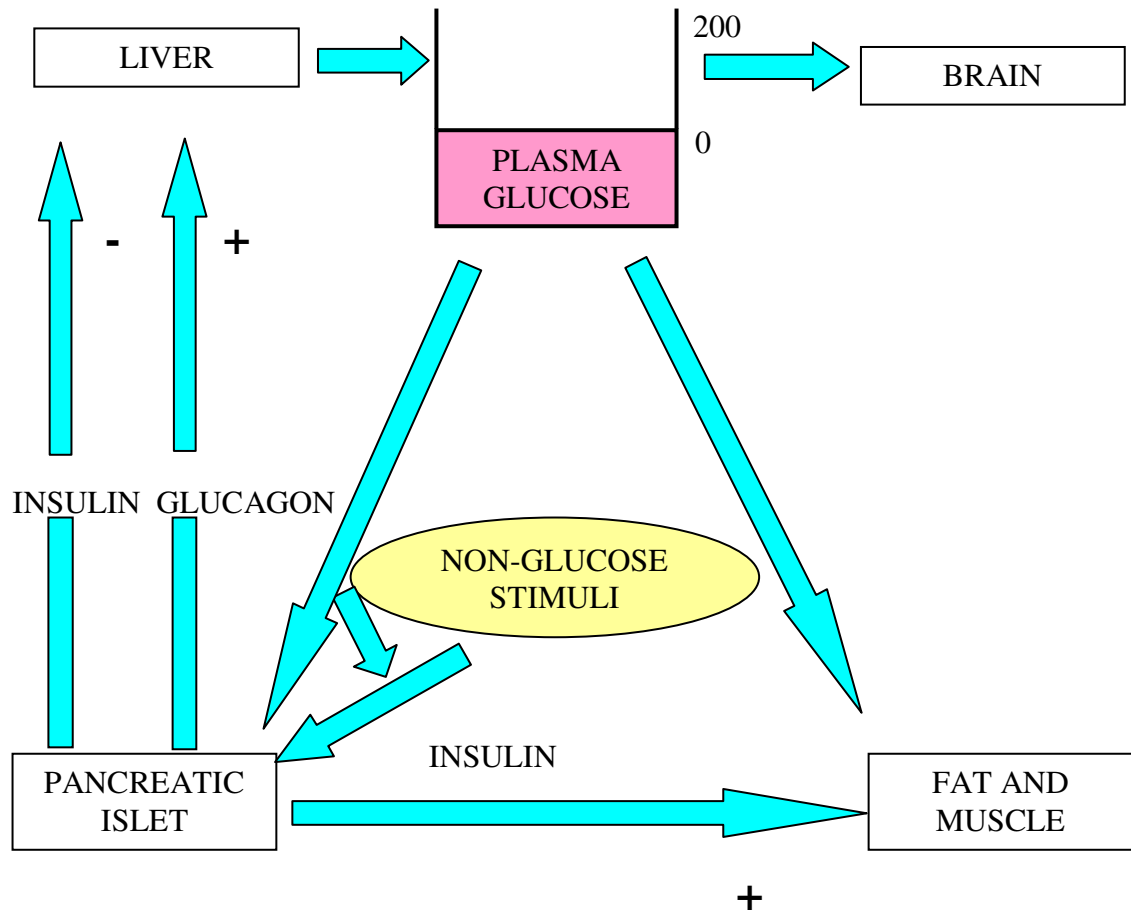


Figure 2.4: A model for the normal steady-state regulation of plasma glucose level. Plasma glucose has direct effects on the pancreas to modulate insulin and glucagon secretion as well as interacting with nonglucose stimuli to modify alpha- and beta-cell responses to these stimuli. During hyperglycemia, insulin secretion is increased and glucagon secretion reduced. When hypoglycaemia prevails, glucagon secretion is enhanced and insulin secretion diminished. Glucagon stimulates hepatic glucose production. Insulin inhibits glucose release by the liver and stimulates glucose use in high-sensitive tissues. Glucose uptake by the brain is insulin-dependent, but in the periphery glucose uptake by fat and muscle is enhanced by insulin. Any changes in hormone or substrate concentration or glucose will be modulated by the loop in order

that glucose use and production remain balanced. The plasma glucose level at which this occurs is determined by the efficiency with which the peripheral tissues take up glucose, the rate of hepatic glucose production, and islet alpha- and beta-cell responsiveness to glucose. (Porte, 1991).

Insulin is produced in the pancreatic beta-cell as the primary biosynthetic product preproinsulin. Insulin is secreted in response to a number of stimuli; glucose is the most important. After a brief circulating time ($t_{1/2}$ 6 to 10 minutes), the hormone interacts with a specific receptor on target tissues to exert its biologic effects.

When applied to the type I diabetic patient, the term “insulin resistance” is more difficult to evaluate. This classification usually denotes a patient who requires a large amount of insulin (> 200 U/day) for control (Shipp, *et al.*, 1965). These patients are unusual and sometimes have high titers of antiinsulin antibodies. However, the great majority of type I diabetic patients do not require more than 100 U/day for control.

In addition to hyperglycemia, changes in pH, counterregulatory hormones, and free fatty acid concentrations can influence insulin action, and as the degree of metabolic control deteriorates, these factors can change in such a way as to impair insulin resistant, (Barrett, *et al.*, 1982) whereas if a type I diabetic patient is maintained in euglycemic control, insulin sensitivity is usually normal (Revers, *et al.*, 1984).

While the exact mechanism of insulin resistance in IDDM patients is unknown, since good metabolic control can reverse the insulin resistance, the defect must be acquired (Revers, *et al.*, 1984). Most likely, the hyperglycemia and related metabolic abnormalities associated with this state are the major factors causing this secondary state of insulin resistance.

The occurrence of any change in glucose production by the liver or glucose use by the peripheral tissues is sensed by the islet and leads to changes in insulin and

glucagon secretion to achieve a new steady state, which minimizes the overall change in glucose level. This steady state returns the glucose concentration toward normal, but complete compensation cannot occur because this would result in the loss of the stimulus responsible for this adaptive change.

The development of a beta-cell lesion in type II diabetes mellitus would reduce plasma insulin levels. Because glucagon secretion is either wholly or partially regulated by the neighbouring beta-cell, an abnormal rise in alpha-cell release of glucagon would also occur (Porte, 1991) as shown in Figure 2.5.

This reduction in insulin and increase in glucagon draining into the liver would be expected to produce an increase in hepatic glucose production. Further, the reduced peripheral insulin level would impair glucose use by both fat and muscle, while glucose use in the noninsulin-dependent tissues proceeds normally.

Due to the reduction in insulin secretion, insulin-mediated glucose uptake cannot increase sufficiently to compensate for the increased rate of hepatic glucose release, and the fasting glucose level tends to rise. This situation is only transient, because the elevation in the fasting plasma glucose level would to increased beta-cell stimulation, thereby producing a more 'normal' plasma insulin level as depicted in Figure 2.6.

The increase in glucose and insulin concentrations results in a reduction in glucagon secretion, but at the new steady state the glucagon level is not appropriately reduced for the degree of glycemia. Concurrent with these changes in islet hormone secretion, glucose production and use are moderated. However, at the new steady state, the rate of hepatic glucose release will remain elevated and total glucose uptake will be increased due to the hyperglycemia.

When hepatic and peripheral insulin resistance develops, the impairment of glucose uptake leads to a further increase in plasma glucose level. This additional hyperglycemia leads to further stimulation of the beta-cell with resultant normal or over

supranormal insulin levels as well as further increase in hepatic glucose delivery and peripheral glucose uptake (Figure 2.7). Although a further reduction in glucagon level occurs, the resultant level is still inappropriately elevated for the degree of hyperglycemia.

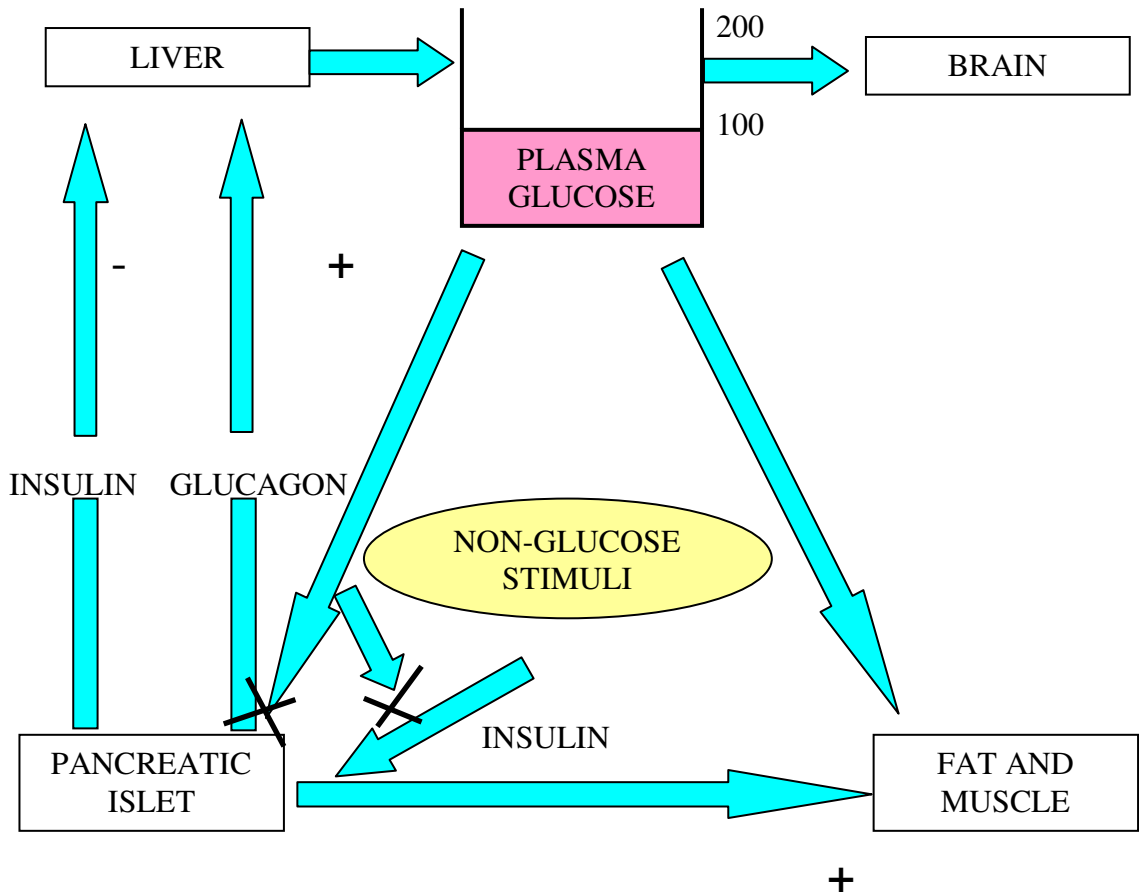


Figure 2.5: Model for the development of hyperglycemia in type II diabetes mellitus. Hypothetical initiation islet lesion of type II diabetes. The impairment of the islet function would be expected to reduce insulin and increase glycogen output, which would result in overproduction of glucose by the liver and underuse of glucose in periphery, with a resultant increase in glucose level (Porte, 1991).

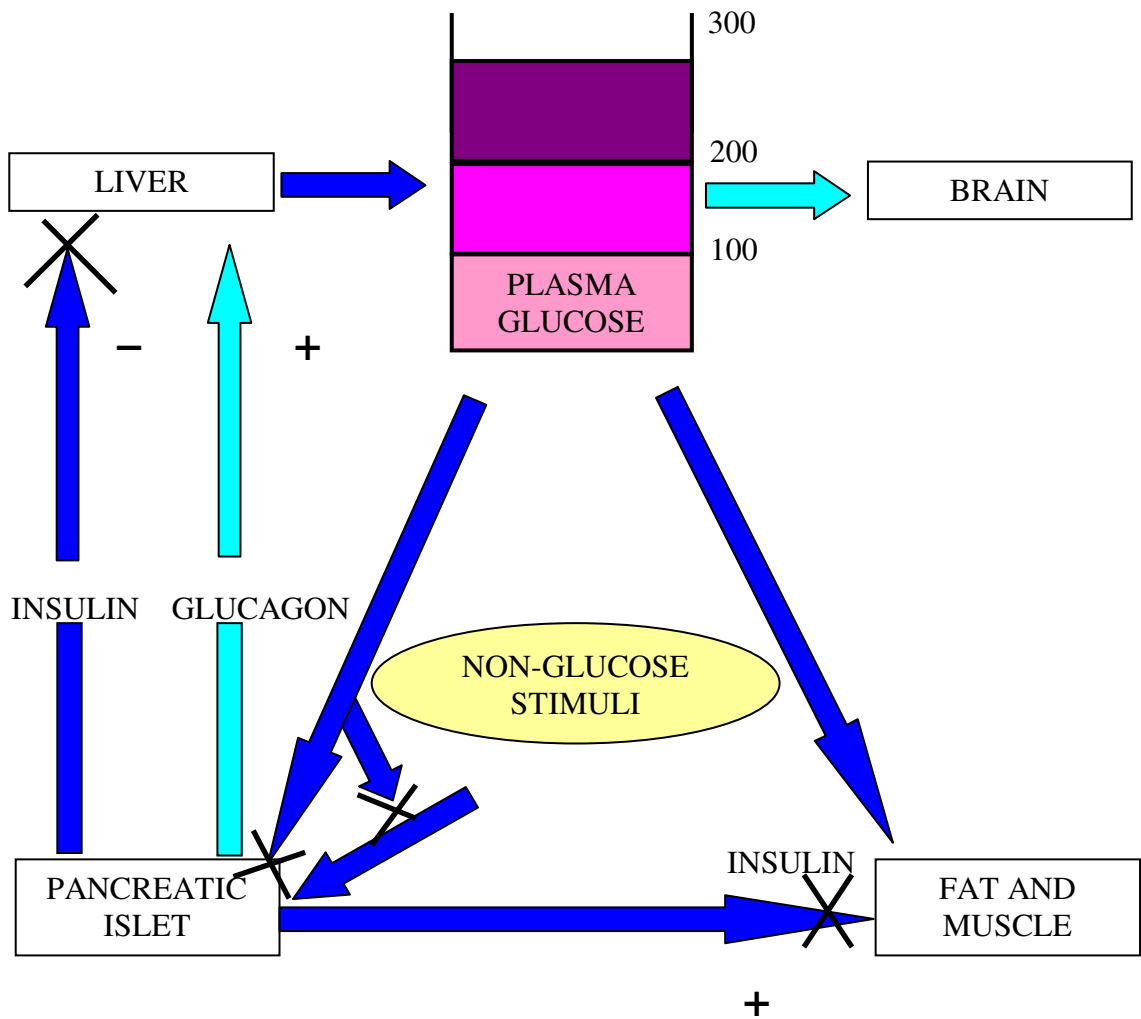


Figure 2.7: Model for the development of hyperglycemia in type II diabetes mellitus. Interaction of islet dysfunction and insulin resistance on basal glucose regulation in type II diabetes mellitus. The impairment of the insulin action in the liver and peripheral tissues requires a marked additional increase in glucose concentration in order that, in the presence of an impaired islet, a new steady state is achieved. Under these conditions, the islet may secrete “normal” or even “supranormal” quantities of insulin while secreting “normal” or “subnormal” amounts of glucagon, despite the presence of islet alpha- and beta-cell dysfunction. The net result is a further increase in glucose production by the liver and glucose use by the peripheral tissues, until the renal threshold exceeded when decompensation occurs (Porte, 1991).

2.11 PLEUROTUS CITRINOPILEATUS AND ITS DIABETIC PROPERTY

Pleurotus citrinopileatus is also known as the golden oyster mushroom and this species is not as prolific as the more commonly cultivated *P. ostreatus* and *P. pulmonarius* in the conversion of substrate mass to mushrooms.

The mycelial growth is cottony, whitish mycelium, often with tufts of dense growth, sometimes with yellowish tones, and occasionally run through the underlying rhizomorphic strands. This mushroom produces pale pinkish buff coloured spores (Ohira, 1990). Its brilliant yellow colour astonishes all who first see it.

This species forms clusters hosting a high number of individual mushrooms, whose stems often diverge from a single base. Its penchant for forming clusters making harvesting easy and prevents damage to individual mushrooms. Its extreme fragility post harvest limits, especially along the thin cap margin, complicating long distance shipping. Cropping yields are not nearly as good compared to other *Pleurotus* spp. However, its rarity and broad range of flavours, especially spicy and bitter at first, this mushroom imparts a strong nutty flavour upon thorough cooking and this makes this species uniquely marketable and pleasurable to grow.

P. citrinopileatus grows quickly through pasteurized straw and sterilized sawdust, and thrives at high temperatures. Coupled with the brevity of time between spawning and fruiting, and its fondness for cottonseed hulls, this mushroom is better suited for cultivation in warmer climates of Asia, the southern United States, or Mexico, or during the summer months in temperate regions.

The blood glucose level of a diabetes patient is usually controlled or regulated by diet, physical exercise (Heled, et al., 2003), and medications. Medications, however, can sometimes result in side effects (Richter and Neises, 2003). Although diabetes has no direct relation to mortality, it jeopardizes one's health (Kagansky, et al., 2003).

Treating STZ-induced diabetic rats with water-soluble polysaccharides, extracted from the fruiting bodies of edible mushrooms in a fermented medium has been shown to possibly enhance their immunity, partly reduce cell damage, enhance insulin secretion, and inhibit increased blood glucose levels (Kurihara, *et al.*, 2003).

Water-soluble polysaccharides, extracted from submerged fermented medium of *P. citrinopileatus* showed antihyperglycemic effect in STZ-induced diabetic rats (Hu, *et al.*, 2006). Studies also showed that the hypoglycemic effects of aqueous extract of *P. pulmonarius* in alloxan-induced diabetic mice and increased oral glucose tolerance in diabetic (OGTT model) mice at dose levels 250mg/kg, p.o (Badole, *et al.*, 2006a).

CHAPTER THREE

3.0 INTRODUCTION

Potentially harmful ROS are produced as a consequence of normal aerobic metabolism. The reactive species is usually inactivated in vivo by a variety of antioxidants. Antioxidants are deployed to prevent generation of ROS or to scavenge those formed. Thus, oxidatively induced tissue damage is minimized. However, deficiency of antioxidant defences may lead to oxidative stress, which might be associated with a variety of disorders including coronary heart disease, neural disorders, diabetes, arthritis, and cancer (Yoshikawa *et. al.*, 2000; Spiteller, 2001).

A number of methods have been developed to measure the efficiency of antioxidants. These methods focus on different mechanisms of the antioxidant defense system such as scavenging or inhibiting free radicals or chelation of metal ions that otherwise may lead to free-radical formation. These free radicals are capable of inducing damage to all cellular molecules, which can lead to diseased states (Yoshikawa *et. al.*, 2000; Spiteller, 2001). Synthetic antioxidants also have been developed to retard lipid peroxidation, but their use as food supplements and therapeutic agents have been hindered due to their possible toxicity.

A number of natural antioxidants have already been demonstrated from different kinds of plant materials, such as oil seeds, cereal crops, vegetables, fruits, leaves, roots, spices, and herbs (Packer & Ong, 1997; Cullen *et. al.*, 1997; Negi & Jayaprakasha, 2003).

Mushrooms are traditionally used in Chinese medicine and are commonly used for pharmaceutical purposes and health foods. Increasing experimental evidence indicates that mushrooms contain a large number of biologically active components that offer health benefits and protection against degenerative diseases. A number of

medicinal mushrooms have recently been reported to possess significant antioxidant activity (Mau *et. al.*, 2001)

Pleurotus species are commonly called Oyster mushrooms. There are about 40 species of this mushroom. *Pleurotus* spp. are widespread throughout the hardwood forests of the world; they are edible, nutritious, and rank second among the cultivated mushrooms of the world. They have shown a number of therapeutic activities, such as antitumor, immunomodulatory, antigenotoxic, anti-inflammatory, antimicrobial, antiviral, hypocholesterolemic, antihypertensive and antiplatelet-aggregating activities (Gregori, 2007). Research on the antioxidant values of *Pleurotus* species by submerged fermentation have not been studied In this study, crude extracts were prepared from mycelia of nine *Pleurotus* species and tested for the antioxidant activity.

The aims of this study were to:

- a) determine the antioxidant activity of methanol extracts of mycelia of nine *Pleurotus* species,
- b) assess total phenolic content in methanol extracts of nine *Pleurotus* species by the Folin-Ciocalteu's method, and
- c) investigate the correlation between antioxidant activity with total phenolic content.

3.1 MATERIALS AND METHODS

3.1.1 Preparation of mycelia biomass

Cultures of nine *Pleurotus* species: *P. citrinopileatus* (KUM 50093), *P. cystidiosus* (KUM 50094) *P. eryngii* (KUM 50087), *P. flabellatus* (KUM 50091) *P. florida* (KUM 50213), *P. florida (hungarian)* (KUM 50092), *P. ostreatus* (KUM 50089), *P. sajor-caju* (KUM 50084) and *P. sapidus* (KUM 50090) were obtained from Mycology Lab, Institute of Biological Sciences, Faculty of Science, University of Malaya, Malaysia. Mycelia was grown on glucose, yeast, malt and peptone (GYMP) agar in plastic Petri dishes for 7 days at $25 \pm 2^\circ\text{C}$ (Appendix A, pp.113). Ten 5 mm diameter discs from the edge of the growing colony were transferred to 500-ml Erlenmeyer flasks containing 100 ml of sterilized GYMP liquid medium. The inoculated flasks were incubated under static condition for 14 days at $25 \pm 2^\circ\text{C}$.

3.1.2 Preparation of methanol extracts

Antioxidant compounds were extracted from the mycelia that were filtered through Whatman No. 1 filter paper, mashed and soaked in methanol for 48 hours at $28 \pm 2^\circ\text{C}$. The methanol extract was then filtered through Whatman No. 1 filter paper. Extracts were then evaporated to dryness under vacuum using rotary evaporator to form a crude extract. Crude extract was kept at 4°C until the analysis of total phenolic content and antioxidant activity.

3.1.3 Determination of total phenolic content

The concentration of total phenolic compounds of crude extracts were determined according to the method developed by Singleton *et al.*, (1999) using the Folin-Ciocalteu reagent (Appendix B, pp. 115) and gallic acid was used as phenolic standard. An aliquot of 0.25 ml of extract at different concentrations from 2 to 10 µg/ml and negative control (methanol) were mixed with 0.25 ml of Folin-Ciocalteu's phenol reagent (10%). After 3 minutes of incubation, 0.50 ml of 10% sodium carbonate (Na₂CO₃) solution was added to the mixture. The reaction mixture was incubated at 37°C for 1 hour. The absorbance was determined at 750 nm with a spectrophotometer. Methanol was used as blank and Quercetin was used as the positive control. All samples were assayed in triplicate.

3.1.4 Antioxidant activity assays

(i) Scavenging activity on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical

The scavenging activity of crude extracts on DPPH radical was measured according to the method Cheung *et al.*, (2003) (Appendix B, pp. 117). A 3.90 ml of methanolic extract solution at each concentration ranging from 0.25 to 0.50 mg/ml and negative control (methanol) was mixed with 0.10 ml of DPPH solution (0.8 %). The mixtures were mixed and allowed to react for 30 minutes at room temperature. The absorbance was determined at 520 nm with a spectrophotometer. Methanol was used as blank and Quercetin as positive control. All samples were assayed in triplicates. The scavenging activity (%) on DPPH was calculated by the following equation:

$$\text{Scavenging activity (\%)} = \left(\frac{\text{Abs of negative control} - \text{Abs of sample}}{\text{Abs of negative control}} \right) \times 100\%$$

EC₅₀ value (mg/ml) is the effective concentration at which 50% of DPPH radical were scavenged. Low EC₅₀ value indicates strong ability of the extract to act as DPPH scavenger. EC₅₀ values were obtained by interpolation from linear regression analysis.

(ii) Ferric reducing antioxidant power (FRAP) assay

The FRAP assay of mycelial extracts were measured according to the method by Benzie and Strain (1996) (Appendix B, pp. 124) and expressed as FeSO₄.7H₂O equivalents. The working FRAP reagent was prepared by mixing 10 volumes of 300 mmol/l acetate buffer, pH 3.6, with 1 volume of 10 mmol/l TPTZ (2,4,6-tripyryldyl-s-triazine) in 40 mmol/l hydrochloride acid and with 1 volume of 20 mmol/l ferric chloride. 0.025 ml of mycelial extract of each concentration of 2.5 to 10 mg/ml was added to wells of 96-well microtiter plate in quadruplicates. 0.175 ml of freshly prepared FRAP reagent incubated at 37°C was added to three of the replicates, while the same volume of acetate buffer was added to the fourth replicate (sample blank). The plate was placed in an automated microplate reader set at 593 nm and temperature was maintained at 37°C for 4 minutes. Absorbance values (A_{sample}) were measured after 4 minutes. Reagent blank values (using 0.175 ml of FRAP reagent, A_{reagent blank}) and sample blank values (using sample and acetate buffer, A_{sample blank}) were taken at the same time after 4 minutes. The changes in absorbance were calculated:

$$[A_{\text{sample}} - (A_{\text{reagent blank}} + A_{\text{sample blank}})]$$

3.1.5 Statistical analysis

All data are presented as the mean \pm SD. The means of the data were subjected to a one way analysis of variance (ANOVA) using the SPSS programme and the significance of the difference between means was determined by the Duncan's multiple range test at 95% least significant difference ($p < 0.05$) (Appendix D, Table 1-8, pp. 134-138). Correlation between variables was determined by linear regression analysis and the differences were considered significant when $p < 0.05$.

3.2 RESULTS

3.2.1 Yield of methanolic extracts from *Pleurotus* species

The yield of crude extracts from mycelia of *Pleurotus* species were shown in Table 3.1. Among the nine *Pleurotus* species extracted *P. cystidiosus* gave the highest yield of extract with 0.362 ± 0.013 g/g of wet weight of mycelium followed by *P. flabellatus* with 0.334 ± 0.023 g/g of wet weight of mycelium and *P. florida (hungarian)* with 0.258 ± 0.014 g/g of wet weight of mycelium. *P. eryngii* produced the least of extract with 0.067 ± 0.010 g/g of wet weight of mycelium.

Table 3.1 : Yield of extracts of *Pleurotus* species extracted from wet mycelium

<i>Pleurotus</i> species	Code	Yield of extracts (g) / g of wet mycelium
<i>P. cystidiosus</i>	KUM 50094	0.362 ± 0.013
<i>P. flabellatus</i>	KUM 50091	0.334 ± 0.023
<i>P. florida (hungarian)</i>	KUM 50092	0.258 ± 0.014
<i>P. sapidus</i>	KUM 50090	0.203 ± 0.011
<i>P. citrinopileatus</i>	KUM 50093	0.173 ± 0.013
<i>P. ostreatus</i>	KUM 50089	0.166 ± 0.012

<i>P. sajor caju</i>	KUM 50084	0.147 ± 0.026
<i>P. florida</i>	KUM 50213	0.123±0.011
<i>P. eryngii</i>	KUM 50087	0.067 ± 0.010

Values expressed are mean ± S.D. of 3 measurements.

3.2.2 Total phenolic content of extracts of *Pleurotus* species

The total phenolic content of the extracts, expressed as mg of GAEs/g of extract are shown in Table 3.2.

Table 3.2: Total phenolic content in extracts of *Pleurotus* species

<i>Pleurotus</i> species	Code	Total phenolic content (mg of GAEs/g of extract / Quercetin*)
<i>P. eryngii</i>	KUM 50087	194.10 ± 1.80 ^a
<i>P. florida</i>	KUM 50213	194.70 ± 1.04 ^a
<i>P. florida (hungarian)</i>	KUM 50092	195.10 ± 0.92 ^a
<i>P. ostreatus</i>	KUM 50089	195.90 ± 1.80 ^a
<i>P. citrinopileatus</i>	KUM 50093	197.10 ± 1.04 ^a
<i>P. cystidiosus</i>	KUM 50094	209.50 ± 0.92 ^b
<i>P. sajor caju</i>	KUM 50084	210.27 ± 1.75 ^b
<i>P. sapidus</i>	KUM 50090	218.00 ± 1.04 ^c
<i>P. flabellatus</i>	KUM 50091	233.53 ± 3.60 ^d
Quercetin		1801.40 ± 6.81 [*]

GAEs = gallic acid equivalents. Values expressed are means ± S.D. of 3 measurements. Means with same letter in the same column denotes not significant ($p > 0.05$).

The total phenolic content of *P. flabellatus* extract was significantly higher than all the extracts of *Pleurotus* species with 233.53 ± 3.60 mg of GAEs / g of extract ($p < 0.05$). This was followed by *P. sapidus* with 218.00 ± 1.04 mg of GAEs / g of extract and *P. sajor caju* with 210.27 ± 1.75 mg of GAEs / g of extract. *P. eryngii* showed the lowest total phenolic content with 194.10 ± 1.80 mg of GAEs / g of extract. There were no significant difference between *P. eryngii*, *P. florida*, *P. florida (hungarian)*, *P.*

ostreatus and *P. citrinopileatus* ($p < 0.05$) (Appendix D, Table 1-4, pp. 134-136). Total phenolic content in Quercetin was 1801.40 ± 6.81 mg of GAEs / g of Quercetin.

3.2.3 Scavenging activity of *Pleurotus* spp. extracts on DPPH radicals

Figure 3.1 showed the scavenging effects of all nine *Pleurotus* species. At a general concentration of 0.45 mg/ml almost all the extracts reached a steady state and the reaction reached a plateau. The scavenging effects of *P. cystidiosus*, *P. florida* (*hungarian*), *P. florida* and *P. citrinopileatus* however increased with the increasing concentration, i.e. concentration dependent even at a concentration of 0.5 mg/ml. The scavenging effect of Quercetin which was used as positive control was also shown in Figure 3.1.

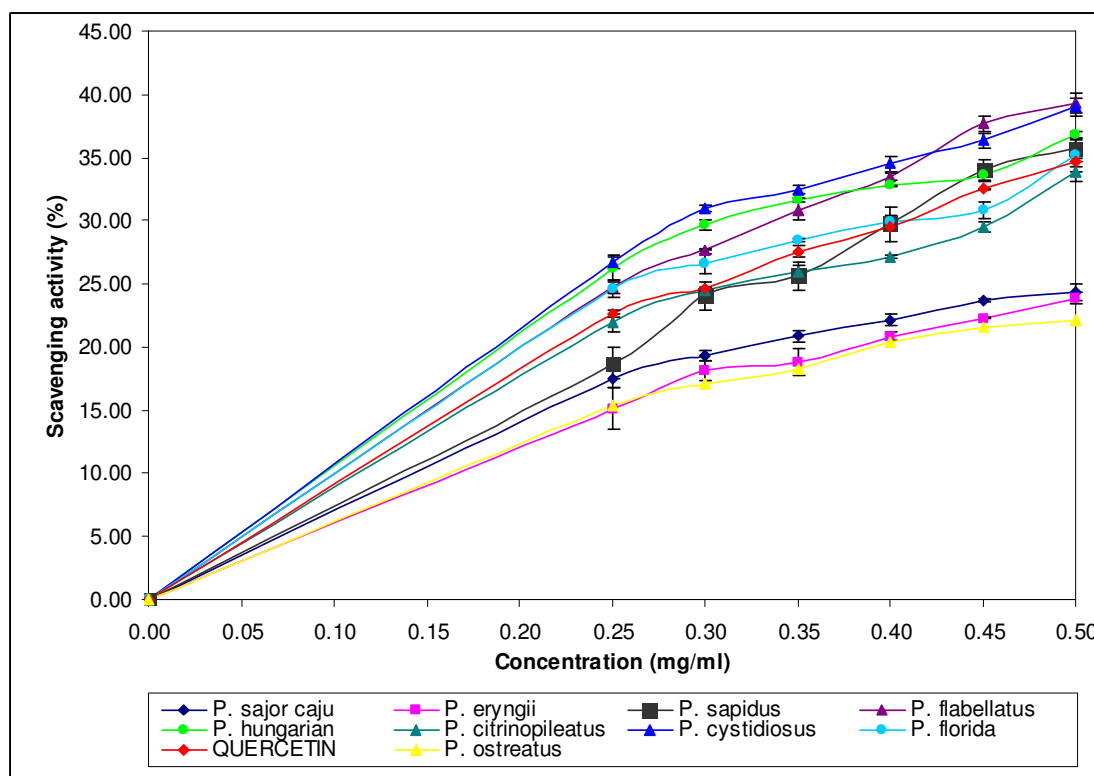


Figure 3.1: Scavenging effect of extracts of *Pleurotus* species on DPPH radical. Values expressed are means of triplicate measurements.

The EC₅₀ value (50% effective concentration) which is defined as the amount of antioxidant necessary to decrease the initial DPPH radical concentration by 50%, was determined from the plotted graph of scavenging activity against the concentration of extracts. The EC₅₀ values of the extracts in DPPH radical scavenging effects could not be determined as the scavenging activity of each extract was below 50%. However, the EC₅₀ values of the extracts in DPPH radical scavenging effects can be obtained by extrapolation of each graph (Appendix B, pp. 119-123).

Table 3.3: The EC₅₀ values of *Pleurotus* species by extrapolation graphs

<i>Pleurotus</i> species	Code	Calculated EC ₅₀ values (mg/ml)
<i>P. cystidiosus</i>	KUM 50094	0.599
<i>P. flabellatus</i>	KUM 50091	0.603
<i>P. florida (hungarian)</i>	KUM 50092	0.637
<i>P. sapidus</i>	KUM 50090	0.679
<i>P. florida</i>	KUM 50213	0.690
<i>P. citrinopileatus</i>	KUM 50093	0.735
<i>P. sajor caju</i>	KUM 50084	0.968
<i>P. eryngii</i>	KUM 50087	1.014
<i>P. ostreatus</i>	KUM 50089	1.068
Quercetin		0.691

Table 3.3 marked the EC₅₀ values obtained from extrapolation graphs of *Pleurotus* species. *P. cystidiosus* had the strongest scavenging activity of DPPH free radicals when compared to other *Pleurotus* species. This is followed by *P. flabellatus*, *P. florida (hungarian)*, *P. sapidus* and *P. florida* which had lower EC₅₀ values when compared to Quercetin which was used as standard. The extract of *P. ostreatus* was relatively a poor free radical scavenger with an EC₅₀ of 1.068 mg/ml.

3.2.4 Ferric reducing antioxidant power (FRAP) of extracts of *Pleurotus* species

The FRAP values of the extracts expressed as μmol of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ equivalents / g of extract were shown in Table 3.4. There was a strong significant difference ($p < 0.05$) (Appendix D, Table 5-8, pp. 136-138) in FRAP values among the extracts. *P. sajor caju* exhibit the highest antioxidant power with $28.20 \pm 0.19 \mu\text{mol}$ of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ equivalents / g of extract, followed by *P. sapidus*, *P. ostreatus*, *P. eryngii* (KUM 50087) and *P. cystidiosus*. The ferric reducing antioxidant power decreases by *P. florida* > *P. flabellatus* > *P. florida (hungarian)* and *P. citrinopileatus* with the lowest antioxidant power with $11.85 \pm 0.23 \mu\text{mol}$ of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ equivalents / g of extract. FRAP value of Quercetin was $53.76 \pm 0.15 \mu\text{mol}$ of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ equivalents / g of Quercetin.

Table 3.4: Ferric reducing antioxidant power (FRAP) values of extracts of *Pleurotus* species

<i>Pleurotus</i> species	Code	FRAP values (μmol of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ equivalents / g of extract / Quercetin*)
<i>P. citrinopileatus</i>	KUM 50093	11.85 ± 0.23^a
<i>P. florida (hungarian)</i>	KUM 50092	12.08 ± 0.16^a
<i>P. flabellatus</i>	KUM 50091	12.09 ± 0.36^a
<i>P. florida</i>	KUM 50213	13.39 ± 0.30^b
<i>P. cystidiosus</i>	KUM 50094	20.81 ± 0.08^c
<i>P. eryngii</i>	KUM 50087	22.39 ± 0.30^d
<i>P. ostreatus</i>	KUM 50089	26.32 ± 0.37^e
<i>P. sapidus</i>	KUM 50090	27.32 ± 0.22^f
<i>P. sajor caju</i>	KUM 50084	28.20 ± 0.19^g
Quercetin		$53.76 \pm 0.15^*$

Values expressed are means \pm S.D. of 3 measurements. Means with same letter in the same column denotes not significant ($p > 0.05$).

3.2.5 Correlation between FRAP, DPPH and TPC

When FRAP values of *Pleurotus* species were plotted against their total phenolic content, there were no correlation found between these antioxidant methods. No correlation between DPPH values of *Pleurotus* species against their total phenolic content was observed.

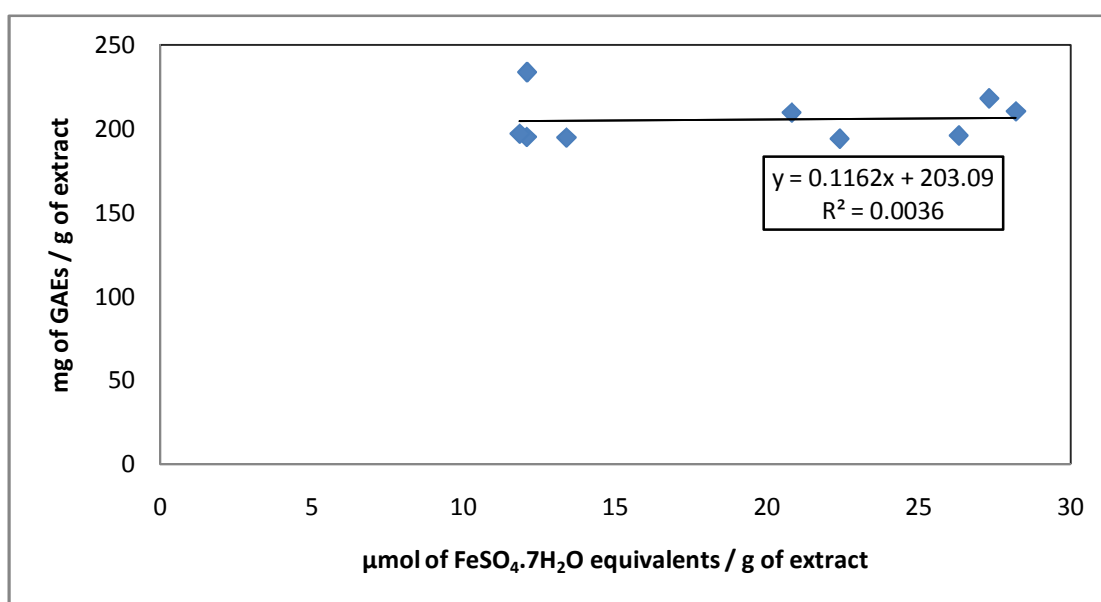


Figure 3.2: Correlation between antioxidant activities determined by total phenolic content and FRAP assay in *Pleurotus* sp. extracts.

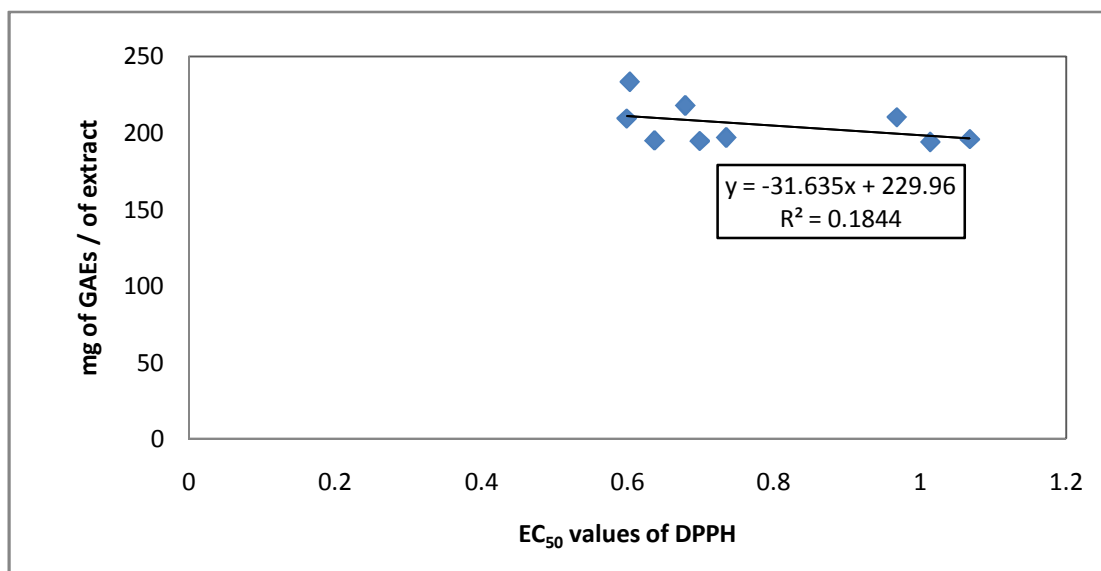


Figure 3.3: Correlation between antioxidant activities determined by total phenolic content and DPPH assay in *Pleurotus* sp. extracts.

3.3 DISCUSSION

Epidemiological studies have strongly suggested that diets play a crucial role in the prevention of chronic diseases such as heart disease, cancer, diabetes and Alzheimers's disease (Willet, *et al.*, 1994; Temple, 2000). Consumption of fruits and vegetables has been associated with reduced risk of chronic diseases. This has been hypothesized to be because they contain phytochemicals that combat oxidative stress in the body by helping to maintain a balance between oxidants and antioxidants. An imbalance caused by overproduction of oxidants leads to oxidative stress, resulting in damage to large biomolecules such as lipids, DNA and proteins. Oxidative damage increases the risk of degenerative diseases such as cancer and cardiovascular diseases (Temple, 2000). Antioxidants reduce oxidative damage to biomolecules by modulating the effects of reactive oxidants (Fraga, *et al.*, 1991; Duthie, *et al.*, 1996).

Mushrooms contain a wide array of phytochemicals that exert health benefits in humans through various mechanisms including antioxidant properties and mediation of hormones. The antioxidant activity of mushroom extracts with stronger inhibition of lipid peroxidation occurring at high concentrations of the extracts in most cases (Cheung and Peter, 2005). The possible mechanisms of antioxidant activity of mushroom extracts includes scavenging of free radicals possibly through hydrogen-holding capacity and oxidation by peroxy radicals (Ali *et. al.*, 1996b).

The total phenolic contents were analysed using the method by Singleton *et. al.*, (1999), without distinguishing specific structures. The total phenolic content represents contributions from free and soluble conjugated phenolics. Conjugated phenolics may still be oxidized and contribute toward total phenolic content and antioxidant activity. Extracts of *P. flabellatus* exhibited the highest total phenolic content and *P. eryngii* on the contrary.

The total phenolic content in dried methanol extracts of wild edible mushroom species from the northeast of Portugal, namely *L. deliciosus*, *T. portentosum* (Ferreira *et. al.*, 2007), *L. gigantus*, *S. imbricatus* and *A. arvensis* (Barros *et. al.*, 2007) were studied. Total phenolic content in extracts of *L. deliciosus* and *T. portentosum* were 17.28 and 10.80 mg of GAEs/g of extract respectively, which were much lower than *P. eryngii* which had the lowest total phenolic content with 194.10 ± 1.80 mg of GAEs/g of extract.

Black and green teas, red wine and cocoa are consumed widely and are known to be rich in phenolic phytochemicals. In particular, theaflavin (TF), epigallocatechin gallate (EGCG), resveratrol and procyanidin in black tea, green tea, red wine and cocoa, respectively, have been considered as major chemo-preventive agents mainly due to their strong antioxidative activities. Cocoa and red wine contained much higher levels of total phenolic contents (611 and 340 mg of GAEs/ g of extract, respectively) when

compared to the extract of *P. flabellatus* (233.53 ± 3.60 mg of GAEs/ g of extract). However, when compared to the extract of *P. flabellatus*, black tea and green tea possessed a lower total phenolic content (124 and 165 mg of GAEs/g of extract) respectively.

Scavenging of stable radicals such as the chromogen radical of DPPH quantified by spectrophotometer in organic media is extensively used for comparison of homologous series of antioxidants. The decrease in color has been correlated to a dose-response curve with a standard antioxidant assay (Arnao, 2000; Brand-Williams, *et al.*, 1995). The assay was further developed as the rate of scavenging and, accordingly, the antioxidant activity was characterized for a series of flavonoids (Madsen, *et al.*, 2000; Butkovic, 2004). DPPH has the advantage of being unaffected by certain side reactions, such as metal ion chelation and enzyme inhibition (Amarowicz *et al.*, 2004).

In the present study, *P. cystidiosus* showed lowest EC₅₀ value and *P. ostreatus* with the highest EC₅₀ value. The EC₅₀ value of *Phellinus linteus* was calculated to be 22.07 µg/ml, where as that of Vitamin C, used as a positive control was 5.11 µg/ml (Song *et al.*, 2003). These EC₅₀ values were very much lower than EC₅₀ value of *P. cystidiosus* which was 0.599 mg/ml. These were in contrast with the EC₅₀ values of methanolic extracts from *Grifola frondosa* with 4.95 mg/ml, *Morchella esculenta* with 3.71 mg/ml and *Termitomyces albuminosus* with 5.04 mg/ml (Mau, *et al.*, 2003) when compared with the highest EC₅₀ value of *Pleurotus* sp, which is *P. ostreatus* with 1.068 mg/ml. Quercetin which was used as a positive control in this study was 0.691 mg/ml, slightly higher than *P. flabellatus*, *P. florida (hungarian)* and *P. sapidus* themselves. Besides mushrooms, vegetables, fruits and plants also exhibit antiradical activities. For instance, sorghum (Kamath, *et al.*, 2004) and *Coleus aromaticus* (Kumaran and Karunakaran, 2006) from India had a very low EC₅₀ value. The EC₅₀ value for 8.2 to 41.1 µg/ml for fresh sorghum grains and 210.0 µg/ml for fresh *Coleus aromaticus*.

Sun and Ho (2005) reported a significant correlation between total phenolics and scavenging ability of buckwheat extracts on DPPH radicals. A study by Yu, *et. al.*, (2002) found no correlation between scavenging activity and the total phenolic content in wheat extracts. Results also indicate that low scavenging ability on DPPH could be due to the presence of phenolic compounds in the mycelial extracts. However, results showed no correlation between total phenolic contents and scavenging ability of the mycelial extracts of *Pleurotus* sp. on DPPH radicals with $R^2 = 0.1844$ as shown in Figure 3.3. However, there was a strong negative correlation between the scavenging ability on DPPH radical and total phenolic content in the extract of fruitbody and mycelium of *H. erinaceus*, ($R^2=0.563$), which indicates that high scavenging ability on DPPH radical is not due to phenolic compounds in *H. erinaceus* extracts (Wong, *et. al.*, 2009).

When compared to commercial and speciality mushrooms, *Auricularia* sp. excluding *T. fuciformis* were good DPPH scavengers. Methanol extracts of *A. mesenterica* and *A. polytricha* showed an outstanding scavenging effect of 100% at 1.0 mg/ml, whereas *A. fuscossuccinea* (brown strain) showed a scavenging effect of 95.4% at 3 mg/ml and *T. fuciformis* was 71.5% at 5 mg/ml (Mau, *et. al.*, 2001). *A. fuscossuccinea* (white strain) scavenged DPPH radical by 94.5% at 0.4 mg/ml (Yang, *et. al.*, 2002). Excellent scavenging effects (96.3 to 99.1% and 97.1%) were also observed with methanol extracts of *Antrodia camphorate* and *A. bransiliensis* at 2.5 mg/ml, respectively. Scavenging effects of methanol extracts of other medicinal mushrooms at 0.64 mg/ml were 24.6, 67.6, 74.4 and 73.5% for *C. versicolor*, *G. lucidum*, antler *G. lucidum* and *G. tsugae*, respectively (Yang, *et. al.*, 2002).

The ferric reducing antioxidant power (FRAP) assay is a simple and inexpensive procedure that measures the total antioxidant levels in a sample. The reducing ability of biological sample was determined by FRAP assay of Benzie and Strain (1996). FRAP

assay measures the change in absorbance at 593 nm due to the formation of a blue coloured Fe^{II}-tripyridyltriazine compound from colourless oxidised Fe^{III} form by the action of electron donating antioxidants.

FRAP has been used to analyze antioxidant status in humans after hyperbaric oxygen treatment (Dennog, *et. al.*, 1999), evaluate patients with chronic renal failure (Erdogan *et. al.*, 2006), compare the effects of different diets on plasma (Lee, *et. al.*, 2000), examine the influence of dental amalgams on saliva (Pizzichini, *et. al.*, 2002) and study the efficiency of ascorbate (vitamin C) in plasma (Benzie and Szeto, 1999). It has also been used to compare antioxidant activity in plant and mammalian lignans (Niemeyer and Metzler, 2003) and plant extracts (Tsai, *et. al.*, 2002), compare the effects of different diets in rats (Aprikian, *et. al.*, 2001) and evaluate meat quality (Sommers, *et. al.*, 2003).

Evaluation of the total antioxidant capacity of mycelial extracts of *Pleurotus* species by FRAP assay was selected because it had not been determined previously by others using this method. When comparing the FRAP values of mycelial extracts of *Pleurotus* species with fresh vegetables and fruits (Pellegrini, *et. al.*, 2003), FRAP values of *P. eryngii* (22.39 μmol of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ equivalents/g of extract) and *P. cystidiosus* (20.81 μmol $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ equivalents/g of extract) were comparable to chilli pepper (23.54 μmol $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ equivalents/g of fresh weight), red bell pepper (20.98 μmol $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ equivalents/g of fresh weight), orange (20.50 μmol $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ equivalents/g of fresh weight) and cultivated strawberry (22.74 μmol $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ equivalents/g of fresh weight).

FRAP values of mycelial extracts of *P. citrinopileatus* (11.85 μmol $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ equivalents/g of extract), *P. florida* (*hungarian*) (12.08 μmol $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ equivalents/g of extract), *P. flabellatus* (12.09 μmol $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ equivalents/g of extract) and *P. florida* (13.39 μmol $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ equivalents/g of extract) were comparable to broccoli

(11.67 $\mu\text{mol FeSO}_4 \cdot 7\text{H}_2\text{O}$ equivalents/g of fresh weight), black grape (11.09 $\mu\text{mol FeSO}_4 \cdot 7\text{H}_2\text{O}$ equivalents/g of fresh weight), pineapple (15.73 $\mu\text{mol FeSO}_4 \cdot 7\text{H}_2\text{O}$ equivalents/g of fresh weight) and red plum (12.79 $\mu\text{mol FeSO}_4 \cdot 7\text{H}_2\text{O}$ equivalents/g of fresh weight) (Pellegrini, *et. al.*, 2003).

FRAP values of mycelial extracts of *P. ostreatus* (26.32 $\mu\text{mol FeSO}_4 \cdot 7\text{H}_2\text{O}$ equivalents/g of extract), *P. sapidus* (27.32 $\mu\text{mol FeSO}_4 \cdot 7\text{H}_2\text{O}$ equivalents/g of extract) and *P. sajor caju* (28.20 $\mu\text{mol FeSO}_4 \cdot 7\text{H}_2\text{O}$ equivalents/g of extract) were comparable to kale or curly kale (26.50 $\mu\text{mol FeSO}_4 \cdot 7\text{H}_2\text{O}$ equivalents/g of fresh weight), raspberry (33.50 $\mu\text{mol FeSO}_4 \cdot 7\text{H}_2\text{O}$ equivalents/g of fresh weight), cloudberry (25.50 $\mu\text{mol FeSO}_4 \cdot 7\text{H}_2\text{O}$ equivalents/g of fresh weight) and dried apricot (32.70 $\mu\text{mol FeSO}_4 \cdot 7\text{H}_2\text{O}$ equivalents/g of fresh weight) (Halvorsen, *et. al.*, 2002).

The antioxidant activity of a compound against a free radical does not necessarily match its reducing ability. Hence, there was no correlation between values obtained with FRAP and total phenolic content in mycelial extracts of *Pleurotus* species ($R^2=0.0036$). This indicates that there are other components besides phenolic compounds in the antioxidant activity of *Pleurotus* species. This result was against with Benzie and Stezo (1999), who found a strong correlation between total phenolic content and FRAP values of teas. Othman, *et. al.*, (2007) have found that cocoa bean expressed a positive correlation between FRAP assay and phenolic content for both ethanolic ($R^2=0.764$) and aqueous extracts ($R^2=0.782$).

Extracts of *P. sajor caju* expressed the highest FRAP value in terms of per gram of extract. Extract of *P. flabellatus* showed the highest antioxidant activity when determined by scavenging effect on DPPH radical and total phenolic content. *P. citrinopileatus* exhibited the lowest FRAP value while *P. ostreatus* showed lowest scavenging effect on DPPH radical and *P. eryngii* in total phenolic content.

Based on the antioxidant assays, it was thus suggested that it could be other compounds besides phenolic compounds that is present in the extracts of *Pleurotus* species that have high total phenolic content but low in FRAP values.

The use of different methods in antioxidant activity assessment was compulsory to obtain a sufficient result of antioxidant activity of a sample. However, there are several methodological limitations for antioxidant determinations (Kaur and Kapoor, 2001). The most widely used methods for measuring antioxidant activity are those that involve the generation of radical species, where the presence of antioxidants determines the disappearance of radicals (Cao, *et al.*, 1993). It is pertinent to use different assays in an efficient extraction medium, instead of relying on a single assay to assess and compare the antioxidant capacity.

3.4 CONCLUSION

Pleurotus species have been cultivated worldwide since they have been proven to be a good source of nutrients and today it have been a great deal of public interest in the use as good antioxidative agent which has a protective effect. From the studies, it shows that *P. sajor-caju* expressed the highest FRAP value while *P. cystidiosus* showed highest scavenging effect on DPPH radical. *P. flabellatus* possessed the highest phenolic content when compared to the rest of the *Pleurotus* species tested.

Hence, when different methods were used in testing antioxidant property in *Pleurotus* species, it showed that each *Pleurotus* species have different unknown compounds, whereby only certain *Pleurotus* species reacted to certain methods used and producing different results. That is why there is no correlation between the total phenolic content with FRAP and DPPH in all nine *Pleurotus* species tested. However, all nine *Pleurotus* species tested did show some antioxidant property when compared to

other species of plants and grains. In general, *Pleurotus* species have excellent antioxidant activity.

As we know, antioxidants are natural substances that can neutralize free radicals. Free radicals are toxic chemicals that can do serious damage to cells and tissues which can eventually lead to cancer and other diseases. So antioxidants remove these dangerous free radicals from the body. It is widely believed that elevated blood glucose level lead to an increase in free radicals. It has also been shown that many people with diabetes have depleted levels of antioxidants in their bodies. Based on literature, *P. citrinopileatus* showed antidiabetic activity and hence, methanolic extract from mycelia of *P. citrinopileatus* of Malaysian strain was studied on STZ-induced diabetic rats in chapter four to determine the antidiabetic properties.

CHAPTER FOUR

4.0 INTRODUCTION

Increasing urbanization, aging populations, increasing obesity, and falling levels of physical activity are all contributing to increases in diabetes worldwide. It is thought that in 2000, the number of people with diabetes worldwide was ~171 million. India, China and the United States have the highest number of people with diabetes in the world (Wild, *et al.*, 2004). It has also been estimated that from 1995 to 2025, the number of people with diabetes in the world will increase by 122% (King, *et al.*, 1998).

Diabetes mellitus is characterized by recurrent or persistent hyperglycemia, and is diagnosed by demonstrating any one of the following: (World Health Organization Department of Noncommunicable Disease Surveillance, 1999).

- ❖ Fasting plasma glucose level at or above 126mg/dL (7.0 mmol/L)
- ❖ Plasma glucose at or above 200mg/dL (11.1 mmol/L) two hours after a 75g oral glucose load as in a glucose tolerance test.
- ❖ Random plasma glucose at or above 200mg/dl (11.1 mol/L).

Control of plasma glucose concentrations is vital to decrease the incidence and severity of long-term diabetic complications (Mandrup-Poulsen, 1998). Currently, dietary changes, oral hypoglycemia agents, or insulin injections are utilized to prevent hyperglycemia. At present, drug therapies either alone or in combination cannot restore normal blood glucose homeostasis, and many limitations exist in their use (Gray and Flatt, 1997). While external insulin is necessary for control of type 1 diabetes mellitus, the use of drug therapy in type 2 diabetes is initiated only after dietary and lifestyle modifications (Clark, 1998).

The characteristic symptoms of diabetes mellitus are excessive urine production (polyuria), excessive thirst and increased fluid intake (polydipsia), blurred vision,

unexplained weight loss and lethargy. These symptoms are likely to absent if the blood sugar is only mildly elevated.

Various dietary regimes have been considered for prevention of hyperglycemia in diabetes (Watkins, *et al.*, 1996). Throughout the world, many traditional plant treatments for diabetes exist and therein is a hidden wealth of potentially useful natural products for the control of diabetes (Gray and Flatt, 1997). Despite this, few traditional antidiabetic plants have received proper scientific screening.

There are many varieties of mushrooms species one of which – *Pleurotus* – is commonly known as the “oyster mushroom” (Miles and Chang, 1997). Easily grown on dead logs, the *Pleurotus* species has a polysaccharide fraction known as β -glucan complex which can be obtained from *Pleurotus* species (Bobek and Galbavy, 2001) possesses a tremendous effect as antidiabetic agent (Konno, *et al.*, 2002).

Pleurotus citrinopileatus is an edible mushroom, which has recently become popular, with a consequent increase in industrial production due to its condition that is successfully grown in tropical climates. Research on the medicinal value of *P. citrinopileatus* grown in Malaysia, a tropical country is minimal and yet to be explored. There have been no studies to investigate if the mycelia of *P. citrinopileatus* grown in Malaysia do have antidiabetic activity. Hence, *P. citrinopileatus* was chosen for antidiabetic study based on literature (Hu, *et al.*, 2006). *P. citrinopileatus* also showed that it have a good antioxidant activity based on previous results shown in chapter 3.

In this study, crude extract was prepared from mycelia grown by submerged fermentation and was fed to rats with streptozotocin (STZ) – induced diabetes and evaluated for hyperglycemia and damaged pancreatic cells.

The aims of this study were:

- a) to investigate the antidiabetic activity of mycelia methanolic extract of *Pleurotus citrinopileatus* on STZ-induced diabetes rats.
- b) to look at the insulin level, pathophysiology of the kidney and catalase activity after 45 days of extract ingestion.

4.1 MATERIALS AND METHODS

4.1.1 Growth of mycelium biomass and preparation of methanolic extract

Pleurotus citrinopileatus culture was obtained from stock culture from Mycology Lab, Institute of Biological Sciences, Faculty of Science, University of Malaya, Malaysia. Mycelium was grown in glucose, yeast, malt and peptone (GYMP) agar in plastic Petri dishes for 7 days at $24 \pm 2^\circ\text{C}$. Ten 5 mm diameter plaques from the edge of the growing colony were transferred to 500-ml Erlenmeyer flasks containing 100 ml of sterilized GYMP liquid medium (Appendix A, pp. 113). The inoculated flasks were incubated in static condition for 14 days at $24 \pm 2^\circ\text{C}$. After the incubation period, mycelium were filtered through Whatman No. 1 filter paper, mashed and soaked in methanol for 48 hours at $28 \pm 2^\circ\text{C}$. The methanol extract was then filtered through Whatman No. 1 filter paper, extracted and evaporated to dryness under vacuum using rotary evaporator to form a crude extract. Crude extract was kept at 4°C until the analysis for antidiabetic activity.

4.1.2 Experimental animals

Six to eight weeks old albino rats weighing 180 – 200g (Sprague – Dawley) were purchased from Centre for Animal Lab, Medical Faculty, University Malaya, Malaysia. The animals were individually housed in cages in an air-conditioned room with controlled temperature of $22 \pm 2^{\circ}\text{C}$ and relative humidity of 45 - 55% under 12 hour light: 12 hour dark cycle. The animals had free access to food pellets and water *ad libitum*. The experimental protocol was approved by the Animal Care and Use Ethics Committee, Faculty of Medicine, University of Malaya, Malaysia.

4.1.3 Induction of experimental diabetes

Prior to induction of diabetes, rats were acclimatized for 48 hours and were subjected to a 16-hours of fasting and blood was sampled (0 day). Diabetes was induced with streptozotocin (STZ) (Sigma) at a single dose of 70 mg/kg intraperitoneally. The STZ was freshly dissolved in 0.1 M citrate buffer, pH 4.5; the injection volume was 1ml/kg (Murali and Goyal, 2002) (Appendix A, pp. 113). Control rats received only the buffer. 48 hours after the injection of STZ, diabetes was confirmed in STZ-treated rats with fasting blood glucose concentration above 200 mg/dL.

4.1.4 Experimental design for glucose levels

On treatment day (48 hours after induction of diabetes), blood glucose level and body weight was measured in non-diabetic (normal) and STZ-induced diabetic rats after a 16 hours of fasting. The rats were divided into 5 groups of six rats each.

- Group 1 (Normal): Normal untreated rats
- Group 2 (Low Dose): STZ-treated diabetic rats administered with 500 mg/kg mycelial extract of *P. citrinopileatus* orally in sterilized distilled water.
- Group 3 (High Dose): STZ-treated diabetic rats administered with 1000 mg/kg mycelial extract of *P. citrinopileatus* orally in sterilized distilled water.
- Group 4 (Drug): STZ-treated diabetic rats administered with 10 mg/kg Glibenclamide orally in sterilized distilled water.
- Group 5 (Untreated): STZ- treated diabetic rats administered with sterilized distilled water.

Two days after the induction of diabetes, rats were subjected prior to 16 hours of fasting and blood was sampled (2 days). Starting from the treatment day, sterilized distilled water and mycelial extract from *P. citrinopileatus* were administered orally by feeding needle once daily for 45 days. Prior to 16 hours of fasting, fasting blood glucose levels and body weights were determined. This was carried out at day 7, 15, 30 and 45. Blood was collected from the tail vein and fasting blood glucose levels were determined using ACCU-CHEK Active glucose monitoring system (Roche Diagnostics, Germany).

After 45 days of treatment, all the rats were decapitated after 16 hours of fasting. Blood was collected in plain tube, centrifuged at 3000 rpm for 15 minutes to obtain serum. The serum was kept at -70 °C until the analysis to determine catalase activity and insulin levels. The liver, kidney and pancreas were removed, washed in saline and fixed in 10% formalin for histology analysis.

4.1.5 Insulin assay

The insulin assay was done by using the DSL-10-1600 ACTIVE Insulin ELISA kit (enzyme linked immunosorbent assay) which is an enzymatically “one step” sandwich-type immunoassay. All reagents and samples were brought to room temperature (25 ± 2 °C) and were mixed thoroughly by gentle inversion before use. Controls and standards were assayed in duplicate and samples were assayed in triplicate. 25 μ L of standards A (0.0 μ IU/ml), B (3.0 μ IU/ml), C (10.0 μ IU/ml), D (50.0 μ IU/ml) and E (100.0 μ IU/ml), control I (10.0 μ IU/ml) and control II (30.0 μ IU/ml) and serum were pipetted into appropriate wells. 100 μ L of insulin antibody-enzyme conjugate solution were added to each well using a semi-automatic dispenser. The 96 well plate was then incubated at room temperature (25 ± 2 °C), shaking at a fast speed (500 -700 rpm) on an orbital microplate shaker for one hour. The plate was then washed by aspirating the liquid from each well and dispensing 0.4 ml washing solution into each well using multichannel pipette. This step was repeated four times before adding 100 μ L TMB Chromogen solution to each well. The 96 well plate was then incubated shaking at a fast speed (500 -700 rpm) on an orbital microplate shaker at room temperature (25 ± 2 °C) for 20 minutes and direct exposure to sunlight was avoided. The colour development was monitored visually to optimize the incubation time. (The colour development may vary than the recommended incubation time depending in the localized room temperature). 100 μ L of stopping solution (0.2 M sulfuric acid) was later added and absorbance of the solution in the wells was taken within 30 minutes, using a microplate reader set at 450 nm. When reading of the absorbance of the microtitration plate was taken, it is necessary to program the zero Standard as a “Blank”.

The mean absorbance for each Standard, Control and unknown was calculated. The log of the mean absorbance readings was plotted on the y-axis versus the log of

concentration on x-axis (Appendix C, pp.128-129). Any sample reading higher than the Standard was ignored and any sample reading lower than the lowest Standard should be reported as such. Repeated freezing and thawing of reagents supplied in the kit and the specimens were avoided. Hemolyzed and lipemic specimens may give false values, hence these specimens were not used.

4.1.6 Catalase activity assay

2250 Unit/mg solid of crystals of bovine liver catalase (Sigma) was suspended in phosphate buffer saline, pH 7.0 (Beers and Sizer, 1952). The solution was then filtered with Whatman No. 1 Filter Paper and a serial dilution was done to obtain a series of standard of 20, 40, 60, 80 and 100 Unit/mg solid. An aliquot of 0.9 ml of 75 mM hydrogen peroxide was added to 100 μ L of standard and absorbance was taken at 240 nm for 30 seconds. This step was repeated for the serum samples as well and was assayed in triplicates (Appendix C, pp. 130-131).

4.1.7 Histology assay (Preparation of tissues and slides procedures)

(i) Fixation

The rats that were sedated were cut open. The pancreas, kidney and the liver were isolated from rats and was cut into small pieces, estimating 2 to 3 mm thick and rinsed with normal saline. The tissues were fixed individually into 10 % formaldehyde (*formalin*) for 24 hours. Solution of formaldehyde (*formalin*) was used to react with proteins and other organic molecules to stabilize cell structures. This solution is buffered and osmotically balanced to minimize shrinkage, swelling, and other collateral

damage. Ideally, fixation should be accomplished extremely quickly to minimize post-mortem changes in cell structure. After 24 hours of fixation, the tissues were transferred and submerged into 70 % alcohol.

(ii) Dehydration and clearing

Because a large fraction of the tissue was composed of water, a graded series of alcohol baths, beginning with alcohol 70% and progressed in graded steps to 95% alcohol were used to remove the water. The tissues were then immersed into 70% alcohol for 30 minutes. After 30 minutes, the tissues were transferred into 85% alcohol for another 30 minutes. After the time had ended, the tissues were submerged into alcohol 95% for another 30 minutes. After 30 minutes, this step was repeated again. The tissues were then treated with terpineol for 30 minutes. This step was repeated again. After 30 minutes, the tissues were transferred into mixture of terpineol : paraffin (1:1) for 30 minutes at 60 °C.

(iii) Infiltration and embedding

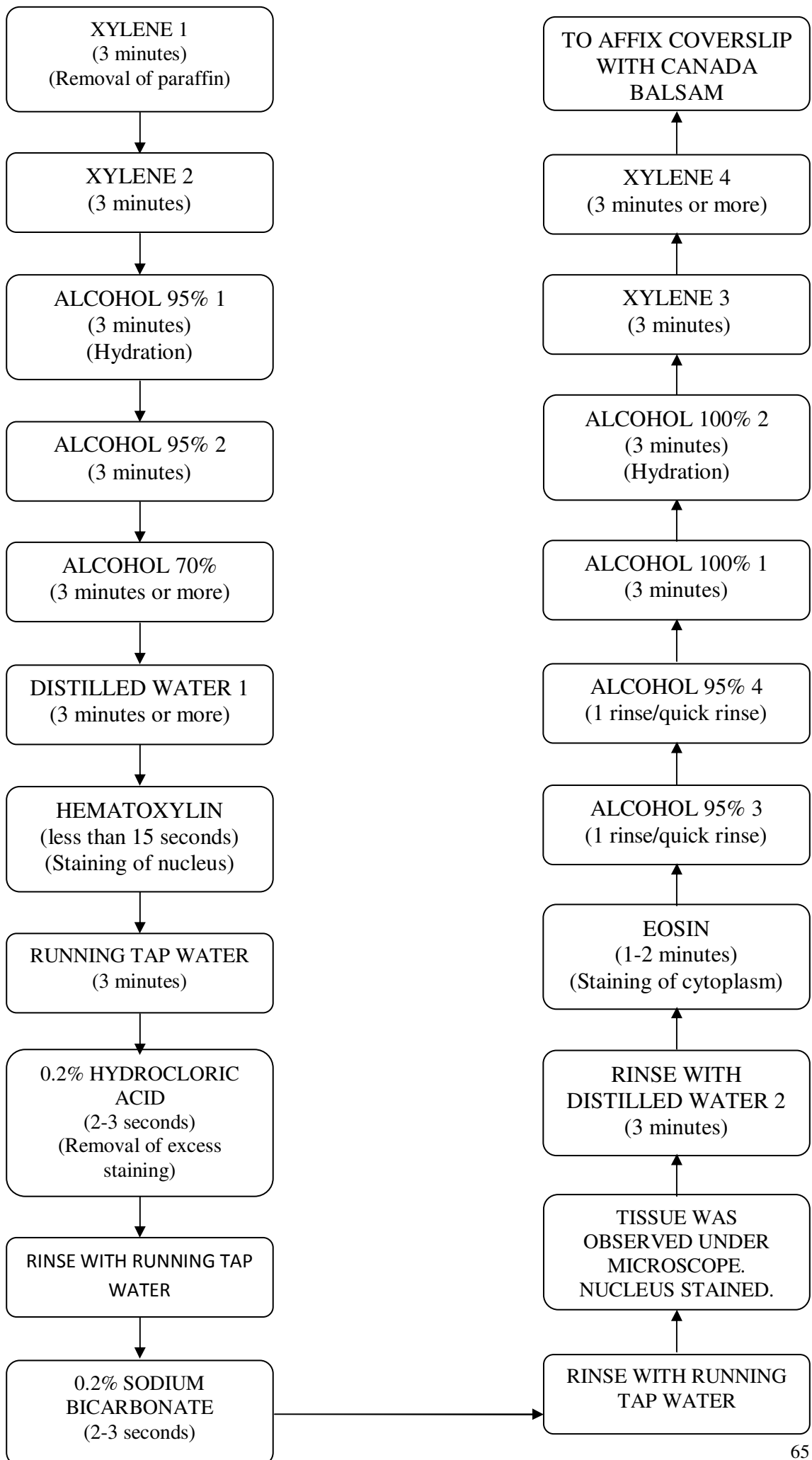
After 30 minutes in terpineol : paraffin (1:1), tissues were immersed in paraffin for 60 minutes. This step was repeated again for another 60 minutes until the tissues were completely infiltrated. Once the tissues were impregnated with paraffin, it was placed into a small receptacle, covered with melted paraffin and allowed to harden, forming a paraffin block containing the tissue.

(iv) Sectioning and mounting

After blocks of tissue was trimmed of excess embedding material, they were mounted for sectioning. The task was performed by a microtome (Leica), and the thickness of each section was 8 μm . The slides were labelled and a drop of Mayor's Albumin was placed on the centre of the slide and it was spread as thin as possible. A drop of distilled water was placed on the centre of the slide. A layer of sectioned tissue was placed on the slide and the slide was heated on a hot plate (40 – 45 °C) to ensure the tissue was fixed to the slide and this method was prolonged until the distilled water was dried up.

(iv) Staining (Hematoxylin & Eosin method)

Staining for light microscopy was performed mostly with water-soluble stains. Therefore, the slides were stained following the flow chart below. After staining, the section was labelled permanently affixed with coverslip by using mounting medium known as Canada Balsam.



4.1.8 Toxicity test

Thirty six animals (SD rats), 18 males and 18 females were assigned equally into 3 groups labelled as vehicle (distilled water), 2 g/kg and 5 g/kg body weight of synthesized compound preparation respectively. The animals were fasted overnight (food but not water) prior dosing. The purpose of starving the animals was to eliminate food inside the gastrointestinal tract that may complicate absorption of the tested substance. Foods were withheld for a further 3 to 4 hours after dosing. The observations were done on mortality and behavioural changes of the rats following treatment. All animals were sacrificed after 24 hours for gross necropsy and histopathological examination.

4.1.9 Statistical analysis

All data are presented as the mean \pm SD. The means of the data were subjected to a one way analysis of variance (ANOVA) using the SPSS programme and the significance of the difference between means was determined by the Duncan's multiple range test at 95% least significant difference ($p < 0.05$) (Appendix D, Table 9-52, pp. 139-165).

4.2 RESULTS

4.2.1 The effect of *P. citrinopileatus* mycelial methanolic extract on fasting glucose levels and body weights

Figure 4.1 showed the mean differences of fasting glucose levels (mmol/L) between days for each group for 45 days and the mean differences of glucose levels (mmol/L) between groups for each day for 45 days. There was a significant decrease in blood glucose levels between day 2 at 14.20 ± 1.20 mmol/L and day 45 at 6.42 ± 1.29 mmol/L for low dose treated rats. For animals receiving high dose of mycelial extract, there was also a significant reduction in fasting blood glucose levels between day 2 at 12.60 ± 1.68 mmol/L and day 45 at 5.52 ± 0.71 mmol/L. At day 30, there was no significant differences between normal group of rats with 4.88 ± 0.24 mmol/L, 6.67 ± 1.55 mmol/L for group of diabetic rats receiving low dose and 6.23 ± 0.81 for group of diabetic rats receiving high dose treatment. (Appendix D, Table 9-26, pp. 139-150) However, the blood glucose levels were significantly lower between the concentrations of the extracts compared to the Glibenclamide drug that acts as positive control.

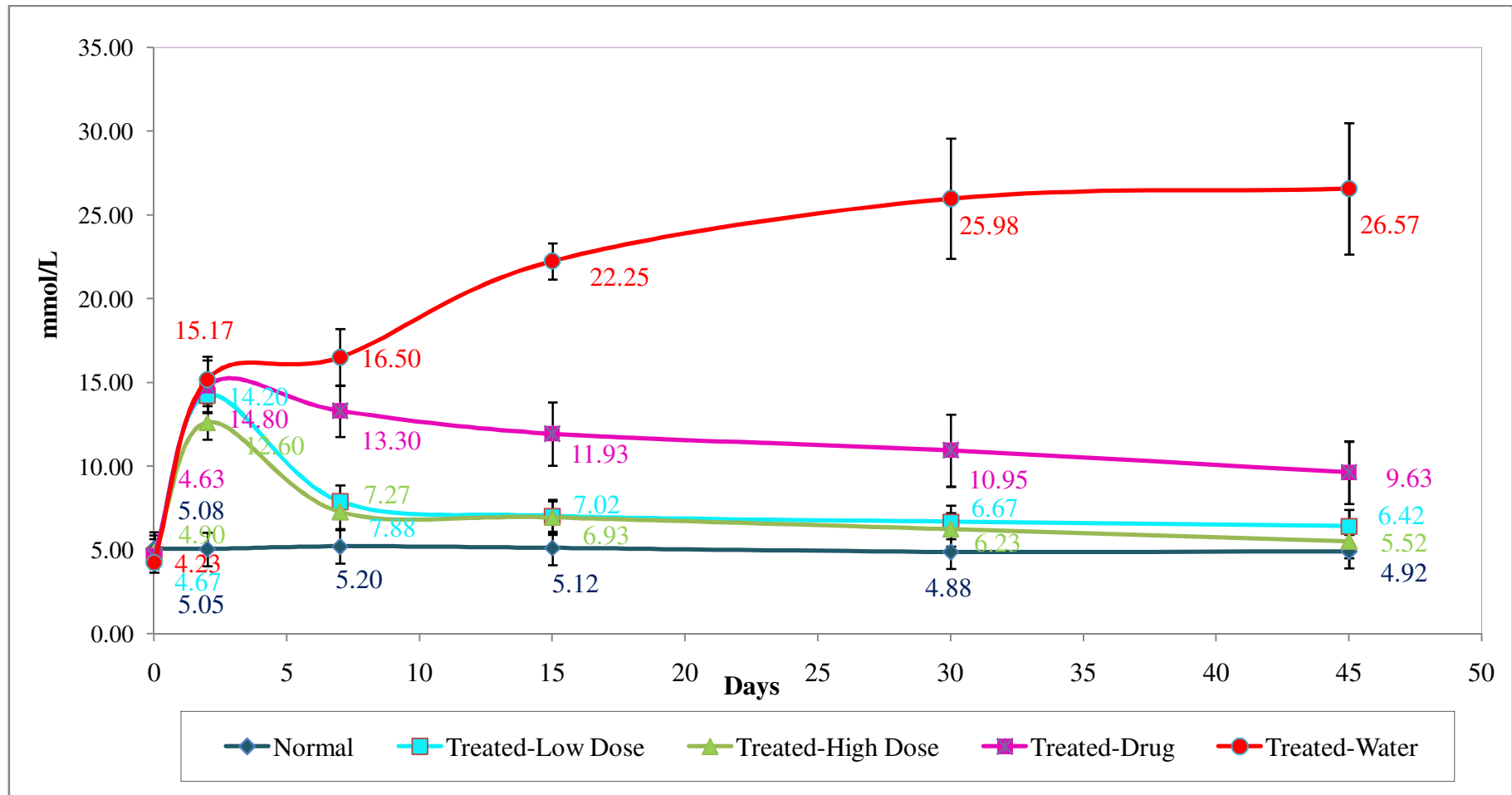


Figure 4.1: Fasting blood glucose levels in STZ-induced diabetic rats for 45 days.

There was no significance difference in body weight of rats treated with low dose of mycelial extract throughout the whole experiment for 45 days. However, the group of rats treated with high dose of mycelial extract gained body weight by the end of 30 days with 275.00 ± 22.36 grams compared to 245.83 ± 18.82 grams at the end of 15 days. Groups of rats treated with Glibenclamide drug showed a decreased in body weight significantly throughout the 30 days of experiment and gained weight at the end of 45 days as shown in Figure 4.2. At day 15, there was no significant difference between group of rats treated with low dose, high dose, drug and untreated rats but at day 30, rats treated with low dose and high dose of mycelial extract gained weight significantly compared to drug treatment rats and untreated rats. However, at day 45, group of rats receiving high dose of extract gained significant body weight of 287.50 ± 13.69 grams compared to low dose treatment rats with 254.17 ± 29.23 grams (Appendix D, Table 27-44, pp. 151-162).

With regards to the changes in physical appearance, after being fed with mycelial extract for 45 days, the color of the eyes of both mycelial extract fed group and glibenclamide fed group were normal when compared to untreated rats. After 45 days, eyes of the untreated rats become almost white. Their lenses also showed obvious lesions of pigmentary degeneration. The untreated rats were thin, the fur was rough and the rats were obviously losing fur. They moved around sluggishly.

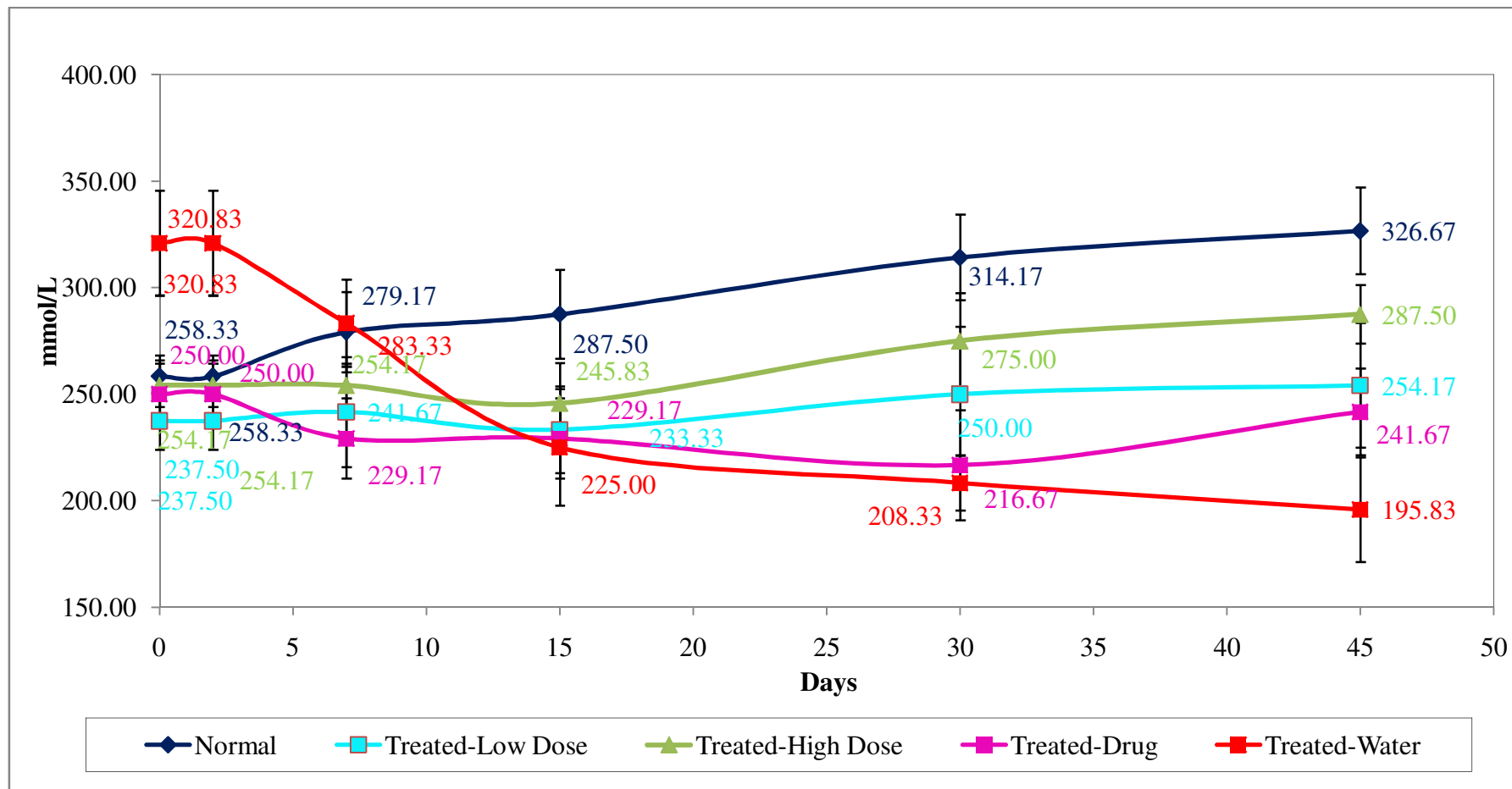


Figure 4.2: Body weight of STZ-induced diabetic rats for 45 days.

4.2.2 Fasting insulin and catalase activity

There was no significant difference in fasting insulin levels between groups of normal, low dose and high dose treated rats at the end of 45 days (Appendix D, Table 45-48, pp. 162-163) Normal rats and rats treated with high dose of mycelial extract had the same insulin level at $1.66 \pm 0.22 \mu\text{IU/mL}$ and $1.66 \pm 0.16 \mu\text{IU/mL}$ compared to drug treated rats with $1.04 \pm 0.10 \mu\text{IU/mL}$. Fasting catalase levels marks no significant difference between normal rats and rats treated with mycelial extracts.

Table 4.1: Fasting insulin levels at the end of 45 days

Group	Fasting insulin levels ($\mu\text{U/mL}$)	Percentage of insulin level (%)
Treated-Water	0.53 ± 0.13^a	31.92
Treated-Drug	1.04 ± 0.10^b	62.65
Treated-Low Dose	1.60 ± 0.07^c	96.39
Treated-High Dose	1.66 ± 0.16^c	100.00
Normal	1.66 ± 0.22^c	100.00

Values expressed are means \pm SD. of 6 measurements. Means with same letter in the same column denotes not significant ($p > 0.05$).

Table 4.2: Fasting catalase levels at the end of 45 days

Group	Fasting catalase levels (U/mL)	Percentage of catalase level (%)
Treated-Water	53.10 ± 1.53^a	52.12
Treated-Drug	96.10 ± 2.85^b	94.33
Treated-Low Dose	100.77 ± 2.34^{bc}	98.91
Normal	101.88 ± 4.07^c	100.00
Treated-High Dose	103.66 ± 2.69^c	101.75

Values expressed are means \pm SD. of 3 measurements. Means with same letter in the same column denotes not significant ($p > 0.05$).

Rats treated with water possessed the lowest catalase activity with 53.10 ± 1.53 U/mL and rats treated with high dose of mycelial extract with high catalase activity level at 103.66 ± 2.69 U/mL, higher than the normal rats by 1.75% (Appendix D, Table 49-52, pp. 164-165).

4.2.3 Behavioral changes and toxicity of *P. citrinopileatus* mycelial extract towards experimental animals

The mycelial extract did not cause any lethality or observable behavioral changes in the experimental animals. Toxicity tests carried out in the preliminary studies indicated that there was no significant modification in the general behavior of the animals up to 5 g/kg of the mycelial extract which is the maximum allowable dose by the Organization for Economic Cooperation and Development (OECD) Guideline 423 for the testing of chemicals (OECD, 2000).

4.2.4 Histology assay

Diffuse intercapillary glomerulosclerosis was observed in the histological slides. It is characterized by an increased amount of eosinophilic material within the mesangial region and a widening of the mesangial matrix in plate 4.2, plate 4.3 but eventually the process becomes both diffuse, involving the glomerulus, and generalized, affecting all glomeruli within the kidney as seen in plate 4.4. With increasing duration of diabetes, the mesangial material expands and coalesces, encroaching on adjacent capillary lumina and reducing the capillary surface area that is available for filtration. As this process progresses, entire glomeruli become hyalinised.

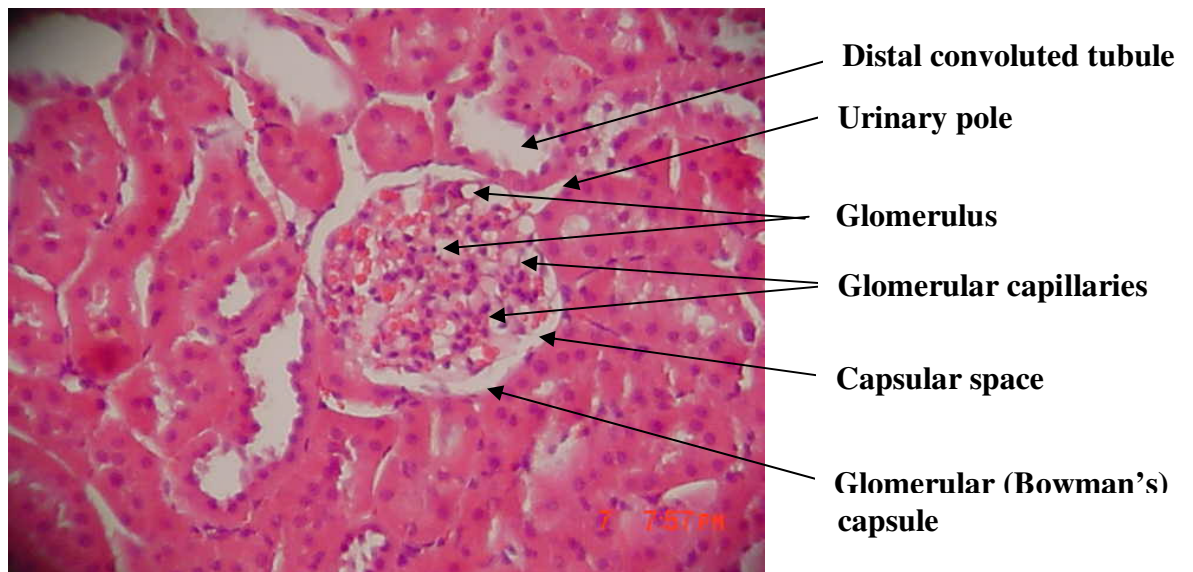


Plate 4.1: Histological section of kidney of normal rats showed normal architectures of glomeruli and tubules and no abnormality detected (H & E stain, X80).

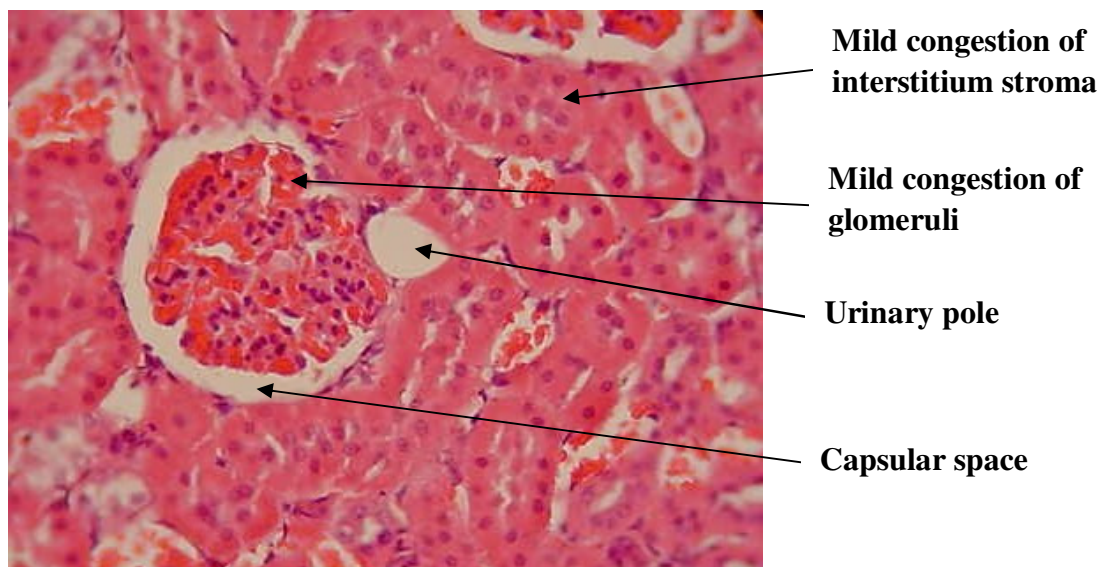


Plate 4.2: Histological section of kidney in STZ-induced diabetes and treated with 1000 mg/kg of mycelial extract of *P. citrinopileatus*. Section showed a very mild congestion of the glomeruli and interstitium stroma of cortex (H & E stain, X80).

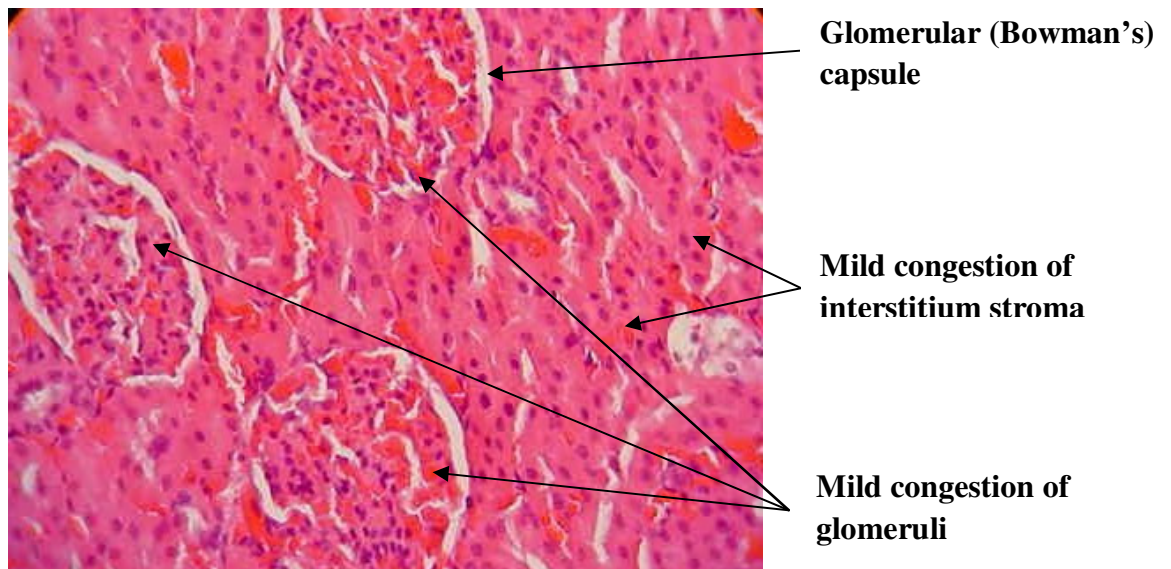


Plate 4.3: Histological section of kidney of STZ-induced diabetes rats and treated with 10 mg/kg Glibenclamide showed mild congestion of the glomeruli and interstitium stroma of cortex (H & E stain, X80).

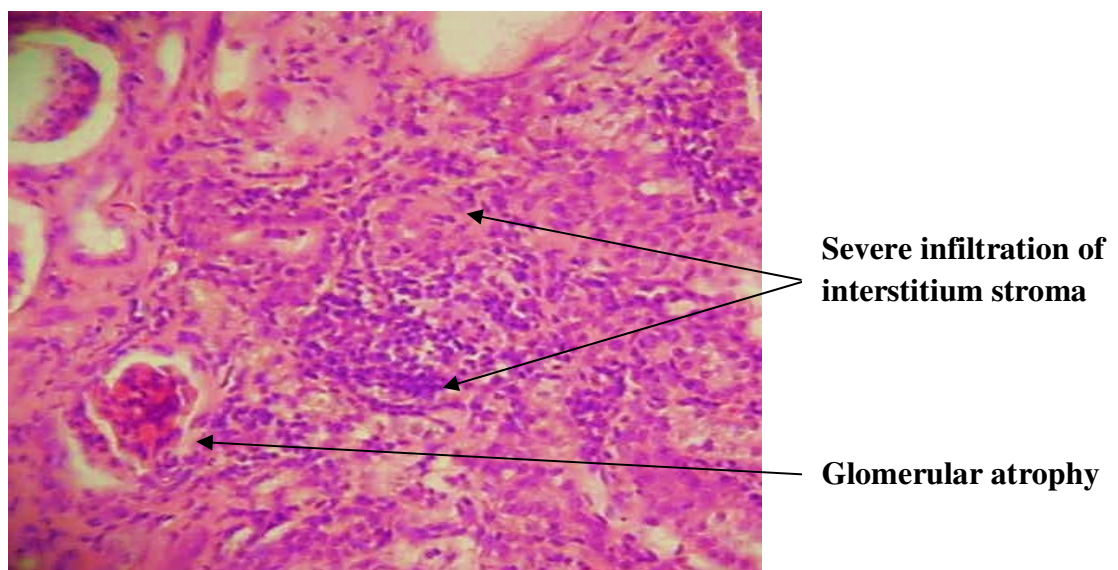


Plate 4.4: Histological section of kidney of STZ-induced diabetes rats showed glomerular atrophy and severe infiltration of interstitium stroma of cortex with chronic inflammatory cells (H & E stain, X80).

4.3 DISCUSSION

Diabetes mellitus is characterized by a reduced capacity of the beta cells of the pancreas to release sufficient insulin to induce the activity of glucose metabolizing

enzymes whether the cells are destroyed as in type 1 diabetes (IDDM) or intact as in type 2 diabetes (NIDDM) (Pannerselvam and Govindaswamy, 2002).

The mycelial extract of *P. citrinopileatus* produced a marked decrease in blood glucose levels at 500 mg/kg and 1000 mg/kg in STZ-induced diabetic rats over 45 days of treatment. The elevation in insulin in the mycelial extracts treated STZ-induced diabetic rats could be because of the insulintropic substances present in the fractions that induce the intact functional beta cells of the Islet of Langerhans to produce insulin, or the protection of the functional beta cells from further deterioration so that they remain active and produce insulin. The hypoglycemic effect of the extract may be because of the increased level of serum insulin and the enhancement of peripheral metabolism of glucose (Skim, *et al.*, 1999). It is evident from the data that both the extract and glibenclamide produced a comparable suppression in glycemia after 7 days and a consistent fall in fasting blood glucose level was recorded after feeding the extract and glibenclamide for 45 days. ANOVA revealed a significant effect of treatment together with a significant treatment-time interaction.

The body weight was found to drop after 7 to 15 days of STZ administration. Thereafter, both the extract and glibenclamide treated rats showed a sign of recovery in body weight gain, although latter showed greater gain in weight. On the contrary, diabetic rats treated with water showed a progressive fall in body weight throughout the experimental period.

Catalase is a hemoprotein which catalyzes the reduction of hydrogen peroxides and protects the tissues from highly reactive hydroxyl radicals (Chopra, *et al.*, 1958). Hydrogen peroxide is formed in cells by controlled pathways and elicits a broad spectrum of cellular response ranging from mitogenic growth stimulation to apoptosis to necrosis at different concentration levels. Locally intense amounts of hydrogen peroxide can also be produced by inflammatory cells to kill pathogens. Hydrogen peroxide at

high concentration is deleterious to cells and its accumulation causes oxidation of cellular targets such as proteins, lipids and DNA leading to mutagenesis and/or cell death. Removal of hydrogen peroxide from cells is therefore necessary for protection against oxidative damage. The catalase activity increased in mycelial extract treated rats and drug treated rats. Rats treated with mycelial extracts and drugs enhances the oxidative stress due to sudden attack of free radicals caused by STZ where glucose levels were increased above the renal threshold, gene expression and repairing mechanism may be in favour of synthesizing more catalase and antioxidants to compensate oxidative and carbonyl stress-mediated cellular damage and possibly the cells try to cope up with the adverse situation. Glucose levels in rats treated with water indicate that the antioxidant barrier was drastically broken down and the cells cannot cope up with the situation.

Hence, catalase defense system showed lower activity in water treated diabetic rats and the results agree well with the earlier published data (El-Missiry, *et al.*, 2004). The decreased activity of catalase may be a response to increased production of hydrogen peroxide and oxygen by the auto oxidation of excess glucose and non-enzymatic glycation of proteins (Argano, *et al.*, 1997). The decreased activity of catalase could also be due to their decreased protein expression levels in the diabetic condition as reported recently in liver (Sindhu, *et al.*, 2004).

Catalase reduces hydrogen peroxide into neutral water. However, hydrogen peroxide itself acts as a precursor of deadly damaging free radical by performing the Fenton reaction with free reactive iron which has been reported to be increased (Lyons and Jenkins, 1997; Kar, *et al.*, 2001). High hydrogen peroxide level in water treated rats but low levels of catalase cannot convert the total hydrogen peroxide level into neutral water. Therefore, hydrogen peroxide concentrations may be increased (by Fenton

chemistry with increased level of free reactive iron) and the thus cellular damage has been found to be significantly increased in this group.

Some natural bioactive substances with hypoglycemic action may be mediated through stimulating insulin synthesis and/or secretion from the islets beta cells (Hsu *et. al.*, 1997; Richard and Polansky 2002). When mycelial extract was digested in the small intestine, it is partially hydrolysed into oligosaccharide and absorbed in this form, which may have an immunoenhancing effect. This could be because some substances, after being absorbed, may cause the inhibition of the enzyme responsible for the metabolism of saccharides, which may lead to a decrease in the absorption of glucose into the blood and a subsequent lowering of blood glucose levels (Yakovlev, *et. al.*, 2002; Xie, *et. al.*, 2002; Kurihara, *et. al.*, 2003). Therefore, mycelial extract appears to protect pancreatic cells or delay their impairment. Because STZ may harm the immune system of cells, the function of the islets of Langerhans should be inhibited, resulting in the appearance of diabetic syndrome.

Mycelial extract of *P. citrinopileatus* is not dose-dependable for fasting blood glucose levels and body weight but time-dependable up to 7 days for fasting blood glucose levels and 30 days for body weight. By the end of 45 days, fasting blood glucose levels, body weight, fasting insulin levels and fasting catalase levels are all not dose-dependable as there were no significant differences between low dose (500 mg/kg) and high dose (1000 mg/kg) at the end of the day.

The development of diabetes renal disease has been related to acquired (metabolic and hemodynamic) disturbances that result from either insulin deficiency or insulin resistance. Both metabolic and hemodynamic alterations play a major role in the development of diabetic nephropathy. Glomerular basement membrane (GBM) thickening is an early histopathologic feature of diabetic glomerulosclerosis. It is not limited to the glomerulus and in poorly controlled diabetics occurs in capillaries

throughout the body, including muscle, skin and retina. The GBM is comprised of a collagen-like protein that is synthesized by the visceral epithelium of the glomerulus (Spiro, 1973). In patients with diabetes, both increased epithelial synthesis and diminished removal by the mesangial cells contribute to the basement membrane thickening.

Even though the STZ drug did damage all the glomeruli and interstitium stroma of cortex, but the cross section of cortex treated with Glibenclamide drug and mycelial extract showed that the tissues suffered mild congestion compared to the cross section of the cortex treated with water. This indicates that the mycelial extract do have wound healing property or perhaps an unknown enzyme that repairs the damage caused by STZ drug.

The basement membranes of the involved tubules often are thickened, and immunofluorescence studies have demonstrated IgG deposition along the tubular basement membrane (Heptinstall, 1983). The interstitial area surrounding the involvement tubules is fibrotic and may contain a mixture of lymphocytes and plasma cells. These interstitial changes progress with increasing duration of the diabetes (Heptinstall, 1983). Ziyadeh and Cohen (1993), suggest that the interstitial changes are a direct representation of a diabetic state. The only tubular lesion that is characteristic of the diabetic state is the Armani-Ebstein lesion, a vacuolization of the proximal tubular cells of the pars recta. These vacuoles contain glycogen and most often are observed in poorly controlled diabetic patients.

The earliest clinically apparent manifestations of diabetic retinopathy are classified as nonproliferative. During this phase a number of pathophysiologic changes associated with diabetes occur in the microvasculature; these include occlusion, dilation, and increased permeability of the small retinal blood vessels (Kohner, *et al.*, 1998). Histological changes that occur prior to the appearance of retinopathy were

degeneration of retinal capillary pericytes (mural cells) and thickening of the capillary basement membrane (Kohner, *et al.*, 1998). The endothelial cell wall thickens with deposition of lipid material and stagnant blood may trap in the lumen. With the further thickening of the wall and obliteration of the lumen, the microaneurysm may then appear yellow or white as seen in the eyes of STZ-induced diabetic rats of untreated group and non in those groups fed with low dose and high dose of mycelial extract and also diabetic rats treated with glibenclamide. These results may partially show that mycelial extract have an antihyperglycemic effect in STZ-induced diabetic rats.

However, as the disease progresses, retinal blot hemorrhages and hard exudates appear. Hard exudates result from leakage of lipoprotein material from retinal capillaries or microaneurysms into the outer retinal layer (Dobree, 1970). The exudate forms preferentially in the posterior retina, especially temporal to the macula, and may extend into the macula, reducing visual acuity. Increased permeability of retinal capillaries and retinal microaneurysms may result in accumulation of extracellular fluid and thickening of the normally compact retinal tissue. Macular edema appearing as diffuse thickening of the posterior retina is associated with retinal ischemia. The diffuse leakage of retinal blood vessels is a result of an extensive breakdown of the normally impermeable blood-retinal barrier of the capillary endothelial cells and was associated with the risk of loss vision.

4.4 CONCLUSION

In the context of the world wide explosion in type 2 diabetes where insulin resistance rather than failure of insulin secretion is regarded as pathogenetic mechanisms, mycelial extract of *P. citrinopileatus* which acted independently for the raise of insulin secretion are extremely important. Supplementation of *P. citrinopileatus*

mycelial extract may be beneficial for correcting hyperglycemia as the level of fasting blood glucose decreased drastically in STZ-induced diabetes rats and in fact, insulin resistance is believed to underlie not only diabetes but also hypertension, obesity, infertility, heart diseases; a far wider spectrum than the diseases related to deficiency in insulin secretion alone. The extract protected the weight loss of rats induced by STZ; similar findings are observed in other plant extracts (Swanston-Flatt, *et. al.*, 1989, Badole, *et. al.*, 2006a). These studies substantiate the usefulness of herbal treatment in the long-term treatment of diabetes. Mycelial extract of *P. citrinopileatus* mushroom also offers protection of beta cells against reactive oxygen species-mediated damage by enhancing cellular antioxidants defense as the elevation of catalase level in rats treated with high dose of mycelial extract and reducing hyperglycemia in chemically induced diabetes. Thus, the oxidative stress was decreased with the elevation of catalase levels. Mycelial extract also contain wound healing property or unknown enzyme that might act in repairing the damaged tissues in the cortex. This explains the mild congestion of the glomeruli and interstitium stroma of the cortex. Any approach to treatment of type 2 diabetes must combine education, diet, exercise, and management of multiple risk factors as nutritive supplement itself is not enough in reducing oxidative stress.

CHAPTER FIVE

GENERAL DISCUSSION, RECOMMENDATIONS AND CONCLUSION

Pleurotus species have been used by human cultures all over the world for their nutritional value, medicinal properties and other beneficial effects. *Pleurotus* mushrooms are a good source of dietary fibre and other valuable nutrients. They also contain a number of biologically active compounds with therapeutic activities. Oyster mushrooms modulate the immune system, inhibit tumour growth and inflammation, have hypoglycemic and antithrombotic activities, lower blood lipid concentrations, prevent high blood pressure and atherosclerosis, and have antimicrobial and other activities (Gunde-Cimerman, 1999).

The aims of this study were to investigate and compare the mycelial extracts of *Pleurotus* species for antioxidant activities as well as for antidiabetic measurement specifically on *P. citrinopileatus*. To my knowledge and from survey of literature, little information was available on these activities of *Pleurotus* species in Malaysia. Thus this is the first bioactivity studies of mycelium processed and extracted in methanol.

A number of natural antioxidants have already been demonstrated from different kinds of plant materials, such as oil seeds, cereal crops, vegetables, fruits, leaves, roots, spices and herbs (Ramarathnam, *et. al.*, 1995; Packer and Ong, 1997; Cullen, *et. al.*, 1997; Kamat, *et. al.*, 2000; Devasagayam, *et. al.*, 2001; Nigris, *et. al.*, 2003; Negi and Jayaprakasha, 2003). Because the role of free radicals has been implicated in a large number of diseases, the antioxidant activity of the mushrooms is of significant importance in exploiting their therapeutic potential. The proof of their antioxidant activity can also explain their mechanism of action. A number of medicinal mushrooms have recently been reported to possess significant antioxidant activity (Ajith and Janardhanan, 2001; Mau, *et. al.*, 2001; Ekanem and Ubengama, 2002).

With regards to the total phenolic content of *Pleurotus* species, mycelial extract of *P. flabellatus* exhibited the highest total phenolic content in terms of per gram of

extract. Mycelial extract of *P. cystidiosus* showed the highest antioxidant activity in terms of scavenging effect on DPPH radical. Mycelial extract of *P. sajor-caju* expressed the highest FRAP value in terms of per gram of extract. *P. eryngii* exhibited the lowest phenolic content when compared with the rest of the *Pleurotus* species which had been studied. *P. ostreatus* was a poor free radical scavenger on DPPH radical with the highest EC₅₀ value. On the other hand, *P. citrinopileatus* had the lowest FRAP value when compared with the rest of the species that was studied. Hence, there was no positive correlation between all three antioxidant studies that had been carried out. However, larger amounts of total phenolic content was found in the extracts, indicates the role of the phenolic compounds in the antioxidant activity of *Pleurotus* species.

The various antioxidant mechanisms of the mushroom species extract maybe attributed to strong hydrogen donating, metal chelating and reducing power abilities, and their effectiveness as good scavengers of superoxide and free radical. Since the *Pleurotus* species are now known to possess antioxidant activity in the mycelial extract and phenolic compounds might be a possible class of antioxidant in this mushroom species. Future work on the isolation and structural characterization of the active components are needed as there is no study so far conducted on the characterization and isolation of the active compounds and the mechanisms by which the protection against disease development.

Total phenols were the major naturally occurring antioxidant components found in methanolic extracts from commercial mushrooms. It had been reported that the antioxidant activity of plant materials was well correlated with the content of their phenolic compounds (Velioglu, *et. al.*, 1998). Therefore, total phenols might be responsible for the antioxidant properties studied in *Pleurotus* species as they exhibit the highest antioxidant properties when compared to the other methods used. Phenols such as BHT and gallate, are known to be effective antioxidants (Madhavi, *et al.*, 1996).

The highest content of total phenols in oyster mushrooms might account for the better found in antioxidant activity, as compared with other commercial mushrooms.

The best known organisms for potential producers of lovastatin from edible higher Basidiomycetes mushrooms are species of the genus *Pleurotus* (Gundecimerman and Cimerman, 1995). The presence of the inhibitor was determined in four species: *P. ostreatus*, *P. cornucopiae*, *P. eryngii* and *P. sapidus*. The highest content of lovastatin was found in the fruiting bodies of the *P. ostreatus*. Therefore, mature fruiting bodies of *P. ostreatus* could be recommended for consumption as a natural cholesterol lowering agent. Lovastatin appears early in the life cycle of the mushroom, in the mycelia from which primordial are being formed.

The reactive species is usually inactivated *in vivo* by a variety of antioxidants. Antioxidants are deployed to prevent generation of ROS or to scavenge those formed. Thus, oxidatively induced tissue damage is minimized. However, deficiency of antioxidant defenses may lead to oxidative stress, which might be associated with a variety of disorders including coronary heart disease, neural disorders, diabetes, arthritis, and cancer (Yoshikawa, *et al.*, 2000 and Spiteller, 2001).

To protect their possible damages to biological molecules, especially to DNA, lipids and proteins, all oxygen –consuming organisms are endowed with a well-integrated antioxidant system, including enzymatic and non-enzymatic components. The superoxidase dismutase (SOD), glutathione peroxidase (GSHPx) and catalase are the major antioxidant enzymes frequently mentioned in the literature. The non-enzymatic components consist of macromolecules, such as albumin, ceruloplasmin and ferritin as well as an array of small molecules, such as vitamin C, E, β -carotene and reduced glutathione (Fang, *et al.*, 2002 and Jacob, 1995).

In the recent years, more attention has been paid to the antioxidants contained in fruits because epidemiological studies revealed that high fruit intake was associated

with reduces mortality and morbidity of cardiovascular disease and some types of cancer and one of possible mechanisms was attributed to the antioxidant activity presented by the fruits. (Lampe, 1999 and Guo and Yang, 2001). Besides classical antioxidants including vitamin C, E and β -carotene, phenolic compounds had been identified as important antioxidants contained in fruits. Some phenolic compounds are even more powerful as antioxidants than vitamin C, E in vitro and significantly bioavailable as demonstrated by animal and human studies (Bravo, 1998; Rice-Evans, *et al.*, 1996; Su, *et al.*, 2003; Cao, *et al.*, 1998 and Mazza, *et al.*, 2002). Synthetic antioxidants also have been developed to retard lipid peroxidation, but their use as food supplements and therapeutic agents have been hindered due to their possible toxicity.

The antioxidants present in six different fractions may have different functional properties, such as reactive oxygen species scavenging (quercetin and catechin) (Hatano, *et al.*, 1989), inhibition of the generation of free radicals and chain-breaking activity, e.g. p-coumaric acids (Laranjinha, *et al.*, 1995) and metal chelation (Van-Acker, *et al.*, 1998). These compounds were normally phenolic compounds, which were effective hydrogen donors, such as tocopherols, flavonoids, and derivatives of cinnamic acid, phosphatidic and other organic acids.

A concerted action of a number of dietary antioxidants might also be expected from the exceedingly complex physical structure that makes up an individual. The human body, its tissues and organ, cells and macromolecules, consist of compartments with a range of physical variables, anatomical subdivisions and water- and lipid-soluble phases. Within these phases and at interfaces between phases, there will be numerous chemical variables such as pH, ionic strength, osmolarity, electrical charge and chemical concentration. These variables will influence the ability of the phases to act as solvents for lipid- and water-soluble antioxidants. In addition, antioxidants with both hydrophobic and hydrophilic characteristics may be distributed between water- and

lipid-soluble phases dependent upon the relative contribution and stereochemistry of hydrophobic and hydrophilic substitutions. Solubility is further modified when an antioxidant is conjugated or bound into more complex substances such as proteins.

Oxidative stress plays an important role in the pathogenesis and the complications of diabetes. Hyperglycemia results in overproduction of oxygen free radicals, which contributes to the progression of diabetes. The development of complications during diabetes is also associated with oxidative stress.

Preliminary phytochemical analysis of *Pleurotus* species showed the presence of proteins, minerals, vitamins and carbohydrates. Such constituents are confirmed also by Food and Agriculture Organization of United Nations (1968). Mushrooms consist of ~20-35% proteins. The glycoproteins constituents have been shown to have antihyperglycemic effect (Kusano, *et al.*, 2001).

Diabetes is a chronic metabolic disorder in which the body cannot metabolize carbohydrates, fats and proteins because of lack of, or ineffective use of the hormone insulin. An understanding of the pathophysiology of diabetes rests upon knowledge of the basics of carbohydrate metabolism and insulin action. Following the consumption of food, carbohydrates are broken down into glucose molecules in the gut. Glucose is absorbed into the bloodstream elevating blood glucose levels. This rise in glycemia and stimulates the secretion of insulin from the beta cells of the pancreas. Insulin is needed by most cells to allow glucose entry. Insulin binds to specific cellular receptors and facilitates entry of glucose into the cell, which uses the glucose for energy. The increased insulin secretion from the pancreas and the subsequent cellular utilization of glucose results in lowered of blood glucose levels. Lower glucose levels then result in decreased insulin secretion.

The presence of hyperinsulinism in type 2 diabetes, insulin resistance has been considered to play an integral role in the pathogenesis of the disease (Yalow and

Berson, 1960). As chronic hyperinsulinemia inhibits both insulin secretion (DeFronzo, *et al.*, 1981) and action (Del Prato, *et al.*, 1994), and hyperglycemia can impair both the insulin secretory response to glucose (Unger and Grundy, 1985) as well as cellular insulin sensitivity (Fink, *et al.*, 1992 and Yki-Jarvinen, 1992), the precise relation between glucose and insulin level as a surrogate measure of insulin resistance has been questioned. Lean type 2 diabetic patients over 65 years of age have been found to be as insulin sensitive as their age-matched nondiabetic controls (Arner, *et al.*, 1991). Moreover, in the majority of type 2 diabetic patients who are insulin resistant, obesity is almost invariably present (Kissebah, *et al.*, 1989). As obesity or an increase in intra-abdominal adipose tissue is associated with insulin resistance in the absence of diabetes, it is believed by some that insulin resistance in type 2 diabetes is entirely due to the coexistence of increased adiposity (Carey, *et al.*, 1996). Additionally, insulin resistance is found in hypertension, hyperlipidemia, and ischemic heart disease, entities commonly found in association with diabetes (Reaven, 1998; Ferrannini and Stern, 1995 and Nabulsi, *et al.*, 1995), again raising the question as to whether insulin resistance results from different pathogenetic disease processes or is unique to the presence of type 2 diabetes (Reaven, 1998; Charles, *et al.*, 1997 and Fagan, 1998).

Various mechanisms of action such as suppression of hepatic gluconeogenesis, stimulation of glycolysis and inhibition of glucose absorption from the intestine, stimulation of insulin release, inhibition of conversion of dietary disaccharides (alpha glucosidase inhibitors) and induction of the transcription of fatty acids by activating a specific sub-class of peroxisome proliferator-activated receptor may be responsible for hypoglycemic activities of antidiabetic agents (Hardy and McNutty, 1997).

At day 30 of treatment, there was no significant difference in fasting blood sugar level between normal group of rats, group of diabetic rats receiving low dose and high dose of mycelial extract of *P. citrinopileatus*. However, blood sugar level were

significantly lower for both groups of rats treated with mycelial extracts compared to group of diabetic rats treated with Glibenclamide drug. There was no significant difference in body weight of rats treated with low dose of mycelial extract throughout 45 days of treatment. However, diabetic rats treated with high dose of mycelial extract gained weight by the end of 30 days of treatment.

Fasting insulin level marked no significant difference between normal, low dose and high dose mycelial extract treated rats by the end of 45 days of treatment. The lack of potentiation by combination of mushroom extract and insulin suggests that the extract is likely to act via pathways (at least terminally) that are utilised by insulin rather than entirely separate pathways. The effect of extract on glucose uptake differed from that metformin, which exerts its effects on glucose transport via insulin-mediated enhanced peripheral glucose uptake (Bailey and Pua, 1986, Prager, *et al.*, 1986). It is thus apparent from the results that mycelial extract of *P. citrinopileatus* showed significant antihyperglycemic effect in STZ-induced diabetic rats.

The antidiabetic effect of mycelial extract of *P. citrinopileatus* could be due to possible enhancement of the peripheral utilization of glucose and /or potentiation of the biological effect of insulin as the level of insulin increases in diabetic rats treated with mycelial extract. It not only halts progression of diabetes (as it reduced weight loss) but also reduced the mortality of STZ-induced diabetic rats and showed potent synergistic antihyperglycemic effect probably due to increased insulin secretion.

The activity of antioxidant enzymes such as superoxidase dismutase, catalase and glutathione peroxidase, which is low in islet cells when compared to other tissues, becomes further worsened under diabetic conditions (Kawamura, *et al.*, 1994). This is due to the damage caused by oxygen free radicals (Gorray, *et al.*, 1993) since the antioxidant defense system is weak under diabetic condition (Grankvist, *et al.*, 1981). Fasting catalase levels exhibit no significant difference between normal rats and rats

treated with mycelial extract. Thus, under the treatment of mycelial extract of *P. citrinopileatus*, the catalase levels were brought near to normal.

Histological section of kidney of STZ-induced diabetic rats treated with high dose of mycelial extract of *P. citrinopileatus* showed very mild congestion of glomeruli and interstitium stroma of the cortex compared to untreated rats with glomerular atrophy and severe infiltration of cortex with chronic inflammatory cells. This provides a suggestion that the mycelial extract might possess wound healing property or unknown enzyme that might repair the damage caused by STZ drug. The formation of advanced glycosylation end products renders the collagen of GBM more resistant to degradation and contributes to the progressive GBM thickening. In summary, increased formation of both normal and abnormal basement membrane, as well as diminished removal, all play a role in the GBM widening that occurs in poorly controlled diabetic individuals. There were no physical changes observed in treated rats compared to untreated rats where the eyes become almost white due to pigmentary degeneration. The untreated rats were thin, moving very sluggishly, the fur was indeed rough and the rats were obviously losing fur.

Many varieties of mushrooms have been reported to possess antihyperglycemic activity through various mechanisms like glucose/insulin metabolism and/or by enhancing peripheral insulin sensitivity (Talpur, *et al.*, 2002 and Talpur, *et al.*, 2002b) or by enhancing insulin release by Islets of Langerhans (Gray and Flatt, 1998 and Ewart, *et al.*, 1975). Insulin releasing and insulin like activity in *A. campestris* (a variety of mushroom) is reported earlier (Gray and Flatt, 1998).

Guanide, a known hypoglycemic substance related to biguanide class of oral antidiabetic drugs, has been detected in edible mushroom of *Pleurotus* species (Windholz, 1983) and might be responsible for the antidiabetic effect on rats used in this experiment. This phytochemical may be responsible for synergistic action of extract

against STZ-induced diabetic rats as well as OGTT. It is generally agreed that glyburide binds to insulin receptors on β -cells. Following this binding, closure of ATP-dependent potassium channels leads to opening of voltage-gated Ca^{2+} channels, an increased Ca^{2+} influx and the subsequent stimulation of insulin containing granules' exocytosis (Boyd, 1988; Groop, 1992; Patane, *et al.*, 2000 and Elmi, *et al.*, 2000) and reduces fasting plasma glucose concentration.

Current therapeutic agents available for type 2 diabetes mellitus include sulfonylureas and related compounds, biguanides, thiazolidenediones, α -glucosidase inhibitors and insulin. A rational approach would be to begin with the agents particularly suited to the stage and nature of the disease, progressing, if necessary, to combination therapy. Pharmacological agents acting through different mechanisms of action should be chosen to improve glucose values while minimizing adverse effects.

A number of *Pleurotus* species have been reported to have immunomodulatory, hyperglycemic, hypolipidemic, anti-inflammatory and antitumor activities (Eisenhur and Fritz, 1991, Chang, 1996; Gunde-Cimerman, 1999). Earlier studies also showed the significant antitumor activity of the methanol extract of *P. ostreatus var. florida* (Jose and Janardhanan, 2000). Polysaccharides isolated from *Pleurotus* species are reported to have immunomodulatory properties, stimulating humoral and cell-mediated immunity and mechanisms of non-specific immunity and hemopoiesis (Hua, *et. al.*, 1994). Mushroom nutraceuticals are of multifunctional value with concerted effects. These dietary supplements will be useful in prevention and treatment of various human diseases (Wasser, *et. al.*, 2000).

The overwhelming majority of mushrooms used for production of dietary supplements are cultivated commercially (and not gathered in the wild). This guarantees proper identification, and pure and unadulterated products. In many cases it also means genetic uniformity.

Mushrooms are easily propagated vegetatively, and thus keep the one clone. They are capable of growing in the form of mycelial biomass in submerged cultures (Pointing, *et al.*, 2000). The mycelium can be stored for a long time, and the genetic and biochemical consistency may be checked after considerable time.

Today, approximately 80% of mushroom products are taken from fruiting bodies either collected in the wild or grown commercially. In both cases, the resulting products are considerably diverse and unpredictable. The quality of mushroom fruiting bodies is strongly dependent on substrate composition and properties of its ingredients, and usually these are far from constant. This is explained by the fact that the main components for mushroom production are of available agricultural and forest origin, such as cereal straw, corn stakes, horse or chicken manure, wood sawdust, etc.

The production of many biologically active substances is connected with maturation processes of fruiting bodies. In the case of triterpenoid concentration in reishi mushroom, it was shown that their highest rate is in the spore scraping obtained from the underside of the mushroom in the 1-2 mm tube region (Stavinoha, *et al.*, 1991). The same problem occurs with the cholesterol-lowering agent lovastatin in oyster mushroom fruiting bodies. Lovastatin was found concentrated primarily in the lamella and basidiospores, but not in stipe or cap tissue, and its amount depends on fruiting body size and age (Gunde-Cimerman and Cimerman, 1995; Gunde-Cimerman, 1999).

Variability of mushroom fruiting body composition is the reason why processing for extraction of polysaccharides from fruiting bodies is not considered commercially feasible, as the physicochemical properties of the products resulting from these processes are not known or regulated (Ohtsuka, *et al.*, 1977).

The cultivation of mushroom for fruiting body production is a long-term process, taking one of the several months for the first fruiting bodies to appear,

depending on species and substrate. By contrast, the growth of pure mushroom cultures in submerged conditions in a liquid culture medium allow one to accelerate the speed of growth and reduce its duration to several days. Optimization of culture medium composition and the physicochemical conditions of growth allow regulation of mushrooms metabolism to obtain a high yield of biomass and large amounts of specific substances of constant composition.

However, there is still a criticism of dietary supplements safety, and most of it is concerned with standardization, that is, producing mushrooms in such a way that there is consistency in chemical composition and effects across batches. This depends on numerous factors including cultivation conditions, purity and quality of ingredients, composition of substrates, stability of active compounds and storage. Mycelial biomass in submerged cultures forms a promising future platform for fully standardized production of safe mushroom-based dietary supplements. Submerged culture mycelium is of more consistent and predictable composition than fruiting bodies. For most substances, this mycelium biomass obtained by submerged cultivation also has higher nutritional value. The culture media in which mycelia grow are made of chemically pure and ecologically clean substances.

The additional advantage of submerged culturing is the fact that most medicinal mushrooms do not produce fruiting bodies under commercial cultivation. According to Miles and Chang (1997), reliable cultivation techniques are known for only 37 mushroom species, but medicinal mushrooms include many mycorrhizal species [e. g., *Boletus edulis* Bull.: Fr., *Suillus granulatus* (L.: Fr.) O. Kuntze] or parasitic species that need several years for development of normal fruiting bodies on trees [*Fomes fomentarius*, *Phellinus igniarius* (L.: Fr.) Quel.]. Such species cannot be grown commercially, but their mycelia can be grown easily and economically with the help of submerged culturing. High stability and standardization of mycelium grown in

submerged cultures is important not only for producing dietary supplements, but also might be beneficial for producing mushroom-based medicines.

Hence, it is concluded that based on results obtained from this investigations, the mycelial extracts of locally available medicinal mushrooms, *Pleurotus* species possessed antioxidant property while mycelial extract of *P. citrinopileatus* exhibited an excellent antidiabetic property. The investigations also concluded that the mushroom, similar to plants, have a great potential for the production of useful bioactive metabolites and that they are prolific resource for drugs.

Future effort should focus on the isolation and elucidation of the compounds from mycelial extract of *Pleurotus* species in order to identify the major active ingredients responsible for the antioxidant and scavenging of free radicals and also lowering blood sugar level by *P. citrinopileatus* observed, or if the active compounds acts synergistically to enhance the antidiabetic property. It is necessary to identify appropriate dosing and potential effects of long term use and interactions with other medications. Clinical efficacy and potential toxicity of compounds in larger trials require further assessment before recommendations regarding their use can be established.

The responsible bioactive compounds belong to several chemical groups, very often they are polysaccharides or triterpenes. One species can possess a high variety of bioactive compounds, and therefore of pharmacological effects.

The spectrum of detected pharmacological activities of mushrooms is very broad. Dependent on increasing knowledge about chemistry, biotechnology and molecular biology of mushrooms as well as an improvement of screening methods, a rapid increase in the applications of mushrooms for medicinal purposes can be expected.

Prerequisite for a use as drug, nutraceutical or other purpose is the continuous production of mushrooms in high amounts and in a standardized quality. A further necessity is the establishment of suitable quality parameters and of analytical methods to control these parameters. Nevertheless, the legal regulations for authorization as drug or as dietary supplements or as food should get more attention.