CHAPTER 1

INTRODUCTION

Oxidation plays an important role for the production of energy to fuel biological processes in many living organisms. However, the uncontrolled production of reactive oxygen-derived free radicals such as superoxide anion radical (O_2), hydroxyl radical ($OH^{\cdot}$) and hydrogen peroxide (H_2O_2) are involved in the onset of many diseases such as cardiovascular diseases, diabetes, rheumatoid arthritis and are implicated in carcinogenesis-induced mutation that leads to tumour promotion (Mau *et al*., 2002). Cancer is the general term for a series of neoplastic diseases that are characterized by changes in a cell leading to abnormal (unordered and uncontrolled) cellular proliferation. This disorder disrupts the normal processes of cell division, which are controlled by the genetic material (DNA) of the cells (Reddy *et al*., 2003).

For the last few years, cancer has become the second largest single cause of death in children, men, and women with approximately 10 million new cases diagnosed in the year 2000, and resulting in over 6 million deaths each year worldwide (Wasser & Weis, 1999c; Parkin, 2001). Several methods are used for treatment of cancer and these include surgery, chemotherapy and radiotherapy. Chemotherapy has been considered as the most effective method of cancer treatment, however most cancer chemotherapeutics severely affect the host normal cells. Therefore, efforts are needed to find natural products that can control the cancer without causing any damage to normal cells (Ajith & Janardhanan, 2003).

Antioxidants act as a major defence against radical-mediated toxicity by protecting the damages caused by free radicals. Inhibition of free radical generation can serve as a facile system for identifying cancer preventive agents and thus may be useful

in treatment of cancer (Hemnani & Parihar 1998). Although almost all organisms possess antioxidant defence and repair systems that have evolved to protect them against oxidative damage, these systems are insufficient to prevent the damage entirely (Simic, 1998).

Some natural substances present in our diet or synthesized in our cells are able to block, trap or decompose reactive oxygen species (ROS) participating in carcinogenesis. Epidemiological studies have shown that consumption of antioxidants and phytonutrient-containing foods may reduce this degenerative process. Natural antioxidants are being extensively studied for their capacity to protect organisms and cells against oxidative damage by inhibiting or scavenging free radicals (Cazzi *et al*., 1997). The demand for natural antioxidant has increased because of the question on the long term safety and negative consumer perception of synthetic antioxidants (Amarowicz *et al.,* 2004; Sakanaka *et al.,* 2004).

Considerable interest has been focused on wild mushrooms since they contain various amounts of antioxidants and phenolic compound that have the potential to protect against the deleterious action of free radicals that may lead to the genetic instability of cancer cells. The substantial range of medicinal mushroom species from which different bioactive compounds can be derived suggested that mushroom could be a source of novel anti-cancer agents. Due to this knowledge, mushrooms are considered as highly valuable bio-engineering resources and use as starting material in the production of drugs (Mizuno, 1999). The antioxidant properties identified in mushrooms can be extracted and used as dietary supplements for daily consumption.

The use of medicinal mushroom extracts in the fight against cancer is well known and documented in China, Japan, Korea, Russia and now increasingly in the United States (Mizuno *et al.,* 1995). However, it is only within the last three decades that chemical technology has been able to isolate the relevant compounds and

extensively screened for medical properties especially for anticancer application (Mizuno, 1999). Hence, search for new antitumour substances from mushrooms has been a matter of great importance.

The number of mushrooms on Earth is estimated at 140,000, yet maybe only 10% (approximately 14,000 named species) are known. Mushrooms comprise a vast and yet largely untapped source of powerful new pharmaceutical products. Basidiomycetes produce large number of secondary metabolites which show antibacterial, antifungal, antiviral, cytotoxic and hallucinogenic activity or which can be the source of plant growth regulators or flavours. Of the 651 species and 7 infraspecific taxa from 182 genera of higher Hetero- and Homobasidiomycetes, the overwhelming majority have been demonstrated to possess pharmacologically active compounds in their fruit bodies, culture mycelia, or culture broth (Reshetnikov *et al*., 2001). Higher Basidiomycetes mushrooms are still far from being thoroughly studied; even the inventory of known species is incomplete, comprising maybe only 10% of the true number of species existing (Hawksworth, 2001).

The mycelium is the synthesizing organ of the mushroom and also creates and feed the fungal bodies. It was shown to exhibit stronger medicinal properties compared to other parts of the mushroom (Mau *et al.,* 2002). If harvested at the right time, it contains more potent concentration of bioactive substances than the fruiting body. Therefore, in this study the *Marasmius* mycelia was used.

The present study was carried out to evaluate crude dichloromethane extracts from selected *Marasmius* spp. This study was undertaken with the following aims:

1. To determine the cytotoxicity of *Marasmius* extracts against human mouth epidermal carcinoma cell line (KB), human epidermal carcinoma of cervix cell line (CaSki), human colon cancer cell line (HT 29), human intestinal colon cancer cell

line (HCT 119), human colorectal cancer cell line (SKOV 3), human breast cancer cell line (MCF 7) and also on human fibroblast cell (normal cell) (MRC 5).

2. To evaluate the antioxidant activity of *Marasmius* extracts using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, ferric reducing ability power (FRAP) assay and metal chelating assay.

CHAPTER TWO

LITERATURE REVIEW

2.1 Overview of Cancer

Cancer is a group of diseases characterized by uncontrolled cell division leading to growth of abnormal tissue. Cancer harms the body when damaged cells divide uncontrollably to form lumps or masses of tissue called tumors. Tumors can grow and interfere with the digestive, nervous, and circulatory systems, and they can release hormones that alter body function (Frenkel, 2003).

The Cancer Incidence in Peninsular Malaysia 2003-2005 Report, published by the National Cancer Registry (NCR), states that a total of 67,792 new cancer cases were diagnosed among 29,596 males (43.7%) and 38,196 females (56.3%). The annual crude rate for males was 100.2% per 100,000 population, and 132.1% per 100,000 for females. The most frequent cancer during this period in Malaysians was breast cancer (18%) followed by large bowel cancer (11.9%) and lung cancer (7.4%).

The development of cancer is a complicated process in which a large number of factors interact to disrupt normal cell growth and division. Cancer can be caused by hereditary and hormones as well as external factors such as chemicals, viruses, diet, and radiation. Whatever the cause of cancer, its development is a multi-stage process involving damage to the genetic material of cells (Waris & Ahsan, 2006).

2.2 Multiple Steps in the Carcinogenesis Process

Carcinogenesis is a process in which cells accumulate multiple genetic alterations as they progress to a malignant phenotype. Carcinogenesis can be divided conceptually into three stages: tumour initiation, promotion and progression.

2.2.1 Initiation

The first stage of carcinogenesis is initiation (Figure 2.1). The initiation involves a mutation, a change in the DNA. This is when the normal cell exposed to a carcinogen undergoes an irreversible change and causing genetic damages such as mutations, or chromosomal aberrations such as those derived from cross-linking of DNA leading to translocation or loss of chromosomal regions that remains unrepaired. For mutation to accumulate, the resulting genetic changes in a damaged cell are reproduced during mitosis, giving rise to a clone of mutated cells to complete the initiation event. This takes weeks, months or years during which time the initiated cell may be phenotypically indistinguishable from other parenchymal cells in that tissue (Pitot, 2002). Those mutated cells depend upon surrounding cells to provide necessary growth stimulation. Initiated cells display altered characteristics, which may include an increased life span, selective resistance to cell toxins, apoptotic stimuli and inhibitors of cell proliferation, alterations in the programming or control of normal cell progression and differentiation (Bower & Waxman, 2006).

2.2.2 Promotion

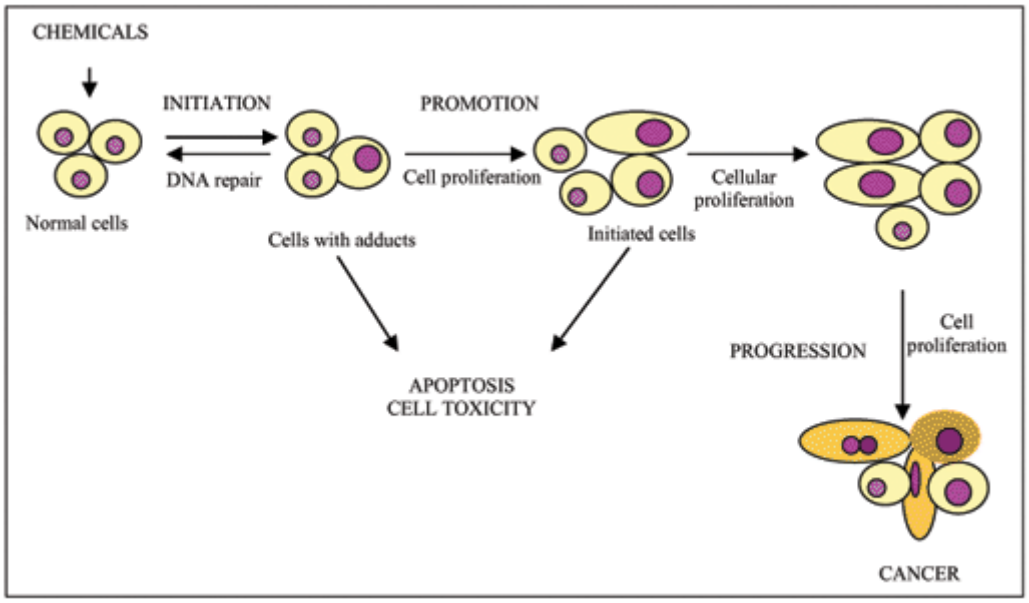
Tumour promotion comprises the selective clonal expansion of initiated cells (Figure 2.1), which form an actively proliferating multi cellular premalignant tumour cell population (Kleinsmith, 2006). Chemicals or agents capable of performing both tumour initiation and tumour promotion activity are known as complete carcinogens. Examples of complete carcinogens are benzo[a]pyrene and 4–aminobiphenyl (Weston & Harris, 2000). When tested alone these compounds illustrate very weak or no carcinogenic activity but markedly enhance the formation of tumour when applied repeatedly following a low or suboptimal dose of an initiating agent (Weinstein, 1988). These agents have the ability to reduce the latency period for tumour formation and can

induce tumour formation in conjunction with a dose of an initiating agent that is too low to be carcinogenic alone.

2.2.3 Progression

Progression is the final stage of carcinogenesis (Figure 2.1). It is irreversible and genetic damages are often found in this stage. In common usage, progression is used frequently to signify the stages whereby a benign proliferation becomes malignant or where neoplasm develops from a low grade to a high grade of malignancy. During progression, neoplasms show progressively increased invasiveness, develop the ability to metastasize and have alterations in biochemical, metabolic and morphologic characteristics. Over time, these cells become more dominant than the normal cells and acquire more aberrant traits through the repetitive process of this cycle resulting in clonal selection. A preference for better surviving traits exists among the cells where cells exhibiting advantageous properties such as increased growth rate, increased invasiveness and more adaptable to new distant sites slowly dominate the cell population.

Tumour cell heterogeneity is an important characteristic of tumour progression. Expression of this heterogeneity includes antigenic and protein product variants, ability to elaborate angiogenesis factors, emergence of chromosomal variants to become independent of external growth factors (autonomous growth or hormone independence), to become independent of surrounding cells and able to destroy biological barriers and escape the immunosurveillance mechanisms. These types of behaviours allow the cells to elude from normal homeostatic mechanisms and possess metastatic potential that enable invasion beyond the immediate location of the primary tumour (Kleinsmith, 2006).



(Source: Mehta, 1995)

Figure 2.1 Schematic model of multistage carcinogenesis

2.3 Cancer Chemoprevention

Cancer chemoprevention is defined as the use of natural, synthetic, or biologic chemical agents to reverse, suppress, or prevent carcinogenic progression to invasive cancer. Chemopreventive agents inhibit both initiation and promotion during carcinogenesis. The inhibition of initiation may occur by preventing the carcinogen from becoming fully active by enhancing DNA repair and/or the activation of tumor suppressor genes. The inhibition of promotion could result by triggering differentiation. Initiation and promotion may also be inhibited by the elimination of transformed malignant clones of cells (Steele, 2003). The success of several recent clinical trials in preventing cancer in high-risk populations suggests that chemoprevention is a rational and appealing strategy (Anne *et al.*, 2004). It is believed that the dietary factors may contribute to as much as one-third of potentially preventable cancers; and the long-term preventive effect of plant based agents for chemoprevention of cancer. Many naturally occurring agents have shown cancer chemopreventive potential in a variety of bioassay

systems and animal models, having relevance to human disease (Ahmad & Mukhtar, 2001).

The most useful cancer chemopreventive agents should have significant ability to reduce tumor incidence, delay tumor onset and prevent tumor progression (Tsao *et al*., 2004). Medicinal plants and natural products rich in antioxidants and bioactive phytochemicals have received growing attention over the past few years as potential chemopreventive agents. A chemopreventive agent must also prove high efficacy and most crucial, is not hazardous to health. In addition to that these agents must meet certain requisites as it is eventually being administered by humans. It must be affordable and easily available, produced, stored and administered (Flora & Ferguson, 2005). As such the consumption of dietary anti-cancer nutrients may be suggested as an important source of chemoprevention.

2.4 *In Vitro* Cytotoxicity Assay

Cytotoxic agents are compounds used to kill cancer cells. Treating cells with a cytotoxic compound can result in a variety of cell fates. The cells may undergo necrosis, in which they lose membrane integrity and die rapidly as a result of cell lysis. The cells can stop actively growing and dividing (a decrease in cell viability), or the cells can activate a genetic program of controlled cell death (apoptosis) (Eisenbrand, 2002).

In the search for new potential chemopreventive and anticancer drugs, the most common screening methods employ cytotoxicity tests against a panel of cancer cell lines. Cytotoxicity test, an alternative method in toxicological sciences, is an *in vitro* study to measure the different parameters involved in the progression of cell death and proliferation. Compared to animal studies, cell-based testing is easier to perform, reproducible, less ethically ambiguous, and is less expensive. There are many *in vitro* methods, which have been developed to test toxicity towards particular tissues. Different cytotoxicity assays will reveal different results depending on the cytotoxicity

assay employed and the test agent used (Weyermann *et al*., 2005). Some examples of the few preferred cytotoxicity assays that are frequently used in numerous studies are Neutral Red (NR) assay, lactate dehydrogenase assay (LDH) and 3-(4,5-dimethylthiaazol-2-yl)-2,5-diphenyltetrazolium bromide tetrazolium (MTT) salt assay.

These are high throughput screening assays, revealing compounds with highest cytotoxicity activity. Although cytotoxic agents are designed to kill cells, such activity should be selective towards tumour cells. Therefore it seems reasonable to utilize, at the primary screening stage, *in vitro* toxicity assays to select the least toxic compounds among the most active ones. The NR assay is used to measure damages in the cell membrane by monitoring the amount of dye uptake into the lysosomes of viable cells (Reddivari *et al*., 2007). The LDH leakage assay is based on the measurement of LDH activity in the extracellular medium (Patel *et al*., 2010). The loss of intracellular LDH and its release into the culture medium is an indicator of irreversible cell death due to cell membrane damage. This assay was originally used to measure neuronal cell death occurring via necrosis, but later, it has been shown to accurately measure neuronal apoptosis in cortical cultures. The MTT assay is one of the widely used methods to assess cell viability. MTT is reduced by mitochondrial dehydrogenases in living cells to a blue-magenta-colored formazan precipitate. The absorption of dissolved formazan in the visible region correlates with the number of intact alive cells. Cytotoxic compounds are able to damage and destroy cells, and thus decrease the reduction of MTT to formazan (Mueller *et al*., 2004).

The cytotoxicity tests that are practical, easily reproduced and predictive are always favoured for many researchers (Fotakis & Timbrell, 2006). Another factor to consider is high sensitivity in detecting early toxicity by revealing statistically significant results between the controls and the treated cells.

2.4.1 *In Vitro* Neutral Red Cytotoxicity Assay

One of the widely used cytotoxicity assay in *in vitro* toxicology to measure cell viability is the neutral red (NR) assay. NR is a supravital dye and is used to estimate the viability of cells. (Fautz *et al*., 1991). This rapid colorimetric test is based on the uptake of the cationic supervital dye NR (3-amino-7-dimethylamino-2-methylphenazine hydrochloride) into viable cells, where it accumulates in lysosomes by non-anionic diffusion and binding electrostatically to anionic sites in the lysosomal matrix (Babich & Borenfreund, 1992). Damage to the cell surface or sensitive lysosomal membranes decrease the uptake of and binding of NR making it possible to differentiate between viable intact cells and dead/damaged cells. The NR assay was developed and successfully used to evaluate the cytotoxic effect of metal salts in mouse BALB/c 3T3 fibroblasts (Borenfreund & Puerner, 1985).

The NR assay is more accurate than any other toxicity assay because the uptake of NR is not biased by occasional microbial contamination, which can lead to an overestimation of cell viability. Other advantages of the NR assay include easy handling; many replicates and the reagents are easily obtained as it involves the usage of common laboratory equipments. Another important fact is that the NR assay can be performed within a short assay time and still retaining its high sensitivity level in estimating cell viability and/or growth (Ciapetti *et al*., 1996).

The cytotoxicity activity was expressed as the IC_{50} value estimated from the graphical interpolation of the dose-response curve, which is defined as the concentration of extract that causes 50% inhibition or cell death (Chiang *et al*., 2003:Geran *et al*., 1972). The extract that gave an ED_{50} value 20.0 µg/ml or less is considered as active. However, extracts that exhibit ED_{50} value of more than 30.0 µg/ml were considered not active and does not require further testing (Geran *et al*., 1972).

2.5 Free Radicals

Free radicals are atoms, molecules or ions with unpaired electrons on an open shell configuration. They can be found exogenously and endogenously. Exogenous free radicals are enhanced by the environment and occupational factors such as lifestyle, exposure to radiation, pollution and pesticides (Martin-Moreno *et al*., 2008). The endogenous free radicals are formed inside the human body as by-products during metabolism. Oxygen is broken down during redox reactions to produce energy via a series of sequential electron reductions that consequently forming reactive molecules such as superoxide radicals, hydrogen peroxides and hydroxyl radicals (Pappa *et al*., 2007). These radicals are important as intermediates in the body's defence against invading microorganisms. However, excessive free radicals could pose danger to the cells for they may damage important macromolecules, kill the cells and subsequently cause damage to tissues (Vimala *et al.,* 2003).

Free radicals are highly unstable and aggressively seek for an electron to stabilize itself again by 'stealing' an electron from the nearest molecule, leaving that particular molecule with insufficient electrons. The latter then becomes unstable and 'steal' electrons from other molecule and the reaction continues resulting in a chain reaction. Each time free radicals 'steal' electrons from a normal cell; the molecular structure of the cell will be damaged causing cell dysfunction or cell death (Kleinsmith, 2006).

These chain reactions known as oxidation if not stopped will create more damages to the body in a long term. When the oxidant particles trespass into a cell seeking for electrons, it breaks the cell membrane and disrupts the normal cellular function (Kleinsmith, 2006). The DNA will also be damaged by these free radicals and this in turn can lead to diseases such as cancer (Miller, 2005).

2.5.1 Types of Free Radicals

Types of free radicals include the hydroxyl radicals (OH^o), the superoxide radical O_2^{-o} the nitric oxide radical (NO^o) and the lipid peroxyl radical (LOO^o) (Miller, 2005). Most free radicals are oxygen-based molecules but there are other types of radicals exist depending on the major atom(s) of the species such as the sulfur and nitrogen-centred species.

Reactive oxygen species are known as ROS. Reactive oxygen species (ROS) form as a natural byproduct of the normal metabolism of oxygen and have important roles in cell signaling and homeostasis (Devasagayam *et al*., 2004). ROS are produced as a normal product of cellular metabolism. While ROS are produced as a product of normal cellular functioning, excessive amounts can cause deleterious effects. ROS damages the cell by interacting with critical macromolecules such as DNA, protein and lipids leading to cell death, mutation and other toxicities. Some of the ROS are the hydrogen peroxide (H_2O_2), superoxide radicals (O_2^-), peroxyl ($RO_2^{\cdot}$), singlet oxygen ($^1O_2$) and hydroxyl radicals ($OH^{\cdot}$) (Miller, 2005).

a) Superoxide, O_2^- and Hydrogen Peroxide, H_2O_2

Superoxide anion (O_2^{-o}), the product of a one-electron reduction of oxygen, is the precursor of most ROS and a mediator in oxidative chain reactions. Dismutation of O_2^{-o} (either spontaneously or through a reaction catalysed by superoxide dismutase (SOD)) produces hydrogen peroxide (H_2O_2), which in turn may be fully reduced to water or partially reduced to hydroxyl radical (OH^o), one of the strongest oxidants in nature. The formation of OH^o is catalysed by reduced transition metals, which in turn may be re-reduced by O_2^{-o}, propagating this process (Liochev & Fridovich, 1999). H_2O_2 is naturally produced in organisms as a by-product of oxidative metabolism and not a free radical. Both O_2^- and H_2O_2 are likely to target at intracellular substances which they

can do direct damage. H_2O_2 passes all the biological membrane easily compared to O_2^- hence spreading its reactivity and possible toxicity (Boots *et al*., 2008).

b) Hydroxyl (OH^o)

OH^o is the most reactive ROS and thus the most damaging because it will react with any molecule it encounters (Galli *et al*., 2005). It is abundantly found in cells and plays an important role in the DNA damage by attacking the nucleotide bases resulting in the breaking of DNA strand (Schyman *et al*., 2008). The main *in vivo* source of OH^o is from the Haber-Weiss reaction (Kehrer, 2000).

$$O_2^o + H_2O_2 \rightarrow O_2 + OH^o + OH^-$$

c) Reactive Nitrogen Species (RNS)

The reduction of oxygen by one electron at a time produces relatively stable intermediates. In addition, O_2^{-o} may react with other radicals including nitric oxide (NO^o) in a reaction controlled by the rate of diffusion of both radicals. The product, peroxynitrite, is also a very powerful oxidant (Beckman & Koppenol, 1996; Radi *et al.* 2002*b*). The oxidants derived from NO^o have been recently called reactive nitrogen species (RNS). Other nitrogen oxides are nitrite (NO^-_2), nitrogen peroxide (NOO^o) and peroxynitrite ($ONOO^o$) (Lopez *et al*., 2007).

2.6 Oxidative Stress

Oxygen is an element indispensable for life. When cells use oxygen to generate energy, free radicals are created as a consequence of adenosine triphosphate (ATP) production by the mitochondria. These by-products are generally reactive oxygen species (ROS) as well as reactive nitrogen species (RNS) that result from the cellular redox process. These species play a dual role as both toxic and beneficial compounds. The balance between their two antagonistic effects is clearly an important aspect of life. At low or moderate levels, ROS and RNS exert beneficial effects on cellular responses

and immune function. At high concentrations, they generate oxidative stress, a deleterious process that can damage all cell structures (Halliwell *et al*., 2007).

In humans, oxidative stress is involved in development of chronic and degenerative ailments such as cancer, arthritis, aging, autoimmune disorders, cardiovascular and neurodegenerative diseases (Valko *et al*., 2007). The human body has several mechanisms to counteract oxidative stress by producing antioxidants, which are either naturally produced *in situ*, or externally supplied through foods and/or supplements.

A particularly destructive aspect of oxidative stress is the production of ROS, which include free radicals and peroxides. ROS can cause oxidative DNA, damage to protein and tumor suppressor genes and enhanced expression of proto-oncogenes (Wei, 1992). Oxidative stress has also been shown to induce malignant transformation of cells in culture (Valko *et al*., 2007). ROS are potential carcinogens because they facilitate mutagenesis, tumor promotion and progression. The growth promoting effects of ROS are related to redox-responsive cell signaling cascades. Sometimes, even normal cells show increased proliferation and expression of growth-related genes if exposed to H_2O_2 or O_2^-. Certain types of cancer cells also produce significant amounts of ROS.

2.7 Oxidative Stress and Associated Diseases

2.7.1 Cancer and Oxidative Stress

The development of cancer in humans is a complex process including cellular and molecular changes mediated by diverse endogenous and exogenous stimuli. It is well established that oxidative DNA damage is responsible for cancer development (Valko *et al*., 2007). Cancer initiation and promotion are associated with chromosomal defects and oncogene activation induced by free radicals. A common form of damage is the formation of hydroxyled bases of DNA, which are considered an important event in

chemical carcinogenesis (Halliwell, 2007). This adduct formation interferes with normal cell growth by causing genetic mutations and altering normal gene transcription. Oxidative DNA damage also produces a multiplicity of modifications in the DNA structure including base and sugar lesions, strand breaks, DNA-protein cross-links and base-free sites. For example, tobacco smoking and chronic inflammation resulting from non-infectious diseases like asbestos are sources of oxidative DNA damage that can contribute to the development of lung cancer and other tumors (Valko, 2004). The highly significant correlation between consumption of fats and death rates from leukemia, breast, ovary and rectum cancers among elderly people may be a reflection of greater lipid peroxidation (Droge, 2002).

2.7.2 Cardiovascular Disease and Oxidative Stress

Cardiovascular disease is of multifactorial etiology associated with a variety of risk factors for its development including hypercholesterolaemia, hypertension, smoking, diabetes, poor diet, stress and physical inactivity amongst others (Bahorun *et al*, 2006). Further *in vivo* and *ex vivo* studies have provided precious evidence supporting the role of oxidative stress in a number of cardiovascular diseases such as atherosclerosis, ischemia, hypertension, cardiomyopathy, cardiac hypertrophy and congestive heart failure (Bahorun *et al*, 2006; Droge, 2002). Atherosclerosis is now understood to be a chronic inflammatory disease characterized by excess accumulation of monocyte-derived macrophages within the arterial wall (Ross, 1999).

Compelling evidence points to oxidative stress as an important trigger in the complex chain of events leading to and promoting atherosclerosis. The expression of chemotactic factors such as monocyte chemotactic protein-1 (MCP-1) is enhanced by oxidative stress and oxidized low density lipoproteins (LDL). Endothelial expression of vascular cell adhesion molecule-1 (VCAM-1), which is regulated through a redox-sensitive mechanism, promotes the adhesion of monocytes to the endothelium. The

release of macrophage colony-stimulating factor (M-CSF) is also stimulated by modified LDL. Expression of these factors results in the attraction and adhesion of monocytes to the arterial wall and the promotion of their differentiation into tissue macrophages. Exposure to the superoxide ion, a ROS, activates the nuclear factor kappa-B (NF-kappa B) regulatory complex and triggers the transcription of several atherosclerosis-related genes (VCAM-1, MCP-1, tumor necrosis factor (TNF), matrix metalloproteinase (MMP)-9 and procoagulant tissue factor (Bourcier *et al*., 1997).

This series of events leads to the accumulation of macrophages in the arterial wall, which then avidly incorporate oxidized LDL to form foam cells. Oxidized LDL, in turn, stimulates the release of interleukin-1 from macrophages (Bourcier *et al*., 1997). The activity of MMPs is also regulated by oxidative stress and appears to be closely linked to smooth muscle cell activation and migration. MMPs have also been implicated in the physiopathology of plaque rupture. Furthermore, ROS can lead to platelet activation and thrombus formation. Therefore, oxidative stress appears to be important in both the early and later stages of the atherosclerotic process.

2.7.3 Neurological Disease and Oxidative Stress

Oxidative stress has been investigated in neurological diseases including Alzheimer's disease, Parkinson's disease, multiple sclerosis, amyotrophic lateral sclerosis (ALS), memory loss, depression (Butterfield, 2002). In a disease such as Alzheimer's, numerous experimental and clinical studies have demonstrated that oxidative damage plays a key role in the loss of neurons and the progression to dementia (Christen, 2000). Patients suffering from Alzheimer disease may be unaware of these cognitive changes. The brain is especially vulnerable to oxidative damage in several aspects due to the fact that it has a high rate of oxidative metabolism, contains elevated concentrations of readily-oxidized polyunsaturated fatty acids, relatively low

level of antioxidants and consists of post–mitotic cells. The production of ß-amyloid, a toxic peptide often found present in Alzheimer's patients' brain, is due to oxidative stress and plays an important role in the neurodegenerative processes (Butterfield, 2002).

2.8 Antioxidant

Antioxidant is associated with the prevention of oxidative damages thus promoting human health in general (Wang & Zeng, 2001). Many naturally derived antioxidants compounds have gained the recognition as free radical and/or ROS scavengers (Gulcin *et al*., 2004). Antioxidants are effective because they are willing to give up their own electrons to free radicals. When a free radical gains the electron from an antioxidant it no longer needs to attack the cellular macromolecules and the chain reaction of oxidation is broken. After donating an electron an antioxidant becomes a free radical by definition. Antioxidants in this state are not harmful because they have the ability to accommodate the change in electrons without becoming reactive.

Antioxidants can be classified as endogenous and exogenous antioxidant based on their sources. The human body naturally produces endogenous antioxidants and these are considered as enzymatic antioxidants. These antioxidants are catalyst, efficiently recycled and small amount is sufficient for protection (Diplock *et al*., 1998). Some examples of endogenous antioxidants are superoxide dismutase (SOD), glutathione peroxidase (GSH.Px) and catalase (CAT).

2.8.1 Endogenous antioxidants

2.8.1.1 Superoxide Dismutase (SOD)

SOD is an endogenously produced intracellular enzyme present in essentially every cell in the body. As an enzyme, SOD has particular value as an antioxidant that can help to protect against cell destruction. It has the distinct ability to neutralize

superoxide, one of the most damaging free radical substances in nature into oxygen and hydrogen superoxide. They are an important antioxidant defense in nearly all cells exposed to oxygen. There are three major families of SOD, depending on the metal cofactor: Cu/Zn (which binds both copper and zinc), Fe and Mn types (which bind either iron or manganese), and the Ni type, which binds nickel.

$$2O_2^{o-} + 2H+ \rightarrow H_2O_2 + O_2$$

2.8.1.2 Glutathione peroxidase (GSH.Px)

The GSH.Px family of enzymes catalyze certain reactions that remove reactive oxygen species such as hydrogen peroxide and organic hydroperoxides. Glutathione exists in reduced (GSH) and as such, the enzyme itself is oxidized into glutathione disulfide (GSSG). In the reduced state, the thiol group of cysteine is able to donate a reducing equivalent ($H^+ + e^-$) to other unstable molecules such as reactive oxygen species. In donating an electron, glutathione itself becomes reactive, but readily reacts with another reactive glutathione to form GSSG. GSH.Px is renewed to the antioxidant network in the human body when GSSG is reduced by NADPH-dependent glutathione reductase (Serafini, 2006).

$$2GSH + H_2O_2 \text{---- GSH.Px} \rightarrow GSSG + 2H_2O$$

2.8.1.3 Catalase (CAT)

CAT is a common enzyme found in nearly all living organisms that are exposed to oxygen, where it functions to catalyze the decomposition of hydrogen peroxide to water and oxygen (Chelikani *et al*., 2004). Hydrogen peroxide is a harmful by-product of many normal metabolic processes: to prevent damage, it must be quickly converted into other less dangerous substances. Catalase is usually located in a cellular

organelle called the peroxisome (Albert *et al*., 2002). The reaction of catalase in the decomposition of hydrogen peroxide is:

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

2.8.2 Exogenous Antioxidants

Exogenous antioxidants are primarily derived from our diet and these substances can act as free radical scavengers and/or quenchers, reducing agent and metal chelators (Mathew & Abraham, 2006). Vitamins such as C and E are crucial antioxidants that help fight oxidative stress and also prevent cancer. Vitamin C acts as a cytotoxic agent to several cancer cell lines and also is an efficient free radical scavenger (Reddy *et al*., 2003). Alternatively, vitamin E is the most important lipid-soluble chain breaking antioxidant in mammalian cells. Other examples of exogenous antioxidants are the phenolic compounds, carotenoids and catechins.

2.8.2.1 Phenolic compounds

Phenolic compounds are vital substances that possess the ability to protect the body from damage caused by free radical induced oxidative stress (Souri *et al*., 2004). Antioxidant action of phenolic compounds is due to their high tendency to chelate metals. Phenolics possess hydroxyl and carboxyl groups, able to bind particularly iron and copper (Jung *et al*., 2003). They may inactivate iron ions by chelating and additionally suppressing the superoxide-driven Fenton reaction, which is believed to be the most important source of ROS. Phenolics (especially flavonoids) are able to alter peroxidation kinetics by modifying the lipid packing order. They stabilize membranes by decreasing membrane fluidity (in a concentration-dependent manner) and hinder the diffusion of free radicals and restrict peroxidative reaction (Blokhina *et al*., 2003).

2.8.2.2 Carotenoids

Carotenoids are a group of red, orange and yellow pigments found in plant foods, particularly fruits and vegetables. Carotenoids have many physiological functions. Their electron-rich conjugated double-bond system is highly reactive towards oxidizing agents and free radicals. Some carotenoids like β-carotene act as a precursor of vitamin A. β-carotene is an effective antioxidant as it is one of the most powerful singlet oxygen quenchers. It can dissipate the energy of singlet oxygen, thus preventing this active molecule from generating free radicals. Its other antioxidant properties include the scavenging of free radicals. Unlike other nutrient antioxidants, β -carotene is efficient at low oxygen pressure. Besides β-carotene, other important dietary carotenoids include a-carotene, lycopene, lutein, zeaxanthin and β-cryptoxanthin. Recent studies have confirmed that lycopene has a powerful role as an antioxidant. This gives carotenoids the ability to protect against oxidative damage and they enhance the vertebrate immune system. Epidemiological studies have shown that people with high β-carotene intake and high plasma levels of β-carotene have a significantly reduced risk of lung cancer. (Britton *et al*., 2008).

2.8.2.3 Catechins

Catechins are some examples of exogenous antioxidants that are obtained through food diet since the human body cannot produce its own vitamin source. Scientifically, the catechin is a bioflavonoid (phyto-chemical 3,3', 4', 5,7 – flavanpentol) found in various plants. Bioflavonoids are a group of chemical substances found in many plants that help keep the cell walls of small blood vessels permeable. It is known that catechin stabilizes collagen; it prevents capillary fragility and abnormal permeability. Catechins are potent metal ion chelators, reactive species trappers and also good donors for hydrogen bonding (Hou *et al*., 2005). The principal catechin found in

plants are epicatechin (EC) epigallocatechin (EGC), epicatechin gallate (ECG), and epigallocatechin gallate (EGCG), EGCG is the most potent catechin (Kuzuhara *et al*., 2008).

2.8.3 Mechanism of Antioxidants Defence

There are three series of line defence mechanism for the antioxidant defence (Noguchi & Niki, 1999). The mechanism of antioxidant defence mechanism can be categorized into first, second and third. In first line antioxidant defence, an antioxidant is introduced to suppress the formation of free radicals. By introducing antioxidant at the initiation stage of free radicals, further oxidative damages can be hindered. For instance, under oxidative stress state, an atom or molecule may break and form free radicals. These free radicals if not stopped will result in further damage to the cell or tissue in the body. Antioxidants involved in this mechanism defence are called preventive antioxidants (Vimala, 2008).

The second line of antioxidant defence involves scavenging of free radicals and also inhibition of chain initiation and propagation of a chain reaction. These antioxidants are known as radical scavenging antioxidants and usually smaller in size. The antioxidants are quickly renewed into the system again and scavenge other free radicals (Vimala, 2008).

Final stage of the antioxidants defence mechanism will terminate the radical chain reaction. The objective is to repair and arrest the oxidative damages that may cause the degeneration of vitamins, enzymes and interruption of the *de novo* pathways. *De novo* pathways form biomolecules from simple precursor molecules in the body which may act as antioxidant and fight against oxidative damages. At this stage the antioxidants are usually large molecules like enzymes and vitamins. These antioxidants sacrifice their own electrons to compensate the unstable radicals by quenching the free

radicals' need to 'steal' electrons from other molecules to stabilize themselves (Vimala, 2008).

2.8.4 Antioxidant Assays

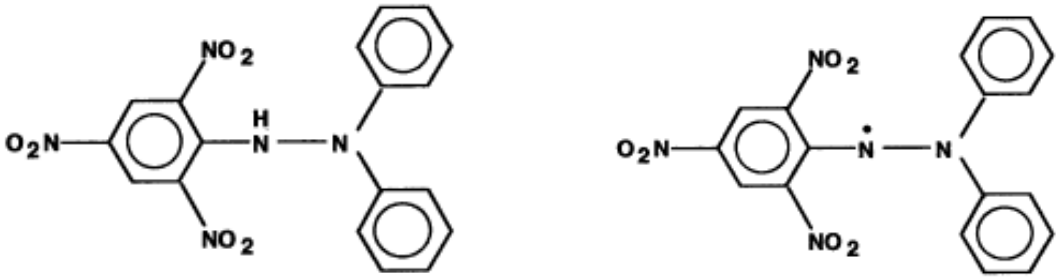
In recent years, numerous methods have been developed to evaluate antioxidant activity of foods, serum and other biological fluids. The bioassay methods are preferred to be simple, precise in determining the effective activity of a single compound or of a complex crude extract (Almela *et al*., 2006). The usage of more than one method is recommended because different methods of evaluation address different mechanism of action (Aruoma, 2003).

2.8.4.1 Diphenyl picrylhydrazyl (DPPH) Radical Scavenging Assay

The diphenyl picrylhydrazyl (DPPH) radical scavenging assay is a rapid, simple and inexpensive method to measure antioxidant capacity of a sample. It involves the use free radical, 2,2-diphenyl-1-picrylhydrazyl or sometimes known as α,α–diphenyl-β-picrylhydrazyl (DPPH). The molecular formula of DPPH is $C_{18}H_{12}N_5O_6$ and its molar mass is 384.33 g/mol. The DPPH assay is widely used to test the ability of compounds to act as free radical scavengers or hydrogen donors. Scavenging of DPPH by antioxidant is due to their hydrogen-donating ability (Singh & Rajini, 2003). The DPPH method can be used for solid or liquid samples and is not specific to any particular antioxidant component but applies to the overall antioxidant capacity of the sample.

The assay is relatively easy since the radical is stable and does not have to be generated. It is convenient, accurate and reproducible antioxidant assay for both natural and synthetic antioxidants. The reaction of specific compounds in extracts, with the DPPH in a methanol solution is measured at ambient temperature thus, eliminates the

risk of thermal degradation of the molecules tested. The stable DPPH radical exhibits a deep violet colour due to its spare electron that is delocalized over the whole molecule. Due to its odd electron, the radical is paragmagnetic and becomes stable and diamagnetic when it accepts an electron or hydrogen radical (Figure 2.2). This yields the reduced form with the loss of the deep violet colour, occasionally giving a residual pale yellow colour due to the picryl group (Molyneux, 2004).



1: Diphenylpicrylhydrazyl (free radical) **2: Diphenylpicrylhydrazine (nonradical)**

Figure 2.2 Structure of the DPPH (1) free radical form and (2) non radical form.

The odd electron in the DPPH free radical gives a strong absorption band at 515 nm, which disappears upon reduction by an antioxidant. However, the reaction mechanism between the antioxidant and DPPH depends on the structural conformation of the antioxidant. The interpretation of the results from the DPPH method is in the form of "efficient concentration" or EC_{50} value (otherwise called the IC_{50} value). EC_{50} value (mg extract/ml) is the effective concentration at which DPPH radicals were scavenged by 50% and is obtained by extrapolation from the dose-response curve by linear regression analysis.

Ascorbic acid normally used as the positive control for the DPPH assay and is a reducing agent that can reduce and thereby neutralize reactive oxygen species such as

hydrogen peroxide. Ascorbic acid can be easily oxidized and destroyed by heat, light, metal and oxygen. For that reason, ascorbic acid solutions must be prepared fresh before use and wrapped with aluminium foil to avoid light exposure. It must be stored in the dark and cold environment at 4^{o}C in a non-metal container.

2.8.4.2 Reducing Power Assay

The Ferric Reducing Ability of Plasma (FRAP) assay is another method for assessing antioxidant power. It was developed by Benzie and Strain in 1996. FRAP assay measures the ferric-to-ferrous ion reduction in the presence of antioxidant. The FRAP assay gives fast and reproducible results. The presence of reductants (antioxidants) in the test sample would result in the reduction of the Fe^{3+}/ferriccyanide complex to the ferrous (Fe^{2+}) form.

The Fe^{2+} can therefore be monitored by measuring the formation of Pearl's Prussian blue at 700 nm. To determine the difference in absorbance, the change in absorbance measurement from the blank to that of the final measurement is calculated and then related to the change in absorbance of a standard iron (II) solution that is tested in parallel. This Fe^{3+}/ferriccyanide system provides a sensitive 'semi-quantitative' determination of diluted polyphenolics in a redox reaction (Amarowicz *et al*., 2004). It can also serve as a significant indicator of the potential of an extract as an antioxidant (Gulcin, 2006a) and has been frequently used in the assessment of antioxidant capacity of various vegetables, plants and other natural products.

2.8.4.3 Metal Chelating Assay

Chelation is a chemical combination with a metal in complexes in which the metal is part of a ring. Organic ligand is called chelator or chelating agent, the chelate is a metal complex. The larger the number of ring closures to a metal atom the more

stable the compound is. This phenomenon is called the chelate effect; it is generally attributed to an increase in the thermodynamic quantity called entropy that accompanies chelation. The stability of a chelate is also related to the number of atoms in the chelate ring (Lippard & Berg, 1994).

Chelating agents can be defined as organic compounds which complex or sequester metal ions. Chelating agents offers a wide range of sequestrants to control metal ions in aqueous systems. By forming stable water-soluble complexes with multivalent metal ions, chelating agents prevent undesired interaction by blocking normal reactivity of metal ions. The chelating agent removes a metallic ion from a solid salt and holds it in solution. By forming a soluble complex from an insoluble compound it is possible to remove unwanted material, washing it away with water. The chelating agent or ligand must have at least two functional groups, bidentate, capable of bonding to the metal atom. In this bond the ligand is an electron-pair donor and the metal an electron-pair acceptor, this is known as co-ordination bonding. The functional groups can be acidic or basic. An acidic group loses a proton (Burgess, 1991).

Chelating reactions work fastest when both the chelating agent and the metal to be chelated are present in solution (Gordon, 1990). A good method of using chelating agents is to combine them with a reducing agent. The reducing agent will transform the coloured, insoluble ferric iron (Fe^{3+}) into a more soluble, colourless ferrous iron, Fe^{2+}. The most commonly used chelating agent in conservation is EDTA (ethylnene diamino tetra acetic acid). EDTA is non ion-selective and has therefore been used for various treatments such as rust removal, removal of basic lead carbonate, chemical stripping bronzes, salt removal from mural paintings and frescoes, iron and copper stain removal from leather and textiles and the removal of various accretions from archeological ceramics and glass. The structure of EDTA consists of six donor atoms capable of co-ordination to a single metal ion and is therefore sexadentate: two basic groups (amino

parts) and four acidic groups (acetic ends). EDTA forms very stable complexes with most metals, including alkaline earth cations calcium and magnesium and some non-metals (Lippard & Berg, 1994).

2.9 Mushroom

Mushrooms are referred to as macrofungi with distinctive fruiting bodies commonly occurring in fungi of the class Basidiomycetes or Ascomycetes (Miles & Chang, 1997). Fungi are eukaryotes having well defined membrane-bond nuclei with a number of definite chromosomes and, as such, clearly distinguishable from bacteria. They are heterotrophic, requiring organic carbon compounds of varying degrees of complexity that distinguishes them from plants that manufacture their own organic food by photosynthesis. All but a few fungi have well-defined cell walls made up of variously linked polymer of glucose (glucans) of glucosamin (chitosan) and N-acetylglucosamine (chitin) through which all their nutrients must pass in a soluble form and, in this respect, they differ from animal cells which lack defined cell walls. Because of these peculiar characteristics, fungi have been separated from the plant kingdom and placed under the kingdom Mycota or Kingdom of Fungi (Dube, 1978).

Mushroom producing fungi are composed of long cellular threadlike structures called hyphae which form strands or a web of tissue in the substrate upon which the fungus feeds under the proper conditions such as combination of temperature, relative humidity, carbon dioxide levels. This tangle of hyphae is termed mycelium. It is biologically important for absorption of nutrients, metabolism and energy production. They accumulate nutrients from the substrate (soil for plants) and colonize substrate. Most often, these microscopic threads are buried in the soil, around the roots of the trees, beneath leaf litter in the tissues of a tree trunk or in some other nourishing substrate and are almost invisible to the naked eye.

The mushroom releases millions of spores, which function like seeds to plants. Mycelia are filamentous and generally unseen with the naked eye. Germinated hyphae form primary mycelia, and then secondary mycelia through plasmogamy (hyphal fusion). When stimulated by temperature, humidity etc, the mycelial colony forms pins under certain conditions and grow to fruitbodies (fruits for plants) (Alexopoulos *et al.,* 1996). Young fruitbodies are called pins (buds for plants). Pins differentiate into cap and stem forming fruitbodies. Under the cap, spores are produced in the gills. Fruitbodies release spores in order to produce the next generation. The fungi responsible for producing such grand fruiting bodies belong to two of the large classes of fungi, Basidiomycetes and Ascomycetes.

2.10 Life Cycle of Mushroom

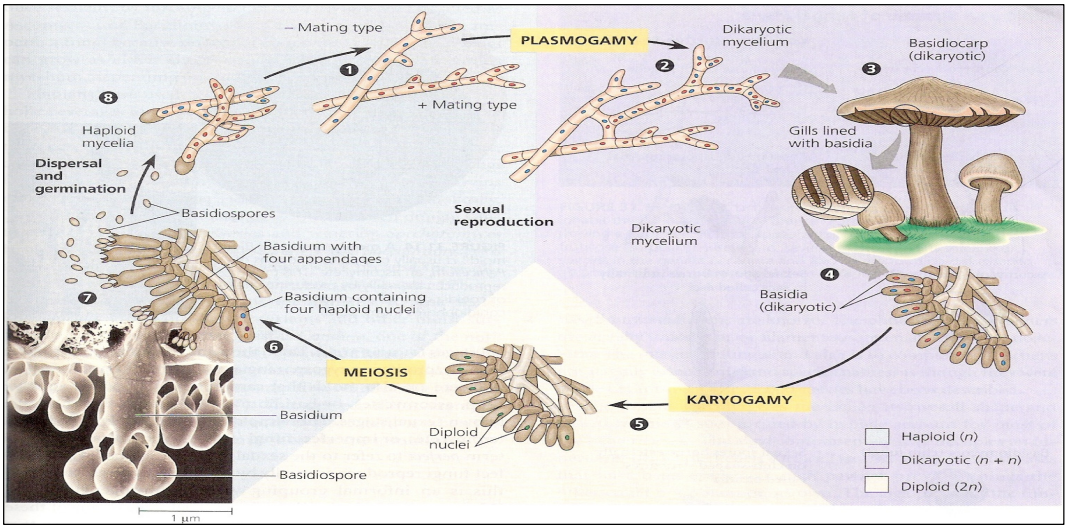
The life cycle of a mushroom can be divided into three essential phases: basidiospore, vegetative mycelium and the fruiting body (Alexopoulos *et al.,* 1996; Kaul, 1997). The life cycle of a club fungus usually includes a long-lived dikaryotic mycelium (Figure 2.2). Periodically, in response to environmental stimuli, this mycelium reproduces sexually by producing elaborate fruiting bodies bodies of various types called basidiocarps (Gr. *basidion* = small base, basidium + *karpos* = fruit). There are many types of basidiocarps, but the commonly known is the hymenial layer made up of hyphae upon which the basidia are produced.

These basidia are commoly club-shaped, terminal portions of hyphae in which compatible nuclei they are the results of plasmogamy, karyogamy, and meiosis, the last two of these processes occur in the basidium, usually four basidiospores typically are produced on each basidium. The basidiospores of most species are balistospores and are discharged forcibly from their basidia by means of an elaborate discharge mechanism.

Basidiospores will typically germinate into monokaryotic mycelium (in which each nucleus is genetically identical).

Basidiospores come in a variety of sizes, shapes and colours and may be either thin or thick walled and smooth or ornamented. In order to form a mycelium, the monokaryotic mycelium must fuse with a mating-type compatible monokaryotic mycelium and form what is called dikaryotic mycelium (two nuclei per cell) (Figure 2.3). The dikaryotic mycelium is the long-lived vegetative stage where the organism gathers nutrients for growth, development and eventually for fruiting body production (Alexopoulos *et al.,* 1996). The fruiting body (basidiome or mushroom) is the component of the life cycle used in classification.

There are various environmental factors associated with fruiting, physical, chemical and biological components in fungi development. Chemical factors include substrate composition, nitrogen, pH and mineral availability, whereas the biological components consist of the interaction between the fungi and other organisms. The physical components include moisture, light, temperature and aeration (Alexopoulos *et al.,* 1996). Asexual reproduction in Basidiomycetes is much less common than in Ascomycetes.



(Source: Alexopoulous *et al*., 1996)

Figure 2.3 The life cycle of a mushroom-forming Basidiomycete

2.10.1 Ecology of *Marasmius*

The diversity of macrofungi in particular *Marasmius* species in Malaysia is poorly known. The genus was first established by Elis Magnus Fries in 1835. More than 1700 epithets have been published to date in *Marasmius* although it is suspected that these represent only about 500 – 600 species worldwide (Desjardin pers. Comm.). The diversity of *Marasmius* is found to be strongly correlated with the diversity of plants in the habitats where they occur, with a higher diversity in tropical areas and fewer species in temperate areas (Lodge *et al.,* 1995). They are wood and litter-inhabiting fungi that colonized leaf litter, twigs, trapped debris, lianas and standing dead wood (Hedger *et al*., 1993).

As *Marasmius* are saprotrophic they serve many important ecological roles in wood and litter decomposition, nutrient cycling, soil genesis and as a food source for myriad molluscs, small vertebrates and arthropods (Dedeyan *et al*., 2000). Favourable temperature and humidity conditions in Asian tropical forests support a wide diversity of *Marasmius* species.

There are about 300 species of agarics in the genus *Marasmius* (family Marasmiaceae), which contains a few edible species. Most *Marasmius* species form small to medium-sized and thin fleshed basidiocarps with thin stalks. Their texture is usually tough and persistent and many are capable of reviving after desiccation, a condition termed marcescent. This allows them to survive the periodic desiccation of the upper litter layers and continue sporulation for up to three weeks (Gilliam, 1975). The dried basidiocarps usually can be revived in water. They cover the forest floor in great numbers. Right after or during rain *Marasmius* species are often seen to spring up from leaves in droves when in fact they were already there, shriveled up and inconspicuous. Among the *Maramius* species, only a few are worth eating. Most are too small or have little flavor.

2.10.2 Taxonomy of *Marasmius*

The genus *Marasmius* belongs to the Basidiomycota, order Agaricales where it was traditionally placed in the large family Tricholomataceae (Singer, 1986). The latest molecular phylogenetic analyses recognize the genus *Marasmius* in its own family, the Marasmiaceae, where it is the sister of the genera *Crinipellis*, *Chaetocalathus* and the newly established genus *Moniliophthora* (Moncavo *et al*., 2002; Wilson & Desjardin, 2005; Matheny *et al*., 2006). Basidiomes of saprotrophic, litter decomposing species of *Marasmius* are commonly encountered throughout Southeast Asia. Although a concerted effort has been focused on the taxonomy and distribution of *Marasmius* from Malaysia species of this diverse and widespread genus have gone mostly undocumented (Corner, 1996).

Marasmius taxonomic macromorphological features include pileus colour and surface features, lamellae attachment, spacing and edge colours, stipe colour, surface ornamentation and basal attachment. As reported by Antonin & Noordeloos in 1993, the main characters that distinguished *Marasmius* from genera of mushrooms are "basidiocarps tough, dry persistent, not withering nor putrescent, reviving after being re-moistened; stipe horny or cartilaginous; lamellae tough, subdistant, with entire sharp edge".

The typical fruitbody of basidiomycetous mushrooms is organized into pileus (or cap), lamellae (or gills) and stipe (or stem). *Marasmius* ornamentation of the pileus is smooth or wrinkled or appearing velvety to pruinose. The majority of pilei are striate to plicate but some are smooth or venose. Pilei are often brightly coloured, ranging from yellow to orange, red or brilliant purple although the majority of species range from white or cream-coloured to various shades of brown (Antonin & Noordeloos, 1993; Desjardin *et al*., 2000). The number of lamellae and presence or absence of lamellulae are important diagnostic characters. Some species form poorly developed lamellae that

are vein-like, whereas others may be intervenose but rarely approaching poroid. The lamellae range in colour from white to cream with an edge that is with the sides or concolorous with the pileus surface.

Most species have a well-developed central stipe, although a few species form poorly developed eccentric to lateral stipes or no stipes at all. Stipes are usually filiform to narrowly cylindrical with surface ornamentation ranging from glabrous to pruinose or hispidulous. In many *Marasmius*, the stipe base is inserted cleanly at the substrate without obvious mycelium, termed insititious whereas in others the stipe base is associated with obvious strigose mycelial hairs and is termed non-insititious (all other sections). Many species form coarse, black, wiry rhizomorphs and absorptive structures that bind litter while others are putative plant pathogens causing horse hair blights (Desjardin *et al*., 2000).

Micromorphological features are paramount in the taxanomy of *Maramius.* The pileipellis is the uppermost layer of cell forming the surface of pileus and is an important character in *Marasmius* taxanomy. The pileus is usually small, convex, umbilicate, striate to plicate, often with a papilla located in the umbilicus, with collariate lamellae and typically a wiry, dark brown to black institious stipe. In *Marasmius*, the pileipellis is a hymeniform layer of erect cells that are either smooth and elevate (sect. *Globulares*) or with cells called broom cells with various types of finger-like or wart-like appendages (other sections). The broom cells may also occur on the edges of the lamellae (cheilocystidia) and stipe (caulocystidia). Other distinctive refractive, sterile cells may occur on the sides of the lamellae (pleurocystidia). Pileus trama and lamellae trama are the hyphal tissues that form the bulk of the pileus and lamellae (Desjardin *et al*., 2000).

2.11 Medicinal Properties of Mushrooms

Although the biggest use of mushrooms has traditionally been used for reasons of their gastronomic and nutritional appeal**,** there has been a great upsurge in activities concerning with the use of mushroom products for medicinal purposes. This interest increased greatly in the 1970's and much research was done to determine the validity of claims of medicinal properties and the nature of the action of compounds present in these mushrooms. In the east (China, Japan, Korea and Russia), the traditional knowledge about medicinal properties of mushrooms is most notable, where the application of mushrooms to maintain health was formally recorded as early as 100AD in China (Cimerman, 1999; Wasser *et al*., 2000). Mushrooms like reishi [*Ganoderma lucidium* (Curt.:Fr.) P.Karst.], shitake [*Lentinula edodes* (Berk.) Sing.], chaga [*Inonotus obliquus* (Pers.:Fr.) Bond. Et Sing.] and others have been collected, cultivated and used for millenia.

The major medicinal properties attributed to mushrooms include anticancer activity, antibiotic activity, antioxidant activity, antiviral activity and immune response–stimulating effects. It has also being confirmed that mushrooms have effective substances for decreasing blood cholesterol and could have hypolipidemic, antithrombotic, hypotensive activities, as well as other applications (Wasser & Weis, 1999c). Extracts from the mushrooms or mycelium are obtained that the value of mushrooms as medicinals can be scientifically determined. Compounds derived from aqueous extracts or from organic solvents can be tested in the laboratory against microorganisms, cells, tissues or tumours for various physiological or biochemical effects. They can be used in animal studies to determine whether they are effective against certain ailments present or induced in the experimental animals.

Extensive clinical studies, primarily in Japan, have clearly demonstrated that a number of species have medicinal and therapeutic value, by injection or oral

administration, in the prevention and treatment of cancer, viral diseases (influenza, polio), hypercholesterolemia, blood platelet aggregation and hypertension (Breene, 1990). Several mushroom species belonging to the *Polyporus* family, *Tricholomus, Agaricus*, are now being regarded as the next candidate producers of medicines (Mizuno & Zhuang, 1995). Finally, clinical tests can be made to determine if the mushroom nutriceuticals is useful in the treatment of diseases of humans. The application of modern analytical techniques can be used to establish a scientific basis for the empirical observations that may have been made centuries before.

There has been renewed interest in the genus *Marasmius* (Desjardin & Ovrebo, 2006) since they have also been found to produce metabolites that have cytotoxic and anti-microbial properties. A preliminary study done on the ethanolic mycelium extracts of *Marasmius purpureostriatus* showed an inhibitory antimicrobial activity against *Staphylococcus aureus, B. cereus, Bacillus subtilis, Escherichia coli, Proteus vulgaris, Pseudomonas aeruginosa, Candida albicans* and *C. parapsilosis* (Yee Shin, 2007). *Maramius oreades* was found to have anti-microbial activity against Gram-positive bacteria such as *Bacillus* and *Enterococcus* spp. (Suay *et al.,* 2000). Another study done has found that extracts of mycelium *Marasmius berambutanus* and *Marasmius leucorotalis* have inhibitory activity against *Schizocsaaharomyces pombe* and *C. albicans* (Yee Shin, 2007).

2.11.1 Anticancer Properties of Mushroom

The medicinal property for which mushrooms have been most extensively investigated is for anti-tumour activity. The involvement and importance of polysaccharides in tumour and cancer treatment were first recognized more than 100 years ago when it was found that certain polysaccharides could induce complete remission in patients with cancer. Ever since anti-tumour activity of macrofungal

poysaccharides was first published by Chihara in the 1960s (Chihara, 1969), the study of the anticancer potential of Basidiomycetes appears to be very promising.

The majority of anti-tumour compounds extracted from mushrooms have been found to be polysaccaharides with strong anti-tumour activity (Kaul, 2002). Unlike traditional antitumour drugs, these substances produce an anti-tumour effect by activating various immune responses in the host and cause no harm to the body (Wasser & Weis, 1999). Extensive studies have been made with a polysaccharide called lentinan, which was originally obtained from hot water extracts of *Lentinula edodes* fruiting bodies. The polysaccharide completely inhibited the growth of sarcoma 180 in mice. Chihara (1993) suggests that lentinan operates as a host defence potentiator due to its ability to augment the host's response by maturation, differentiation and proliferation of lymphoid cells and other cells important in host defence mechanisms. T-lymphocytes are responsible for cellular immunity and lentinan stimulates T-lymphocytes production which is normally suppressed in the presence of cancer cells.

Lentinan also leads to an increase in interleukin-1 (IL-1), which acts on leucocytes to activate the immune system. Thus lentinan leads to maturation, differentiation and proliferation of immunologically important cells of the host defence mechanism. As a result of these, there is an enhancement of the host's capacity to respond to lymphokines such as interleukin-2. Lentinan potentiates the induction of different types of anti-tumour effector cells such as killer T-cells, NK cells and cytotoxic macrophages. Since then, scientists have subsequently isolated anti-tumour mushroom polysaccharides from *Ganoderma lucidum* (Lin *et al*., 2004), *Poria cocos* (Jin *et al*., 2003), *Lentinus edodes* (Cheung, 2008).

A metabolite from the surface culture of *Marasmius conigenus* (Pers.: Fr.) P. Karst. (now accepted as a synonym of *Baeospora myosura* (Fr.) Singer) known as marasmic acid was found to have the anti-microbial and cytotoxic activity (Kupka *et al.,*

1983). This sesquiterpenoid inhibited the ascetic form of Ehrlich carcinoma RNA and DNA syntheses (Kupka *et al.,* 1983). Kavanagh *et al.,* (1949) reported marasmius acid an anti-bacterial substance produced by *Marasmius conigenus* has antibacterial and anti-fungal activity against *S. aureus* and *E. coli*. Fabian *et al.,* (1999) discovered that sesquiterpene isolated from *Marasmius* species collected from tropical regions exhibited strong cytotoxicity to different cell lines. In addition to that, *Marasmius* sp. also contains anti-tumour or immunomodulating polysaccharides that were active against sarcoma 180 solid cancer cells and Ehrlich solid cancer cell (Wasser, 2002).

Bioactive polysaccharides can be isolated from mycelium, the fruiting body, and sclerotium which represent three different forms of a macrofungi in the life cycle. Lentinan from *Lentinus edodes*, schizophyllan from *Schizophyllum commune* and polysaccharide-peptide (PSP) and protein-bound polysaccharide (PSK) from *Trametes versicolor* are in clinical use especially in Japan and China, for the adjuvant tumour therapy (immunotherapy) (Ooi & Liu, 2000;Cui & Chisti, 2003) in addition to the major cancer therapies like surgery, radiotherapy and chemotherapy. Application of lentinan (parenteral) in addition to chemotherapy lead to prolongation of survival time, restoration of immunological parameters and improvement in life quality in patients with stomach cancer, colon cancer and other carcinomas in comparison to patients who had chemotherapy alone (Hazama *et al.,* 1995).

PSK and PSP are used clinically for immunotherapy in addition to the major cancer therapies like surgery, radiotherapy and chemotherapy and have been developed as pharmaceuticals in Japan and are now commercially available worldwide. PSK was commercialized by Kureha Chemicals, Japan. After extensive clinical trials, PSK was approved for use in Japan in 1977, and by 1985, it ranked 19th on the list of the world's commercially most successful drugs (Yang *et al*., 1992). About 10 years after PSK, PSP appeared on the market. Both compounds have been isolated from *Coriolus versicolor*.

In addition to clinically tested PSK and PSP, numerous other extract preparations of *Coriolus versicolor* are on the market as neutraceuticals and traditional medicines. Neutraceutical PSP preparations are sold worldwide in the form of capsules, ground biomass tablets, syrups, food additives, and teas (Cui & Chisti, 2003).

Medicinal mushrooms produce beneficial effects not only as drugs but also as a novel class of products by a variety of names: dietary supplements, functional foods, nutriceuticals, mycopharmaceuticals, and designer foods that produce healthy benefits through everyday use as part of a healthy diet (Chang 2006; Wasser & Akavia 2008). A mushroom nutriceutical is a refined, or partially refined, extract or dried biomass from either mycelium or the fruiting body of a mushroom, which is consumed in the form of capsules or tablets as a dietary supplement and has potentially therapeutic applications. Regular intake may enhance the immune response of the human body thereby increasing resistance to disease and in some cases causing regression of the disease state. Thus, acting as immunopotentiators, medicinal mushroom preparations modify host biological responses (also known as BRMs).

By 1990, about 80% of drugs were either natural products or analogs inspired by them. “Blockbuster drugs” like antibiotics (penicillin, tetracycline, and erythromycin), antiparasitics (avermectin), antimalarials (quinine, artemisinin), lipid control agents (lovastatin and analogs), immunosuppressant for organ transplants (cyclosporin, rapamycins), and anticancer drugs (taxol, doxorubicin) revolutionized medicine (Li & Vederas 2009). Many of the above mentioned drugs were discovered from components found in fungi.

2.11.2 Antioxidant Properties of Mushrooms

In recent years, the use of some synthetic antioxidants has been restricted because of their possible toxic and carcinogenic effects. This concern has resulted in an

increased interest in the investigation of the effectiveness of naturally occurring compounds with antioxidant properties. Foods rich in antioxidants have been shown to play an essential role in the prevention of diseases among which are cardiovascular diseases, neurodegenerative diseases, Parkinson's and Alzheimer's diseases (Di Matteo & Esposito, 2003), inflammation and problems caused by cell and cutaneous aging (Ames *et al*., 1993). Natural antioxidants are being extensively studied for their capacity to protect organisms and cells from damage brought on by oxidative stress, the latter being considered a cause of ageing and degenerative diseases (Cazzi *et al.,* 1997).

Mushrooms are a very good source of antioxidants. In view of this fact they are evaluated for their medicinal properties and potential benefits. In a study done by Lim (2004), *Marasmius ruforotula, Marasmius guyanensis* and *Marasmius berambutanus* were also found to have antioxidant properties with over 50 % scavenging activity of the DPPH radical at a concentration of 12 – 21 mg/ml, 10 – 32 mg/ml and 22 – 28 mg/ml respectively compared to positive control of ascorbic acid with 0.078 mg/ml concentration. The total phenolic content for *Marasmius berambutanus*, *Marasmius ruforotula* and *Marasmius guyanensis* ranged from 2 – 28 mg/ml, 10 – 21 mg/ml and 23 – 40 mg/ml, respectively. The phenolic compounds may contribute to the antioxidant potential of the species studied. The natural antioxidants present in mushrooms and other biological materials have attracted considerable interests because of their presumed safety and potential nutritional and therapeutics effects (Amarowicz *et al*., 2004; Sakanaka *et al*., 2004).