2.0 LITERATURE REVIEW

2.1 History of mushroom

Medicinal mushrooms have long and rich history of use. More than 2,000 years ago, Paleolithic people knew and used mushrooms as powerful medicines to fight illness. In the world of pre-world Shaman, spirits caused diseases and medicinal compounds were administrated to appease or treat them. Later in future, the mushroom was used as same tool as a practical treatment for microbial infection.

The medicinal use of mushrooms has a very long tradition in the Asian countries, whereas their use in Western hemisphere has been slightly increasing only since last decades (Lindequisite *et al.,* 2005). In Asia, mushroom in traditional Chinese medicine have seen a summation of more than 3,000 years of use for the prevention and treatment of diseases (Chang, 2006). Historically, medicinal mushroom species harvested from the forest where they grew on dead or living trees and forest litter. In order to function as a medicinal supplement, they were prepared as hot water extracts or concentrated in a powder form.

In addition, according to a study, the market value of medicinal mushrooms and their derivative dietary supplement worldwide was nearly US$1.2 bilion in 1991 (Chang, 1993), US$3.6 billion in 1994 (Chang, 1996), US$6.0 billion in 1999 (Wasser *et al.,* 2000) and US$9.0 billion in 2001 (Chang & Buswell, 2003).

2.2 Mushroom's medicinal values

Mushroom is a macrofungus with a distinctive fruiting body, which can be hypogeous or epigeous, large enough to be seen with unaided eyes. These mushrooms are

grouped under Basidiomycetes, but there are still some species of ascomycetes belongs to mushroom. As a whole, mushrooms constitute at least 14,000 and as many as 22,000 known species. However, the number of mushroom species in earth is estimated to be 140, 000, suggesting that only 10% are known (Lindequisite *et al.,* 2005).

Subsequently, the whole mushroom (fresh fruiting bodies), extracts, and isolated compounds are suitable for the use as medicinal properties. The material of the mushroom can obtained from the wild, cultivation in farms and harvesting via fermentation with liquid or solid substrates (Chang, 1996). Generally, extracts are prepared by extraction of mushrooms with suitable solvents. However, pure compounds could also be obtained by isolation from the natural or cultivated material or by chemical synthesis.

Recent studies showed that, 85% of all medicinal mushrooms products are derived from the fruiting bodies, which have been either commercially farmed or collected from the wild, for example Lentinan and various products from *Ganoderma lucidum*. Only ~ 15% of all products are based on extracts from mycelia (Lindequisite *et al.,* 2005).

Thus prerequisition for use as a drug, nutraceutical or other purpose is the continuous production of mushrooms (fruiting bodies or mycelium) in high amounts and in a standardized quality. Based on Chang's (1996) opinion, mycelial products are 'wave of the future' because they ensure standardized quality and year around production. However, 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 supplement or food should get more attention. Finally, the nutritional value of mushrooms should be taken into account.

2.3 Biological activities of *Auricularia* species

The *Auricularia auricula f. auricula* had potent anticoagulant action in plasma. This mushroom is widely used as a medicinal and food supplement in Korea and China. Methanol extracts of *A. auricula* inhibited lipid peroxidation and decreased liver damage in benzol pyrene-treated mice (Chang *et al.,*1998). In addition, methanol extracts of other edible mushrooms such as *Auricularia mesenterica* have antimutagenic activity (Ham *et al.,*1997). At the same time, the *Auricularia polytricha* , as a source for food and medicinal values, showed antioxidant activity via preventing lipid oxidation, scavenging radicals and chelating metal ions in vitro (Mau *et al.,* 2001). Furthermore, this *A. polytricha* also exhibits antinociceptive characteristics, via obtaining four compounds, which, can reduce acetic acid-induced writhing in mice (Koyama *et al.,* 2002).

2.3.1 *Auricularia auricula-judae* (Fr.Quel)

The order Auriculariales comprises some 50 European species in a single family, the Auriculariaceae (Index Fungorum). There are *Auricularia polytricha*, *Auricularia auricular* and *Auricularia auricula-judae* in the genus Auricularia. The species *Auricularia auricula-judae* belongs to the kingdom Fungi, division Basidiomycetes, class homobasidiomycetes, order Auriculariales, family Auriculariaceae and genus Auricularia (Yuan *et al.,* 1998). *Auricularia auricula-judae* commonly known as judae's ear or jelly ear fungus. This jelly ear fungus is conspicuously ear shaped, ranging from purple to dark brown or black in color with a gelatinous, elastic and rubbery texture. These facultative parasites are mostly found on dead elder trees and on elms.

The fruitbodies of *A. auricula-judae* have been traditionally used both as a food and drug supplement in China. In Japan, the mushroom known as Kikurage has a high content

of indigestible polysaccharides or dietary fiber (Hobbs, 2000). Generally, the fruitbodies consist high content of carbohydrates (approximately 630g kg^{-1} in dried fruitbodies), protein and minerals (Ca, P, and Fe) which, are higher than those in *Tremella* sp (commonly called as white fungus) (Hobbs, 2000). The protein is plentiful with Lysine and Leucine, so the mushroom is acknowledged as a healthy food. Each 100 g of *A. auricula-judae* contains 10.6 g protein, 357 mg calcium, 201 mg phosphorus and 185 mg iron. In addition, it also contains carotene, vitamins B1 and B2, mannan, glucuronic acid, lecithin, and cephalin (Yoon *et al.,* 2003). The fruitbodies of *A. auricula-judae* are rich in polysaccharides. In recent years, *A. auricula-judae* was found to have many biological activities; this compound possesses antioxidant activity, decreases blood sugar and could improve well being (Acharya *et al.,* 2004). Pharmacologically, the polysaccharides have been used as anticoagulant and to lower cholesterol (Yoon *et al.,* 2003). As for the cultivation, the total production of *Auricularia* spp. in 1997 exceeded 485,000 tonne (fresh weight). This value is an increase of 85,000 tonne or 18% over 1990 levels (Chang 1999). Thus, this shows that, the world population of consuming this *Auricularia* spp has increased.

2.3.2 Biological Activities of *A. auricula-judae*

There are reports regarding the hypoglycaemic and hypocholestrolemic effects of *A. auricula-judae*. This can be seen by the study of its effectiveness in reducing the blood glucose levels in KK- Ay mice that are genetically obese and have diabetic syndrome as hyperglycaemic, hyperinsulinemia, glucosuria and several insulin resistance (Yuan *et al.,* 1998). The first study was done on the water-soluble polysaccharide, which was extracted from fruitbodies of *A. auricula-judae*. While the second study was practiced on three

different groups of polysaccharides all found in the fruiting body; a mixture of crude polysaccharides extracts, acidic polysaccharides fraction and neutral polysaccharides fractions (Yuan *et al.,* 1998). Both studies showed that, *A. auricula-judae* have hypoglycaemic effect.

Another study showed the effect of *A. auricula-judae* on rats fed with a hypercholestrolemic diet with or without the addition of *A. auricula-judae* (Cheung 1996). The effect observed after four weeks was a significant decrease in the serum cholesterol concentration (17%) and a decrease in the serum LDL (low-density lipoprotein) by 24%. However, the HDL (high-density lipoprotein) concentration and the total liver cholesterol were not affected. By this study, it is concluded as *A. auricula-judae* showed hypercholestrolemic activity in rats. The function could lead to its use as a preventive for the problem of vascular sclerosis (Cheung, 1996). It is also stated that *A. auricula-judae* possess antitumor, cardiovascular, hypocholesterolemia, antiviral, antibacterial and antiparasitic effects (Wasser *et al.,* 1999).

2.4 Cancer

Cancer is a group of diseases in which cells are aggressive, invasive and metastatic. Aggressive cancer cells grow and divide without respect. Invasive leads to cancer cells, which, invade or destroy adjacent tissues. Therefore, cancer is known as a chronic genetic disease, which has become a leading cause of death in many countries throughout the world. Cancer may affect people at all ages, even fetuses, but for common varieties tends to increase with age (Cancer Research, 2007). The formation of neoplasm occurs due to abnormal proliferation of genetically altered cells. These neoplastic cells have developed a partial autonomy and continue to replicate apparently due to non responsiveness to the

normal growth regulatory influences. The neoplasm may stimulate their own growth by secretion of autocratic growth factors as well as the production of angiogenic factors, which will enhance the blood supply and thus increase nutrient supply to the area (Nagle, 1997).

The neoplastic growth or known as tumor, defined as any mass formation or swelling including those resulting from non-neoplastic causes such as inflammation. Benign neoplasms are known as tumors, which solid neoplasm that has self limiting growth and does not invade other tissues nor metastasize; usually non-cancerous (Jemal *et al.,* 2005). Pre malignancy is a non invasive neoplasm that may not form an obvious mass, but has the potential to progress to cancer if left untreated. Pre malignant neoplasms may show distinctive microscopic changes such as dysplasia or atypia.

The most important characteristics of malignant cells are the abnormalities in genes, which control cellular proliferation and lead to unrestrained growth (Rollins *et al.,* 2000). Studies showed that, two genes namely; protooncogens and tumor suppressor genes regulate the normal cells growth control. The protooncogens has the role of accelerating cell proliferation, while the tumor suppressor genes functions to inhibit or retard cell proliferation (Kleinsmith, 2006).

Oncogenes promote the cell growth via many ways. Many can produce hormones, a "chemical messenger" between cells, which encourage mitosis, the effect of which depends on the signal transduction of the receiving tissues, or cells (Doll and Peto, 1981). Hormone receptor on a recipient cell is stimulated; the signal is conduct from the surface of cell to the cell nucleus to effect some changes in gene transcription regulation. Some oncogenes are a part of signal transduction themselves, therefore controlling the sensitivity to such hormones (Newmark *et al.,* 2001). Quiescent counterpart of oncogenes, protooncogenes can be mutated and this will modify their functions and expression (Jamel *et al.,* 2005).

Due to this process, the protooncogenes become oncogenes which upsets the normal balance of cell cycle and leads to uncontrolled growth (Huang, 2002). However, the chances of cancer cannot be reduced by removing protooncogenes from the genome (Huang, 2002).

Tumor suppressor genes code for anti proliferation signals and proteins that suppress mitosis cell growth. Generally, tumor suppressors are transcription factors that are activated by cellular stress or DNA damage. Often DNA damage will cause the presence of free floating genetic material as other signs, and will trigger enzymes and pathways which lead to the activation of tumor suppressor genes. The p53 protein, one of the ultimately important studied tumor suppressor genes, is a transcription factor activated by many cellular stressors including hypoxia and ultraviolet radiation damage (Newmark *et al.,* 2001). p53 functions as a transcription factor in nucleus and regulates the cell cycle, cell division and apoptosis in cytoplasm. In addition, the p53 has been shown to regulate the shift from the respiratory to the glycolytic pathway (Matoba *et al.,* 2006).

2.4.1 Carcinogenesis

Based on the epidemiological studies, cancers in human populations develop as a multistage process initiation, promotion, conversion, and progression (Sutherland, 1999; Weston and Harris, 2000). Initiation is a process which involves uptake of a carcinogenic agent followed by distribution and transport to organs and tissues where metabolism occurs (Wood *et al.,* 2005). This happens in chemically induced carcinogens. Normally, adduct formation between carcinogens and its derivatives with a nucleotide in DNA detected (Weston and Harris, 2000). For mutation to accumulate, the resulting genetic changes in a damaged cell will be brought forward to offspring during mitosis, giving rise to a clone of

mutated cells to complete initiation event (Kleinsmith, 2006). Initiation is important but not sufficient to lead tumor formation in carcinogenesis. If the surrounding normal cells undergoes terminal differentiation, those mutated cells will be eliminated. In other words, these initiated cells needs selection place to expand its growth and for tumor (Jamel *et al.,* 2005). Furthermore, different carinogens will induce different kind of neoplasm (Table 2.1).

Table 2.1: Carcinogens and associated neoplasm.

Agents	**Neoplasm**
Aflatoxin	Hepatoma of liver
Alcohol	Carcinoma of mouth, pharynx, esophagus and larynx
Alkylating agents	Bladder carcinoma and leukemia
Aromatic amines	Bladder carcinoma
Asbestos	Mesothelioma of pleural surfaces
Benzene	Leukemia
Cadmium	Prostate carcinoma
Estrogens	Carcinoma of endometrium and vagina
Polycyclic Hydrocarbons	Carcinoma of skin and lung
Tobacco smoking	Carcinoma of mouth, pharynx, esophagus, lung bladder and lip
Vinyl chloride	Angiosarcoma of liver
UV light	Squamous carcinoma of skin and lip
Hepatitis viruses	Hepatoma of liver

(Doll and Peto, 1981)

The expansion of initiated cells to build an actively proliferating multi-cellular premalignant tumor cell population is known as tumor promotion. This is the subsequent process from initiation. Promotion refers to increament in the number and/ or size of preneoplastic foci after exposure of a tissue to a tumor promoter. The promotion of tumor

normally equipped with inflammation or increased level of differentiation. The level of differentiation also accompanied by changes in cellular morphology and in expression of certain biochemical markers (Newmark *et al.,* 2001). The tumor promoting agents are compounds, which describes very weak or no carcinogenic activity when tested alone; but eventually enhance the formation of tumor when applied repeatedly following a low or suboptimal dose of an initiating agent (Kleinsmith, 2006). The chemicals or agents which have the ability on performing both tumor initiation and tumor promotion activities are known as complete carcinogens, examples being benzo[o]pyrene, 4-aminobiphenyl, DAB and ditetraamina (Weston and Harris, 2000).

Conversion is a process of transformation in preneoplastic cell into one that contains the malignant phenotype (Weston and Harris, 2000; WHO, 2007). Conversion process of the tumor begins with the invasion of cells outside their normal tissue boundaries into surrounding tissues and changes in the nuclear morphology which often can be observed (Kleinsmith, 2006). This process can be speed up via increasing the rate of cell division and exposure to DNA damaging agents, which can be mediated by the activation of protooncogens and tumor suppressor genes inactivation (Weston and Harris, 2000).

Progression is an irreversible process, which occurs when initiated cells exhibiting increased proliferative with survival potential and thus outgrow other normal cells in the tumor leading to an expansion of clones with more uncontrolled characteristics (Kleinsmith, 2006). The aggressive or uncontrolled characteristics acquired are the capacity to induce angiogenesis, to become independent of surrounding cells. It is also able to destroy biological barriers and escape the immunosurveillance mechanisms. These characteristics allow cells to elude from normal homeostatic mechanisms and process

metastatic potential that enable invasion beyond the immediate location of the primary tumor (Nowell, 1986; Conti, 1997; Weston and Harris, 2000).

2.5 Types of cancer in study

Breast cancer is most common, most feared malignancy in women living in developed countries (WHO, 2007). In developed countries the age standardized incidences rates are around 100/100,000 women with mortality rates about 25/100,000. These rates are up to 5-fold higher than those reported from Asian regions, which have the lower incidence rate of breast cancer (Greenlee *et al.,* 2000).

Colon cancer is one of the commonest cancers in the United States and the Western world. The incidence also recently has increased rapidly in less-developed parts of the world that have adopted Western customs and diet. Colon cancer is the end result of a combination of genetic and environmental changes. The degree to which these two etiologic factors play a role in individual patients with colon cancer varies widely. For example, familial adenomatous polyposis, an autosomal dominant hereditary disease with 90% to 100% penetrance with essentially all affected individuals developing colon adenoma, and later colon cancer , is thought to have almost a 100% genetic basis (Distler *et al.,* 1997).

Ovarian cancer is often asymptomatic in its early stages and symptoms that do occur are often not of the type that would alert most women or their health care providers. Thus, most diagnoses occur at advanced stages of disease, when survival rates are 69 percent for regionally advanced stages and 30 percent for stages with distant metastasis (Women Health Report, 2007).

2.5.1 Cervical cancer

An estimated 11,150 cases of invasive cervical cancer are expected to be diagnosed in the United States in 2007 and 3,670 women are expected to die from the disease (Women Health Report, 2007). Incidence and mortality rates have decreased steadily over the past five decades, largely due to the widespread use of the Pap smear which detects cervical cancer and precancerous lesions (Zur *et al.,* 2000). The Pap smear has made cervical cancer one of the most preventable cancers, but older, poorer, and less educated women are less likely to be screened and screening is not available in many low-resource regions of the world. Worldwide, cervical cancer has a significant impact, with nearly 500,000 new cases and nearly 250,000 deaths reported annually (Women Health Report, 2007).

2.5.2 Human Papillomavirus

Cervical cancer, a world wide health problem has been directly linked to genital infection by Human Papillomavirus (HPV), and HPV DNA. HPVs are mucosal trophic viruses infecting basal squamous epithelial cells, with the productive phase of the viral life cycle elaborated in the upper squamous epithelial cell layers. Most HPV infections are transient but in minority patients persistent viral disease underlies neoplastic progression and emergence of invasive malignancies (Zur *et al.,*1994).

Although oncogenic HPV infections are common and usually clear within 1 to 2 years, infection with certain HPV subtypes is now recognized as the major cause of cervical cancer. A group of approximately 15 HPVs cause virtually all cases of cervical cancer worldwide, with HPV types 16 and 18 accounting for approximately 70 percent of all cases (Women Health Report, 2007). Investigators have identified a possible link between immune response and development of cervical cancer. They showed that

combinations of mutations in two genes associated with immune response, HLA and KIR, may influence a women's susceptibility to developing precancerous lesions after HPV infection (Women Health Report, 2007). Mutation combinations that appeared to confer resistance to lesion development were associated with inhibition of natural killer (NK) cell activity. Combinations that seemed to confer susceptibility were associated with activation of NK cells. Further research is needed to investigate the role of the inflammatory process and NK cells in the development of cervical cancer.

HPV contains two genes in their early region, E6 and E7,and high risk viral types encode E6 and E7 oncoproteins. Several studies support functional role for the E6 and E7 oncogenes in tumorigenesis. Both oncogens are expressed consistently in cervical cancer tissues obtained from patients (Zur *et al.,* 2002). Cell transfection of high risk HPV16 or 18 E6 and E7 oncogens transforms established cell lines and immortalizes primary cells (Barbosa *et al.,* 1989; Howley *et al*.,1989 ; Villa *et al.,*1991). The potential importance of E6 and E7 function in cervical carcinogenesis has also spawned vaccine development targeting viral oncoprotein epitopes (Adams *et al.,* 2001).

2.6 Chemotherapy

Chemotherapy is the treatment of cancer with drugs ("anti cancer drugs") that can kill the cancer cells. The term "chemotherapy" usually refers to cytotoxic drugs which affect rapidly dividing cells generally, in contrast with targeted therapy. Chemotherapy drugs interfere with cell division with various possible ways, e.g. with the duplication with DNA or the separation of newly formed chromosomes (Baron *et al.,* 2006). Most forms of chemotherapy target all rapidly dividing cells and are not specific for cancer cells, although some degree of specificity may come from the inability of many cancer cells to repair

damaged DNA. Hence, chemotherapy has the potential to harm healthy tissues that have a high replacement rate. These intestinal cells usually repairs themselves after chemotherapy. In addition, the treatment of some leukaemias and lymphomas requires the use of high-dose chemotherapy, and total body irradiation (TBI). This treatment ablates the bone marrow, or peripheral blood and reduces body ability to recover and repopulate the blood. Hence, stem cell harvesting for bone marrow and peripheral blood carried out before the ablative part of the therapy, to enable safety after the treatment that given (Baron *et al.,* 2006).

2.6.1 Chemoprevention

Principally chemotherapy distinct from cancer chemoprevention where chemoprevention aims to block the initiation of the carcinogenic process, as well as to inhibit tumor promotion or tumor progression whereas chemotherapy aims to kill the existing cancer cells (Fujiki *et al.,* 1998). Many high quality clinical trials support the use of such chemoprevention in defined circumstances (Vogel *et al.,* 2006). Daily use of tamoxifen, a selective estrogen receptor modulator (SERM), typically for 5 years, has been demonstrated to reduce the risk of developing breast cancer in high risk women about 50% (Chlebowski *et al.,* 2006). A recent study reported that the selective estrogen receptor modulator raloxifene has similar benefits to tamoxifen in preventing breast cancer in high risk women, with a more favorable side effect profile (Vogel *et al.,* 2006)

2.7 *In vitro* Neutral Red Cytotoxicity Assay

The neutral red (NR) cytotoxicity assay involves the uptake of a weak cationic dye, namely neutral red, via non-ionic diffusion into the lysosomes of living cells after the cells were exposed to test compounds in a variety of concentrations. Neutral red dye accumulates intracellularly in lysosomes to bind with anionic sites in the lysosomal matrix of the viable cells. Thus, lysosomal fragility occurs due to the alteration of the cell surface or the sensitive lysosomal membrane and this process is irreversible. This change happens due to the xenobiotics, which can cause such changes by decreasing uptake and binding of NR dye. Based on the criteria, that damaged and dead cells wiil loose their ability to retain NR dye, it is possible to distinguish between viable, damaged, or dead cells using NR assay. Thus, this assay can be applied to determine the cytotoxicity effects of a series of extracts (Borenfreund and Puerner, 1985; Borenfreund and Puerner 1986; Riddell *et al.,* 1986).

Furthermore, lysosomes plays role to extract out the amount of NR dye and was quantitated using spectrophotometer. It is later, compared with NR dye recovered from untreated control cell culture. The results expressed in ED_{50} values that can be obtain from dose response curves. The ED_{50} values are established using different concentrations of each extract tested against each cancer cells. The ED_{50} values points to the effective dose (µg/ml or mmol/L) required to inhibit the proliferation of cell culture by 50% (Chapuis *et al.,* 1988; Liebsch and Spielmann, 1995).

2.8 Immunocytochemistry

Immunocytochemistry staining is a sensitive and specific method that is used specifically to detect and localize viral antigens in human neoplasms using specific labeled

antibodies (IHC, Guide Book,2006). The technique started at 1942 by Coons in order to detect pneumococcal antigens in the livers and the spleens of experimentally infected mice using fluorescein labeled anti-pneumococcal antibody. Subsequently, this immunofluorescence method been applied successfully to detect many different bacterial and viral antigens *in vivo* and *in vitro*. The early immunochemistry approach used polyclonal antibody, which was often used in short supply and sometimes leads to significant variations among many. This problem solved with the introduction of monoclonal antibody, which now plays important role as an immunositological reagent. There are two ways to detect viral antigen. The direct method uses only one virus specific antibody which is directly labeled with an indicator (fluorescein or alkaline phosphatase) and the indirect method which uses two antibodies, one virus-specific antibody (primary antibody) to bind the viral antigen and the second antispecies antibody to be labelled with the indicator , which binds to primary antibody (Lakeman, 1997).

The two-step indirect immunoperoxidase is a method, which is applicated in the immunostaining study. In this study, the test samples are incubated with primary specific antibody against the viral antigen. Then the Horse-Raddish peroxidase (HRP) enzyme is added to form antibody-HRP complex. Then, the test samples are exposed to a 3'-diaminobenzidine tetrahydrochloride (DAB) substrate. Upon reaction with HRP, the DAB substrate forms a nondiffusing and insoluble dark brown precipitate at the site of the viral antigen (Gay and Docherty, 1986; Espinoza *et al.,* 1992; Forghani *et al.,* 1992).

However, there are few important factors, which can majorly affect the staining results. Effective fixation for the antigen must be choosen because it is crucial due to the detection of the antigen cannot be done by immunocytochemistry method if the viral antigen is denatured or altered by the fixative (Gay and Docherty, 1986). Acetone is an excellent preservative of immunoreactive sites and can be used at varying times and

temperatures (5-15 min at room temperature, 12-20 min at 4 ˚C, 3-30 min at -60 ˚C, or -20˚C overnight) (Forghani *et al.,* 1992; Farmilo *et al.,* 1990). The naturally occurring endogenous peroxidase in test samples should be destroyed via pretreatment with hydrogen peroxide (H_2O_2). The endogenous peroxidase activity which will react with the DAB substrate chromogen and lead to false-positive staining should be eliminated prior to incubation with primary antibody (Kleinsmith, 2006).

The sensitivity and specificity of an immunochemistry assay depends on the antibody titer and dilutions, as well as incubation time and temperature. The antibody titer refers to the highest dilution of an antibody that result in maximum specific staining with least amount of background. The antibody titers may vary from 1:100 to 1:2000 for polyclonal antibody; from 1:10 to 1:1000 for monoclonal antibodies in cell culture supernatants; and up to 1:1,000,000 for monoclonal antibodies in ascites fluid (Boenisch, 2001). An ideal antibody should have high titer with high affinity to bind viral antigen and should not react with the host cell or growth medium components (Forghani *et al.,* 1992).

The incubation time, temperature and antibody titers are interdependent; thus a change in any of the factor will affect the others. The incubation time and antibody titer are inversely related where a shorter incubation time is required for optimal results when a higher antibody titer is used. It is recommended, to fix a consistent incubation time for antibody to avoid variations in the overall quality and intensity of staining. The equilibrium in antigen-antibody to avoid reactions can be achieved more quickly at higher temperatures whereby increasing incubation temperature allows for a greater dilution of the antibody or a shorter incubation time (Boenisch, 2001).

In addition, the HRP peroxidase can react with DAB substrate chromogen in the presence of H_2O_2 to yield brown, alcohol-soluble precipitate at the site of the antigen. The HRP peroxidase will first form an enzyme-substrate complex which will then be oxidized

by the electron donor which provides a 'driving' force in the continuing catalysis of H_2O_2 to yield brown, alcohol – soluble precipitate at the site of the antigen. The manipulation of the incubation time and DAB concentration should be made to control the staining intensity over the entire sample. Overstaining will reduce the contrast between stained and unstained areas in the sample and thus may lead to difficulty in the interpretation of the results (Gay and Docherty, 1986; Forghani *et al.,* 1992; Boenisch, 2001).

2.9 Antioxidant activity

Generally the antioxidant effect is closely associated with cancer prevention where carcinogens can be inactivated by antioxidant 'scavengers' (beta carotene, vitamin E, butylated hydroxytoluene) or electrophilic trapping agents (phenols such as ellagic acid) (Bertram *et al.,* 1987). The most commonly used antioxidants in the present time are butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG) and tert-butylhydroquinone (TBHQ) (Sherwin, 1990). These synthetic antioxidants have been widely used nowadays to reduce rancidity of fats and oils in food, but concern about possible adverse effects such as liver swelling, carcinogenesis, and changes of liver enzyme activity is still increasing. Recent findings showed that, these synthetic antioxidants were responsible for liver damage and carcinogenesis in laboratory animals (Grice, 1986; Wichi, 1988). Therefore, the development and utilization of more effective antioxidants of natural origin are desired.

Mushrooms contain various polyphenolic compounds which is recognized as an excellent antioxidant due to their ability to scavenge free radicals by single electron transfer (Hirano *et al.,* 2000). Some common edible mushroom such as *Grifola frondosa*, *Termitomyces*, *Pleurotus sajur_cajo* which are widely consumed in Asian culture, have

currently been found to possess antioxidant activity, which is well correlated with their total phenolic content (Mau 2004; Cheung & Cheung, 2005).

Auricularia species has been reported to exert strong antioxidant effects (Mau *et al.,* 2001; Fan *et al.,* 2006). Mau *et al.,* (2001) reported as, all methanolic extracts from red, jin, and snow ears showed excellent antioxidant activities in the conjugated diene method at 5.0 mg/ml. It is not good scavengers for hydroxyl free radicals but were good chelators for ferrous ions (Mau *et al.,* 2001). Meanwhile the polysaccharides in the fruit bodies of *Auricularia auricula* (black woody ear) was extracted and tested by formulating into bread. The result showed that up to 9% of AAP (*Auricularia auricula* polysaccharides) flour could be included (IC_{50} =100mg/ml) in bread formulation without altering the sensory acceptance of the blended bread. The incorporation of AAP in bread markedly increases the antioxidant property of the bread as tested by DPPH free radical scavenging method (Fan *et al.,* 2006).

Furthermore, *in vitro* studies of *A. auricula-judae* polysaccharides showed stimulation of DNA and RNA synthesis by human lymphocytes, providing support for *Auricularia*'s traditional use as an immune tonic. Numerous, animal studies showed that *A. auricula-judae* is a potent anti bacterial, antioxidant and lowering cholesterol.