# CYTOPROTECTIVE AND GENOPROTECTIVE EFFECTS OF AQUEOUS EXTRACTS OF PROCESSED *PLEUROTUS SAJOR-CAJU* FRUITING BODIES ON HYDROGEN PEROXIDE-INDUCED DAMAGE OF HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS

**MOHAMAD FAIRUS BIN ABDUL KADIR**

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Name of Candidate : Mohamad Fairus Bin Abdul Kadir (801016-01-6227)

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# ABSTRACT

*Pleurotus sajor-caju* authority is one of the mushroom species that is widely grown in Malaysia. The present study was undertaken to evaluate the antioxidant, cytotoxic, cytoprotective, genotoxic and genoprotective activities of crude water extracts of this mushroom. Crude aqueous extracts were prepared from the fruiting bodies that had been processed at different temperatures such as P1: blanching (95°C ± 2°C), P2: sun drying (30°C ± 2°C), P3: freeze drying (-46°C ± 3°C) and different temperatures of oven drying (P4: 45°C, P5: 50°C, P5: 55°C and P6: 60°C ± 2°C respectively). Antioxidant capacity of all the extracts was determined using ferric reducing power (FRAP) and DPPH free radical scavenging assays and the total phenolic content (TPC) was also determined. The TPC per gram of extract in descending order was P2, P4, P7, P6, P5, P3 and P1. Sun dried samples had the highest antioxidant activity compared to other processing method. The correlation analysis showed mild to moderate positive correlation between TPC, FRAP and DPPH. Cytotoxicity and cytoprotective activities were determined by assessing the viability of human peripheral blood mononuclear cells (PBMC) viability using MTT (3-(4, 4-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) assay. None of the processed mushroom extracts were cytotoxic to the PBMCs. Hydrogen peroxide ($H_2O_2$), an oxidant; was used to induce cytotoxicity in PBMC. At a concentration of 0.3mM, $H_2O_2$ showed 25% inhibition of cell viability compared to untreated cells. At 100µg/ml, the extracts P3 and P4 exhibited the highest cell viability (10.24±0.36% and 8.20±1.06% increase respectively) compared with untreated PBMCs, while 20µg/ml of phytohaemagglutinin (PHA) only showed 6.66±0.52% increase in cell viability compared with untreated PBMCs. All the mushroom extracts exhibited mild protection against $H_2O_2$ induced toxicity. The highest protection of 10.47±0.87% and 15.37±0.99% at 80 and 100µg/ml respectively was by

sun dried extracts, whereas at 10µg/ml vitamin C exerted 6.50±1.94% protection. Genotoxicity in PBMC was assessed using single cell gel electrophoresis (COMET assay). None of the mushroom extracts tested had genotoxic effects on the cells. The percentage of head DNA was more than 80% which was similar to control cells treated with water. The cells incubated with 0.2mM $H_2O_2$ caused 50% DNA damage. The genoprotective effect of the mushroom extracts was determined by assessing the COMET tail in PBMC, which had been incubated in the presence of $H_2O_2$ and the extracts. The P1 mushroom extract (80µg/ml) exhibited approximately 80% protection against $H_2O_2$ induced damage. The extracts P2, P3 and P4 of the fruiting bodies of *P. sajor-caju* showed similar degrees of protection. Thus, it can be concluded that the processing methods do affect the antioxidant level which in turn may affect the cytoprotective and genoprotective activities of *P. sajor-caju* against cellular or DNA damage induced by $H_2O_2$.

# ABSTRAK

*Pleurotus sajor-caju* adalah merupakan salah satu spesies cendawan yang diusahakan secara meluas di Malaysia. Kajian ini dijalankan bagi menilai aktiviti- aktiviti seperti antioksidan, sitotoksik, sitoprotektif, genotoksik dan genoprotektif (sekiranya ada), di dalam ekstrak mentah air cendawan ini. Ekstrak mentah air disediakan daripada cendawan yang telah diproses pada suhu yang berbeza seperti, P1: celuran (95°C ± 2°C), P2: pengeringan di bawah cahaya matahari (30°C ± 2°C), P3: pembekukeringan (-46°C ± 3°C) dan pengeringan pada suhu – suhu berbeza dalam ketuhar panas (P4: 45°C, P5: 50°C, P5: 55°C and P6: 60°C ± 2°C). Keupayaan antioksidan bagi semua ekstrak dikenalpasti menggunakan asei kuasa penurunan ferum (FRAP) dan pelupusan radikal bebas DPPH dan jumlah kandungan fenol (TPC). Jumlah kandungan fenol (TPC) per gram ekstrak dalam turutan menurun adalah seperti berikut; P2, P4, P7, P6, P5, P3 dan P1. Sampel P2 menunjukkan aktiviti antioksidan yang paling tinggi berbanding dengan kaedah pemprosesan yang lain. Analisa hubungkait antara TPC, FRAP dan DPPH menunjukkan hubungkait yang sangat positif. Aktiviti sitotoksik dan sitoprotektif dijalankan dengan mengenalpasti jumlah kehadiran sel darah mononuklear (PBMC) dengan menggunakan asei MTT (3-(4, 4-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide). Kesemua ekstrak *P. sajor-caju* menunjukkan ianya tidak toksik kepada PBMC. Hidrogen peroksida ($H_2O_2$), sebagai oksidan, digunakan untuk mengaruh sitotoksik pada PBMC. Pada kepekatan 0.3mM, $H_2O_2$ menunjukkan perencatan bilangan sel sebanyak 25% berbanding dengan sel yang tidak dirawat dengan $H_2O_2$. Pada 100µg/ml, ekstrak P3 dan P4 menunjukkan kehadiran jumlah bilangan sel yang tertinggi iaitu peningkatan sebanyak 10.24±0.36% dan 8.20±1.06% masing – masing, berbanding dengan sel yang dirawat dengan air sahaja. Manakala,

20µg/ml fitohemaglutinin (PHA) hanya menunjukkan peningkatan kehadiran jumlah sel sebanyak 6.66±0.52% berbanding dengan sel yang tidak dirawat (rawatan air suling sahaja). Kesemua ekstrak *P. sajor-caju* menunjukkan aktiviti perlindungan terhadap sel pada tahap yang memuaskan bertentangan dengan toksisiti yang diaruh oleh $H_2O_2$. Tahap perlindugan sel yang tertinggi boleh dilihat pada kepekatan 80µg/ml dan 100µg/ml yang menunjukkan peratusan perlindungan sebanyak 10.47±0.87% dan 15.37±0.99% masing – masing oleh P2, manakala pada kepekatan vitamin C sebanyak 10µg/ml peratusan perlindungan pada sel darah mononuclear adalah sebanyak 6.50±1.94% sahaja. Genotoksik pada PBMC dikaji dengan menggunakan teknik gel elektroforesis satu sel (asei COMET). Kesemua ekstrak tidak genotoksik pada sel darah mononuclear normal manusia. Rata – rata sel yang dirawat dengan ekstrak *P. sajor-caju* memamerkan peratusan kepala DNA pada komet lebih daripada 80% iaitu lebih kurang sama dengan set kawalan. Sel yang diinkubasi dengan 0.2mM $H_2O_2$ mampu memusnahkan 50% DNA sel darah mononuclear manusia. Kesan genoprotektif ekstrak *P. sajor-caju* dikenalpasti dengan menggunakan kaedah COMET pada PBMC yang diinkubasikan dengan kehadiran $H_2O_2$ dan ekstrak. Ekstrak P1 (80µg/ml) menunjukkan lebih kurang 80% perlindungan terhadap sel yang diaruh dengan $H_2O_2$. Tambahan lagi, ekstrak P2, P3 dan P4 *P. sajor-caju* juga menunjukkan tahap perlindungan yang hampir serupa dengan P1. Maka, boleh disimpulkan di sini bahawa kaedah pemprosesan memberi kesan kepada tahap antioksidan di mana ia mungkin memberi kesan yang baik kepada aktiviti sitoprotektif dan genoprotektif pada *P. sajor-caju* bagi melawan kemusnahan selular atau DNA yand diaruh oleh $H_2O_2$.

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Last but not least, I dedicate this thesis as my offering to my parents Mr. Abdul Kadir and Mrs. Yatuma Alee for bringing me up with love and care whilst instilling in me morality, culture, teaching me the importance of education and virtue of prayers. Such upbringing is the reason for me to be able to complete this thesis with the grace of god.

**TABLE OF CONTENTS**

## LIST OF TABLES

**Pages**

**LIST OF FIGURES**

**Pages**

## LIST OF ABBREVIATIONS

| | |
|---|---|
| α | alpha |
| AIDS | Acquired immune deficiency syndrome |
| ALS | Alkaline labile site |
| AZT | Azidothymidine |
| BC | Before Christ |
| β | beta |
| $CO_2$ | Carbon dioxide |
| DAPI | Diamino-2-phenylindole |
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxyribonucleic acid |
| DPPH | 1, 1-Diphenyl-2-picrylhydrazyl |
| $EC_{50}$ | Effective concentration-50 |
| EDTA | Ethylenediaminetetra-acetic acid, di-sodium salt |
| ELISA | Enzyme-linked immunosorbent assay |
| EtBr | Ethidium bromide |
| FBS | Fetal bovine serum |
| FDA | Food and Drug Administration |
| $FeSO_4.7H_2O$ | Iron (II) sulphate heptahydrate |
| FRAP | Ferric reducing antioxidant power |
| g | gram |
| *g* | Gravity |
| GAEs | Gallic acid equivalents |
| GC/MS | Gas chromatography/Mass spectrometry |

| | |
|---|---|
| h | hour |
| $H_2O_2$ | Hydrogen peroxide |
| HDL | High density lipoprotein |
| HIV | Human immunodeficiency virus |
| HPLC | High pressure liquid chromatography |
| $IC_{50}$ | Inhibition concentration - 50 |
| kg | kilogram |
| LDH | lactase dehydrogenase leakage |
| LDL | low density lipoprotein |
| LEM | medium of *L. edodes* |
| LMP | Low melting point |
| mg | milligram |
| ml | milliliter |
| mM | milimolar |
| MTT | 3 - (4, 4 - dimethylthiazol – 2 – yl) – 2, 5 – diphenyl tetrazolium Bromide |
| NaCl | Natrium chloride |
| $Na_2CO_3$ | sodium carbonate |
| NK | Natural killer |
| nm | Nanometer |
| NMP | Normal melting point |
| $O_2$ | Oxygen |
| $O_2^{\bullet}$ | Superoxide anion |
| OH• | Hydroxyl radical |
| $ONO_2^{\bullet}$ | Peroxynitrate |

| | |
|---|---|
| ˚ C | Degree celcius |
| PBMCs | Peripheral blood mononuclear cells |
| PBS | Phosphate buffer saline |
| PHA | Phytohaemaglutinin |
| PSK | Polysaccharide-K |
| RNA | Ribonucleic acid |
| RO | Reverse osmosis |
| ROS | Reactive oxygen species |
| RPMI | Roswell Park Memorial Institute |
| SSB | Single strand break |
| SEM | Standard error mean |
| SDS | Sodium dodecyl sulphate |
| SPSS | Statistical package for social sciences |
| spp | species |
| ssDNA | Single stranded deoxyribonucleic acid |
| TPC | Total phenolic content |
| TPTZ | 2, 4, 6 – Tripyridyl – S – Triazine |
| UV | ultraviolet |
| VLDL | very low density lipoprotein |
| v/v | volume/volume |
| w/v | weight per volume |
| % | percentage |
| µg | Microgram |
| µL | Microliter |
| µM | Micromolar |

# CHAPTER 1

# INTRODUCTION

# CHAPTER 1

# INTRODUCTION

## 1.1 Introduction

Since the beginning of human civilization, the use of natural products with therapeutic properties has been common. For ages, mineral, plant, and animal products were the main sources of drugs (Rates, 2001). The term natural products commonly refers to herbs, herbal concoctions, dietary supplements, traditional Chinese medicine, or alternative medicine (Holt and Chandra, 2002). Natural products are generally either of prebiotic origin or originate from microbes, plants, or animal sources. Components of natural products include terpenoids, polyketides, amino acids, peptides, proteins, carbohydrates, lipids, nucleic acid bases, ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) (Croteau *et al*., 2000).

Nature has provided many remedies for human kind over the years, including natural products for preventive or therapeutic intervention of diseases. Chinese herb guides documented the use of herbaceous plants as far back in time as 2000 BC (Holt and Chandra, 2002). Extracts from the basidiomycetes *Mycenapura* and *Nidula candida* may be useful in the treatment of leukemia. Treatment of diseases with medicine of plant origin is an integral part of many cultures throughout the world (Bhattaram *et al*., 2002). Today, many people are surprised to find natural products still play an important role as sources of medicine. Many pharmaceutical agents have been discovered by screening natural products from plants and microorganisms (Rocha *et al.*, 2001).

The structural diversity of plants and other natural products makes them a valuable source of novel lead compounds against newly discovered therapeutic targets (Harvey, 1999). The belief that natural medicines are much safer than synthetic drugs has gained popularity in recent years and this has lead to the tremendous growth in phytopharmaceutical industry (Bhattaram *et al*., 2002). Biological activities of natural sources have been screened extensively in search of new anticancer, antiviral, and fertility/antifertility drugs (Phillipson, 2001). The extraction of bioactive agents from natural sources is one of the most intensive areas of natural product research today.

In ancient times, mushrooms were used in folk medicine throughout the world. Edible mushrooms have been used to maintain health, increase longevity and consumed as food (Manzi and Pizzoferrato, 2000). Nowadays, mushrooms are increasingly consumed for their nutritional and medicinal properties. Attempts have been made in many parts of the world to explore the use of mushrooms and their metabolites for treatment of a variety of human ailments (Jose and Janardhanan, 2000).

Kosem *et al*. (2007) have reported the antioxidant activity and cytoprotective effect of *Garcinia mangostana*, may play a vital role as chemopreventive activities via a reducing mechanism and inhibition of intracellular oxidative stress which may lead to reduced cell or DNA damage. Recently, studies have been carried out to evaluate the antioxidant activities of commercial mushrooms (Mau *et al*., 2001 and Mau *et al*., 2002). In mushrooms it has been shown that phenolic compounds are responsible for the radical scavenging effects (Yang *et al*., 2002) and these are the major naturally occurring antioxidant components found in mushrooms (Cheung *et al*., 2003). Antioxidants are substances that may protect cells from the damage caused by unstable molecules known as

free radicals. Further, it has been reported that the various processing techniques to preserve the perishable edible mushrooms may affect the antioxidant properties of mushrooms, too (Vikineswary *et al.*, 2008). Thus, this study was undertaken to investigate the effects of different processing methods of *P. sajor-caju* on the antioxidant activity of the mushroom, if any, and its effects on protecting the cells against damage or DNA damage.

Manzi *et al*. (1999a) reported that mushrooms are healthy food, low in calories and fat, rich in proteins, chitin, vitamins and minerals. This chemical composition along with many other components render the mushroom as therapeutic foods that are useful in preventing diseases such as hypertension, hypercholesterolemia and cancer (Manzi *et al*., 2001). In addition, Hasler (1998) reported that mushrooms contained high concentrations of minerals, rich in vitamin D (ergosterol), thiamin (B1), riboflavin (B2), and niacin (B3) as well as all the essential amino acids.

They have also become one of the most desirable foods in Oriental cuisine because of their low caloric value, characteristic smell, taste and texture. Thus, mushrooms are considered as highly valuable bio-engineering resources for development of food material (functional foods) as well as for use of starting material in the production of drugs (Mizuno, 1999). Functional compounds (polysaccharides) in mushrooms have recently been highlighted as they are able to lower cholesterolemia, modulate the immune system and inhibit tumoral growth (Zhang *et al*., 2001).

In Japan, hundreds of mushroom species have been studied for the past 20 years. Most of the medicinal mushrooms such as *Ganoderma lucidum* (reishi), *Lentinula edodes* (shiitake) and *Grifola frondosa* (maitake) show a common property of enhancing immune function by stimulating cell-mediated immunity. These mushrooms seem to stimulate cells in the immune system, called T-cells, which appear to have significant cancer-fighting properties. Weng and Chen (1996) had reported that three different anticancer drugs extracted from mushrooms have been approved by the Japanese equivalent of FDA--the Health and Welfare Ministry. They are lentinan, derived from *L. edodes*; schizophyllan, derived from *Schizophyllum commune* (suehirotake); and polysaccharides-K (PSK), derived from *Trametes versicolor* (kawaratake) (Yang *et al*., 1992). Polysaccharides-K was reported as one of the best selling anticancer drug in the world (Yang *et al*., 1992).

There are approximately 200 species of mushrooms that have been identified to markedly inhibit the growth of different kind of tumors (Wasser and Weis, 1999). *Pleurotus sajor-caju* (oyster mushroom) is a class of "Edible Fungal Food" that has been discovered to have definite nutritive and medicinal values. As a good source of nonstarchy carbohydrate, quality protein with presence of most essential amino acids (Bano and Rajarathnam, 1988), minerals (Breene, 1990) and vitamins (Stamets, 1993), *Pleurotus spp.* have been shown to modulate the immune system, have hypoglycemic activity, antithrombotic effect and reduce blood pressure and blood lipid concentrations and to inhibit tumor growth, inflammation, and microbial action (Cimerman, 1999). *Pleurotus* is becoming popular in many of the European and Southern Asian countries due to its characteristic texture and pleasant flavor (Bano and Rajarathnam, 1988).

Mushrooms are however, highly perishable commodities. Therefore, processing is essential not only to preserve their nutritional and medicinal properties but also increase shelf-life (Vikineswary *et al.*, 2008). Food processing is a technique used to transform raw ingredients into food for consumption by humans and to increase shelf-life or storage period compared to fresh food. Common food processing techniques include removal of water by sun drying, oven drying, fermentation, cooking, deep frying and pasteurization (Rodriguez-Amaya, 1997). Benefits of food processing include toxin removal, preservation, increase food consistency and ease distribution and marketing tasks. However, it also has some disadvantages. For example, the content of vitamin C which is a well known antioxidant, is destroyed by heat and lowers the vitamin C content compared to fresh ones, as its stability is greatly influenced by temperature, oxygen, and metal ion content (Ryley and Kajda, 1994; Marcy *et al*., 1989).

As discussed above, the processing of the fruiting bodies of *Pleurotus sajor-caju* either by cooking or drying could possibly cause loss of nutrients and their protective effects. Wong *et al*. (2009) and Kho *et al*. (2009) reported that processing of mushroom can affect the antioxidant level and medicinal properties such as antimicrobial activity. Kho *et al.* (2009) showed that the different methods of processing *Auricularia auricula-judae* fresh fruiting bodies such as oven drying and freeze drying could affect the antioxidant potential. Similarly, Wong *et al.* (2009) reported that the different processing method employed for the preparation of extracts from *H. erinaceus* affected the selected bioactive properties of the mushroom. In some cases, heat treatment caused no change or even improved the content and activities of naturally occurring antioxidants. Choi *et al*., (2006) reported that heat treatment significantly enhanced the overall antioxidant activities of Shiitake mushroom.

To date, *Pleurotus* spp. are marketed fresh, canned or dried. There has been no investigation on the effects of processing, on medicinal properties of *Pleurotus* spp. Therefore, the present study was carried out to evaluate antioxidant properties and cytoprotective and genoprotective activities of processed *Pleurotus sajor-caju* grown in Malaysia.

The objectives of the present study were to:-

a) assess the antioxidant and polyphenol levels in the crude water extracts of *Pleurotus sajor-caju* subjected to different processing methods.

b) screen for cytoprotective activity of crude aqueous extracts from *Pleurotus sajor-caju* subjected to different processing methods using human peripheral blood mononuclear cellular viability assessment.

c) screen for genoprotective activity of crude aqueous extracts from *Pleurotus sajor-caju* subjected to different processing methods in human peripheral blood mononuclear cells by using single cell gel electrophoresis (Comet assay).

# CHAPTER 2

# LITERATURE REVIEW

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 Free radicals

A major development in carcinogenesis research caught the attention of scientists in the mid 1980s with the discovery of DNA damage and the mutation induced by reactive oxygen species (ROS). These ROS can be generated endogenously during normal cellular metabolism or acquired from exogenous sources such as ultraviolet (UV) radiation, air pollutants and etc. (Marnett, 2000). Damaged bases or strand breaks can be produced by excessive levels of oxygen radicals generated during the reduction of $O_2$ which in turn attacks DNA bases or dexyribose residues (Ward *et al*., 1987). Attempts in replication of the altered or damaged DNA leads to mutation or apoptosis (Johnson *et al*., 1996).

The identity of the oxidants responsible for the production of oxidized DNA bases is still the focus of numerous studies. The hydroxyl radical (HO•) is an obvious candidate because it is extremely reactive and adds to DNA bases or abstracts hydrogen atoms to produce many of the products that occur in the genome (Ward, 1988; Grisham and McCord, 1986). According to Marnett (2000), the HO• plays a role in the endogenous oxidation of DNA but this HO• generated does not diffuse into the nucleus of the adjacent cells. The reactivity of HO• is so great that it does not diffuse more than one or two molecular diameters before reacting with a cellular component (Marnett, 2000). In order to

oxidize DNA, the HO• must be generated immediately adjacent to the nucleic acid molecule (Marnett, 2000). It is likely that $H_2O_2$ serves as a diffusible, latent form of HO• that reacts with a metal ion in the vicinity of a DNA molecule to generate the oxidant (Henle and Linn, 1997 and Cadet *et al*., 1999).

Another oxidant that can generate many of the products observed with HO• is peroxynitrite ($ONO_2^-$) (Beckman *et al*., 1990). Peroxynitrite is the coupling product of nitric oxide and superoxide and is an extremely strong oxidant (Koppenol, 1992). The pattern of products generated by DNA oxidation by $ONO_2^{\bullet}$ is complex and mirrors the diversity of oxidized DNA detected in tissues (Burney *et al*., 1999). Interestingly, $ONO_2$• is more reactive toward 8-oxo-deoxyguanosine (8-oxo-dG) than to unmodified DNA bases (Burney *et al*., 1999). 8-oxo-dG has been detected previously in DNA treated with a variety of oxidants and is easily measured by HPLC and GC/MS (Dizdaroglu and Gajewski, 1990). $ONO_2$• has the ability to diffuse within cells and may be taken up by some cells via anion transporters (Radi, 1998). This may provide a link between inflammation and the induction of mutation by virtue of the ability of $ONO_2$• to oxidize DNA. If these free radicals are not neutralized, they can quickly bind to our cells vital proteins, carbohydrates and even our DNA, potentially altering its structure. This may result in a decrease in immune function or a change in the genetic makeup of cell, which could affect the cell's ability to reproduce normally (Maynard, 2001). These, uncontrolled productions of ROS is also involved in the onset of many common illnesses such as cancer, rheumatoid arthritis, and cardiovascular as well as in degenerative processes of aging (Halliwell and Gutteridge, 1985). In order to preserve genomic stability, a tightly regulated network of intracellular antioxidative and DNA repair pathways has evolved (Sasaki, *et al*., 2002).

## 2.2 Genotoxic effects of DNA damage by ROS/oxyradicals

Genotoxic processes may lead to irreversible changes in the structure of the genetic material of cells. This effect is considered as an important process in cancer development. The term 'genotoxic' was used for the first time in 1973 by Hermann Druckrey during a conference on 'Evaluation of genetic risks of environmental chemicals' in Sweden. He stated: 'In order to describe the components of chemical interaction with genetic material, the term genotoxic is proposed as a general expression to cover toxic, lethal and heritable effects to karyotic and extrakaryotic genetic material in germinal and somatic cells' (Weisberger and Williams 2000).

A variety of techniques for measuring carcinogen interaction with DNA has been developed since the first discovery of mutation and DNA damages. The techniques including binding of radio-labelled chemical, one of the reliable and predictive hepatocyte DNA repair assay (Williams *et al.*, 1998) and the $^{32}$P-postlabeling method of Randerath and Gupta (Reddy *et al.*, 1984). Hence, these procedures can be used to classify carcinogens that are DNA reactive, i.e., genotoxic. Conversely, a substantial number of carcinogens were found to lack genotoxicity and this knowledge was embodied in a key distinction between genotoxic and epigenetic carcinogens based on the underlying mechanism of action (Weisberger and Williams, 2000).

## 2.3 Antioxidants

Antioxidants are substances that delay or prevent the oxidation of cellular oxidizable substrates (Yuan *et al.*, 2005). A multitude of natural antioxidants have already been isolated from different kinds of plant materials such as seeds, cereal crops, vegetables, fruits, leaves, roots, spices and herbs. The body produces a variety of antioxidants to maintain healthy systems and defray cellular destruction. Because antioxidants stop this damaging process, thus supplements and foods containing them are often termed "anti-aging".

Antioxidants have been thought to be important in preventing a variety of disease progression caused by UV radiation from sunlight, cosmic radiation, chemicals, and pollutants and internally generated free radicals. 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, 1988). Jeng *et al*. (2002) reported that when the mechanism of antioxidant protection becomes unbalanced by factors such as aging, deterioration of physiological functions, resulting in diseases and acceleration of aging.

The damage of DNA (the cell’s blue print) by the free radicals is prevented with the presence of antioxidant. This could give antioxidants an important role and potential to reduce the genetic instability of cancer cells and thus may be useful in treatment (Maynard, 2001; and Reddy *et al*., 2003). The demand for natural antioxidant from botanical sources, especially edible plants increased because of the question on the long-term safety and negative consumer perception of synthetic drugs (Amarowicz *et al*., 2004; Sakanaka *et al*.,

2004). Ascorbic acid, most commonly known as vitamin C is the most abundant of water soluble antioxidants available and acts primarily in cellular fluid. Vitamin C is important to the body's biosynthesis of collagen, catecholamine and carnitine and is integral to proper formation of proteins, neurotransmitters, hormones, DNA and RNA in the body (Hathcock, 2004).

Kim *et al*. (1994) and Jeng *et al*. (2002), reported that some Chinese herbs used in traditional medicines exhibited significant antioxidant activity and key ingredients in their medicines also included mushrooms. Some common edible mushrooms, which are widely consumed in Asian culture, had recently been found to possess antioxidant activity and this correlated with their total phenolic content (Cheung *et al*., 2003). Mushrooms accumulate a variety of secondary metabolites, including phenolic compounds, polyketides, terpenes and steroids. Phenolic compounds were found to have antioxidant activity which could inhibit low density lipoprotein (LDL) oxidation (Teissedre and Landrault, 2000). Cheung *et al*., (2003) reported that phenolic antioxidants, such as variegatic acid and diboviquinone were found in mushrooms.

## 2.4 Mushroom

Mushrooms represent one of the world's greatest untapped resources of nutritious food. The number of mushroom species on the earth is estimated to be 140 000 and only 10% are known. Mushrooms which are primarily basidiomycete fungi are rich in protein, minerals, and vitamins, and they contain an abundance of essential amino acids (Mattila *et al*., 2002). Therefore, mushrooms can be a good food supplement. However, many people are

apprehensive about mushrooms as a food source. Ignorance has led many to become skeptical about whether food of fungal origin can hold any great nutritional promise. It seems much education is needed before full advantage can be taken of this readily available, nutritionally rich food source (Chang and Buswell, 1996).

### 2.4.1 Nutritional values of mushrooms

The nutritional values and organoleptic components of a number of commercial mushrooms have been well studied (Yang *et al.*, 2001). Medicinal mushrooms from Asia may be among the best examples of foods containing cancer-fighting nutrients. It is estimated that there are 140,000 different kinds of mushrooms, of which 700 are used for food. Mushrooms are rich sources of minerals, vitamin D (ergosterol), thiamin (B1), riboflavin (B2), and niacin (B3) as well as all essential amino acids; while still being low in fat and calories. Only recently have some of the more exotic varieties appeared in markets; *Lentinula edodes* (shiitake), *Agaricus bisporus* (Portabello), *Flammulina velutipes* (enoki), *Pleurotus* spp. (oyster) and *Boletus edulis* (porcini) are now popular gourmet cuisine ingredients. The 2006 Urban Rural Interface Conference was a great opportunity for producers and consumers to learn more about fruits, vegetables, and mushrooms that provide major benefits to health, as well as new ways to market these products (Hasler, 1998).

Among the edible and medicinal mushrooms *Ganoderma lucidum*, widely used for the promotion of health and longevity is reported to have antioxidant and genoprotective (protects the DNA from oxidative damage) properties (Wachtel-Galor *et al.*, 2004). Oxidation of DNA can produce a number of molecular alterations, including cleavage, cross-linkage between DNA and proteins and oxidation of purines. Unless corrected by the DNA repair machinery, those alterations can lead to mutations, carcinogenesis, and senescence (Deshpande and Irani, 2002).

In order to protect genomic stability, tightly regulated networks of intracellular pathways have evolved. The salient intracellular mechanisms responsible for modulating oxidative stress include the thioredoxin system and antioxidant enzymes, such as catalase, glutathione peroxidise and superoxide dismutase. Low molecular weight compounds, including selenium and phytochemicals, such as ascorbic acid (Vitamin C), α-tocopherol (Vitamin E) and β-carotene have also been found to protect against oxidative stress (Lindsay and Astley, 2002).

### 2.4.2 Antitumor activity of mushrooms

Mushroom-derived beta-glucan has been used in Asia for centuries for health purposes. A review article on mushroom-derived beta glucans showed that different mushrooms exhibited differing effects so that some mushrooms appear to act more strongly against some health challenges than others (Brochers *et al*., 1999). Beta glucans can make the natural killer (NK) cells work more effectively as they actually bind to the NK cells and

increase the proportion of target-binding lymphocytes and of the damaged target cells in the conjugates (Renzo *et al*., 1991).

For almost 40 years, mushrooms have been intensively investigated for medicinal effect in *in vivo* and *in vitro* model systems, and many new antitumor and immunomodulating polysaccharides have been identified and put into practical use (Mizuno *et al*., 1996, 1999; Wasser and Weis 1999; Ikekawa 2001). Mushroom polysaccharides are known to stimulate natural killer cells, T-cells, B-cells, and macrophage-dependent immune system responses (Wasser, 2002). *G. frondosa*, a well known mushroom in Japan showed clear anti-tumor effect against both MM-46 and IMC carcinomas in mice (Mayell, 2001). Mushroom polysaccharides are present mostly as glucans with different types of glycosidic linkages, such as (1-3), (1-6)-beta-glucans and (1-3)-alpha-glucans, but some are true heteroglycans (Wasser, 2002).

Polysaccharides are the best known and most potent mushroom derived substances with antitumor and immunomodulating properties as shown in Table 2.1. The proposed mechanism by which most mushroom polysaccharides exert antitumor effect is via activation of the immune response of the host organism. They are regarded as biological response modifiers (Wasser and Weis 1999), which includes a) cancer preventing activity; b) immune –enhancing activity; and c) direct tumor inhibition activity (Brekhman, 1980). The natural antitumor polysaccharides isolated from mushrooms include acidic and neutral ones with different types of glycosidic linkages, while some are bound to protein or peptide residues such as polysaccharide-protein and peptide complexes (Cun *et al.*, 1994).

### 2.4.3 Other bioactivities of mushrooms

Medicinal mushrooms have a variety of biological activities such antimicrobial, antiviral, hepatoprotective, antidiabetic, and many others. For instance, *G. lucidum* is a medicinal mushroom that has antidiabetic, antioxidant, immunomodulatory, antitumor and antimetastatic activities (Kimura *et al*., 2002).

Mushrooms need antibacterial and antifungal compounds to survive in their natural environment. It is therefore not surprising that compounds with strong antimicrobial activities could be isolated from many mushrooms and that they could be of benefit for human (Lindequist *et al*., 2005). The purified materials from mycelium extracts of edible mushroom, *Hericium erinaceum,* showed antimicrobial effects against *Escherichia coli, Bacillus subtilis, Staphylococcus aureus, Aspergillus niger, Candida albicans* and *Microsporum gypseum* (Dong *et al*., 2000). Cochran (1978) reported that edible mushrooms such as *L. edodes, Coprinus comatus* and *Qudemansiella mucida* have antifungal activity. He also reported that mushroom species such as *Boletus frostii, Calvatia gigantean, Chlorophyllum molybdites* and *Agaricus campestris* protect mice against poliomyelitis (antiviral).

An antifungal peptide (eryngin) with a molecular mass of 10 kDa was isolated from fruiting bodies of the mushroom *Pleurotus eryngii* (Wang and Ng, 2004) was reported to have the ability to inhibit mycelial growth in *Fusarium oxysporum* and *Mycosphaerella arachidicola*. Japanese researchers reported that lentinan isolated from *L. edodes*, in combination with the drug azidothymidine (AZT) was more effective than AZT itself in suppressing the proliferation of the AIDS virus (Tochikura *et al*., 1988).

On the other hand, there are some evidences on the hepatoprotective effects of mushroom extracts such as *G. frondosa, Dendropolyporus umbellatus, S.commune, T versicolor, Tremella fuciformis* and *Wolfiporia cocos* (Wasser and Weis, 1999). There are other studies which showed that fractions isolated from the fruiting body of *A. blazei* exhibit antimutagenic, anticarcinogenic, and immunostimulative activities (Shimura *et al*., 1983; Itoh *et al*., 1994 and Osaki *et al*., 1994). Various fractions of *A. blazei* inhibited benzo-(a)pyrene induced mutagenicity, and that linoleic acid was found to be the main substance associated with such activity (Osaki *et al*., 1994). Anti-HIV activities were reported for mycelial culture medium of *L. edodes* (LEM) and water-soluble lignin in LEM (Tochikura *et al*., 1988 and Suzuki *et al*., 1989).

**Table 2.1: Source, type and bioactivity of some macrofungal polysaccharides (Source: Zhang *et al*., 2007).**

| Fungi Source | Polysaccharide sources | Type | Main bioactivity |
|---|---|---|---|
| *Agaricus brasiliensis* | Fruiting body, mycelium | Glucan, heteroglycan, glucan protein, glucomannan-protein complex | Antitumor |
| *Armillariella tabescens* | Mycelium | Heteroglycan | Antitumor |
| *Auricularia auricula* | Fruiting body | Glucan | Hyperglycemia, immunomodulating, antitumor, antinflammatory, antiradiative |
| *Clitopilus caespitosus* | Fruiting body | Glucan | Antitumor |
| *Cordyceps* sp | Fruiting body, mycelium, culture broth | Glucan, heteroglycan | Antitumor, immunomodulating, hyperglycemia |
| *Dictyophora indusiata* | Fruiting body | Heteroglycan, mannan, glucan | Antitumor, hyperlipidemia |
| *Flammulina velutipes* | Fruiting body, mycelium | Glucan-protein complex, glycoprotein | Antitumor, antinflammatory, antiviral, immunomodulating |
| *Ganoderma lucidum* | Fruiting body, culture broth | Heteroglycan, mannoglucan, glycopeptide | Hyperglycemia, immunomodulating, antitumor, antioxidantive, anti-decrepitude |
| *Ganoderma applanatum* | Fruiting body | Glucan | Antitumor |
| *Grifola frondosa* | Fruiting body | Proteoglycan, glucan, galactomannan, heteroglycan, grifolan | Immunomodulating, antitumor, antiviral, hepatoprotective |
| *Hericeum erinaceus* | Fruiting body, mycelium | Heteroglycan, heteroglycan-peptide | Hyperglycemia, immunomodulating, antitumor |
| *Inonotus obliquus* | Fruiting body, mycelium | Glucan | Antitumor, immunomodulating |
| *Lentinula edodes* | Culture broth, fruiting body | Mannoglucan, polysaccharide-protein complex | Antitumor, immunomodulating |

| **Fungi Source** | **Polysaccharide sources** | **Type** | **Main bioactivity** |
|---|---|---|---|
| *Morchella esculenta* | Fruiting body | Heteroglycan | Hyperglycemia, antitumor |
| *Omphalia lapidescens* | Fruiting body | Glucan | Antinflammatory, immunomodulating |
| *Peziza vericulosa* | Fruiting body | Proteoglycan, glucan | Antitumor, immunomodulating |
| *Phellinus linteus* | Fruiting body | Glucan | Antitumor |
| *Pleurotus citrinopileatus* | Fruiting body | Galactomannan | Antitumor |
| *Pleurotus tuber-regium* | Sclerotium, mycelium | β-D-glucan | Hepato-protective, anti-breast cancer |
| *Pleurotus ostreatus* | Fruiting body | Glycoprotein | Antitumor, hyperglycemia, antioxidant |
| *Polyporus umbellatus* | Mycelium | Glucan | Antitumor, immunomodulating |
| *Polytictus versicolar* | Fruiting body, culture broth, mycelium | Heteroglycan, glycopeptides, krestin | Immunomodulating, antitumor, hyperglycemia, antinflammatory |

## 2.5 *Pleurotus* spp

Mushrooms of genus *Pleurotus* are the most popular fungal food. Worldwide interest in the mushrooms has increased because of their relative ease of cultivation and the ability to grow on a variety of agricultural residues. The first sustained commercial production of *Pleurotus* spp. on straw began in Europe in the early 1960s with large-scale industrial production beginning in the 1970s (Zadril, 1978). This fungus is industrially produced as human food, and it accounts for nearly a quarter of the world mushroom production (Chang, 1996) and also occupies the second most important position in the world mushroom market, led by the button mushroom *Agaricus bisporus* (Baars *et al.*, 2000; Royse, 1996). It is also used for the bioconversion of agricultural, industrial, and lignocellulose wastes (Ballero *et al.*, 1990 and Puniya *et al.*, 1996), as an agent for bioremediation (Axtell *et al.*, 2000) and as organic fertilizer (Abdellah *et al.*, 2000).

Oyster mushrooms which fall under the genus of *Pleurotus*, family *Pleurotaceae* and the order *Agaricales*, are one of the most popular edible mushrooms especially in Oriental region such as China and Japan. The oyster mushrooms were first cultivated in the United States at the beginning of the last century, before being introduced to Europe and India (Guzman, 2000). More than 1000 species of *Pleurotus* have been described throughout the world; however, only approximately 50 species are recognized as *Pleurotus* (Guzman, 2000). The genus can be recognized by their white spores, the stem that attached at the side of the cap, or at least off center, and the fleshy or tough texture of the cap and the ability to grow at temperate climates.

At the beginning of 20th century, the method of *Pleurotus* cultivation on tree stumps and logs was first introduced (Purkayastha and Chandra, 1985). Today, *Pleurotus* spp. can be grown on various agricultural waste materials especially on lignocellulosic materials using different technologies. This white rot fungus is capable of degrading both lignin and cellulose. *Pleurotus* spp are primary agents of decomposition. They have the ability, therefore, to directly break down cellulose and lignin bearing materials without chemical or biological preparation (composting) (Kaul, 2002).

*Pleurotus* spp. became one of the choice edible mushrooms cultivated in tropics (Quimio, 2001) and widely consumed as food in the east and increasingly in the west (Cimerman, 1999). One of the reasons for its success is that oyster mushrooms have good nutritional (Cimerman, 1999) and medicinal values (Bano and Rajarathnam, 1988). They are good sources of nonstarchy carbohydrates, dietary fiber (that can help in reducing the plasma cholesterol), essential amino acid, minerals and vitamins of B group, and folic acid (Bano and Rajarathnam, 1988). The proximate analysis or composition of four *Pleurotus* spp. showing significant difference in the values of crude protein and fat is presented in Table 2.2.

**Table 2.2: Proximate composition of *Pleurotus* spp.**

| ***Pleurotus* spp** | **Moisture (%)** | **Composition (%)*** | | | **Total Carbohydrates +** | **Energy Value Kcal (%)** |
|---|---|---|---|---|---|---|
| | | **Crude protein** | **Fat** | **Ash** | | |
| *P. sajor-caju* | 88.75 | 26.94 | 2.88 | 11.82 | 58.36 | 379 |
| *P. florida* | 91.50 | 37.19 | 3.72 | 10.98 | 48.11 | 385 |
| *P. sapidus* | 88.35 | 35.87 | 2.41 | 12.35 | 49.37 | 373 |
| *P. ostreatus* | 89.25 | 27.38 | 2.27 | 13.44 | 56.91 | 371 |

*Dry weight basis (Bano and Rajarathnam, 1988)

+ calculated by difference from *

Considering the essential amino acid index, biological value, *in vitro* digestibility, nutritional index and protein source, *Pleurotus* spp. fall between high grade vegetables and low grade meats (Bano and Rajarathnam, 1988). The amino acid profile of *Pleurotus* spp. is presented in Table 2.3. All the values are expressed as g amino acid/100g crude protein. In all, 17 amino acids including all the essential amino acids were detected in significant quantities.

**Table 2.3: Amino acid composition of *Pleurotus* spp.**

| **Amino acids** | ***P. sajor-caju*** | ***P. florida*** | ***P. sapidus*** | ***P. ostreatus*** | **Hen's egg Protein** |
|---|---|---|---|---|---|
| **Alanine** | 10.237 | 6.388 | 9.124 | 7.775 | 5.920 |
| **Arginine** | 2.463 | 1.534 | 1.694 | - | 6.096 |
| **Aspartic Acid** | 1.237 | 1.534 | 2.032 | 4.294 | 9.616 |
| **Cystine** | 0.650 | 0.554 | 0.735 | 0.380 | 2.432 |
| **Glutamic acid** | 7.83 | 4.918 | 3.644 | 5.975 | 12.736 |
| **Glycine** | 4.371 | 4.882 | 3.130 | 5.165 | 5.312 |
| **Histadine** | 1.025 | 1.134 | 1.122 | 4.203 | 2.432 |
| **Isoleucine** | 3.572 | 2.416 | 3.098 | 2.792 | 6.288 |
| **Leucine** | 8.665 | 6.578 | 4.153 | 6.433 | 8.896 |
| **Lysine** | 5.435 | 3.196 | 2.152 | 3.286 | 6.976 |
| **Methionine** | 2.043 | 1.840 | 1.398 | 1.235 | 3.360 |
| **Phenylalanine** | 6.035 | 5.820 | 5.333 | 5.992 | 5.728 |
| **Proline** | 2.375 | 2.912 | 2.237 | 2.720 | 4.160 |
| **Threonine** | 2.900 | 5.038 | 3.201 | 2.554 | 5.120 |
| **Tyrosine** | 2.272 | 1.426 | 1.580 | 1.524 | 4.160 |
| **Serine** | 0.148 | 0.110 | 0.322 | 0.270 | 7.648 |
| **Valine** | 6.350 | 3.430 | 4.731 | 4.728 | 6.848 |
| **Total Aromatic** | **8.307** | **7.246** | **6.913** | **7.516** | **9.888** |
| **Sulphur containing amino acid** | **37.930** | **30.298** | **26.381** | **25.024** | **51.216** |
| **Total amino acid** | **67.769** | **53.710** | **49.685** | **56.026** | **103.136** |

Data presented in gram of amino acid/100g crude protein (Khanna *et al*., 1992)
(-) Not detected

Zhang *et al*. (2001) reported that the modified fractions of *P. tuber-regium* exhibited antitumor activities on solid tumour cell lines *in vitro*. In addition, other *Pleurotus* species such as *P. passeckerianus* and *P. mutilis* which contain anticarcinogenic and antiviral agent pleuromutilin, showed an active antiviral reaction against PR8 influenza virus (Jong and Donovick, 1989). Antiviral agents have also been obtained from water extracts of *P. ostreatus* mycelium (Cimerman, 1999).

Cimerman (1999) reported that antibiotic substances had been isolated from the water extracts of the fruiting bodies of *P. griseus, P. palmatus, P. sapidus* and *P. ulmarius*. These *Pleurotus* spp. showed a good antibiotic activity against *Staphylococcus aureus* and cholesterol lowering activity in rats. The hypocholesterolemic effect of oyster mushroom is comparable to the effect of *L. edodes*. The addition of 5% dried oyster mushrooms to a high cholesterol diet effectively reduced cholesterol accumulations in the serum and liver of rats. Cimerman also reported that the distribution of cholesterol was more towards the presence of high-density lipoprotein (HDL), reduced production of very low-density lipoprotein (VLDL) and low density lipoprotein (LDL), reduced cholesterol absorption and HMG CoA reductase activity in liver.

### 2.5.1 *Pleurotus sajor-caju*

Known as Houbitake in Japan, this mushroom was first found by an Indian Scholar, at the foot of Himalayan Mountain. This species was then distributed to China through India and Australia (Zhuang *et al.*, 1993). *P. sajor-caju* grows mostly on stumps and trunks of a wide range of deciduous trees, particularly Populus, Alnus, Salix and Betula (Wasser and Weis,

1999). *P. sajor-caju* is tolerant of a tropical temperature of 28 – 30ºC, although it fruits faster and produces larger mushrooms at 25ºC during the cooler month of the year or in the highlands of the tropics (Quimio, 2001).

*P. sajor-caju* is cultivated using cotton waste as substrate (Chang, 1999). Currently, it is cultivated in China using bark and trunks of banana trees, and rice straw (Zhuang *et al*., 1993). This species is now popularly grown in the tropical South East Asian countries, including Malaysia and India (Tan and Wahab, 1997; Quimio, 2001). *P. sajor-caju* is considered as a delicacy and treasured for its flavours and taste (Mizuno and Zhuang, 1995; Zhuang *et al*., 1993 and Chang, 1999). The fresh fruiting bodies of *P. sajor-caju* contain 85% - 90% of moisture, low lipid levels and practically no starch but alpha glucan is present (Mizuno and Zhuang, 1995).

*P. sajor-caju* contains about 21% protein (dry weight) and eight kinds of amino acids essential to humans where lysine and threonine concentrations are high as compared to other types of fungi. *Pleurotus sajor-caju* contains various vitamins such as vitamin C (33mg), vitamin $B_1$ (0.2 – 0.3 mg), vitamin $B_2$ (1.1 - .14 mg) and niacin (18.2 – 21.3 mg) per 100 g, respectively, in dry matter (Mizuno and Zhuang, 1995; Zhuang *et al*., 1993). They also contain various minerals such as zinc (9.31 mg), ferum (7.94 mg), phosphorus (716.31 mg), calcium (23.66), magnesium (157.67), potassium (2687 mg) and sodium (750.77 mg) respectively per kg wet weight (Caglarirmak, 2007).

It has been demonstrated that this mushroom is not only a gustatory delight but also may be useful for the development of antitumor drugs and other therapeutic agents (Zhuang *et al*., 1993). Its frequent intake may reduce the cholesterol level in blood; prevent cancers and exert carcinostatic activity based on immunoactivation (Mizuno and Zhuang, 1995; Zhuang *et al*., 1993). Zhuang *et al*. (1993) also demonstrated that *P. sajor-caju* exhibited high antitumor activity in mice with Sarcoma 180 that were injected with *P. sajor caju* intraperitoneally. An extract containing mannogalactan, a polysaccharide comprising xylose, mannose and galactose was extracted from the fruiting bodies of *P. sajor-caju* which have high antitumour activity (Zhuang *et al*., 1993). The methanolic extract of *P.sajor-caju* possessed significant hydroxyl radical scavenging, lipid peroxidation inhibiting activities and significant inhibitory effect on solid tumour induced by EAC cells (Jose *et al*., 2002). The IC 50 value for hydroxyl-radical scavenging and lipid peroxidation inhibition was 476µg/ml and 960µg/ml respectively (Lindequist *et al*., 2005).

# CHAPTER 3

# MATERIALS & METHODS

# CHAPTER 3

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## 3.1 Preparation of mushroom extracts from fruiting bodies of *P. cajor-caju*

Approximately 7 kg of the mature fruiting body of *P. sajor-caju* was obtained from a local farmer in Malaysia. The fruiting bodies were divided into seven, one kilogram portions and each portion was sliced. The portions were subjected to the following processing temperatures: blanching for 10 minutes at 95-100ºC (P1), oven dried at 45ºC (P2), 50ºC (P3), 55ºC (P4) and 60ºC (P5) for 16 hours, sun dried for two days (P6) and freeze dried (P7). The temperatures of processing are shown in Table 3.1 below. The weights after each processing technique were recorded.

The processed samples were extracted with hot water. Three volumes of water were added into a container together with the processed samples. Then, the containers were placed into boiling (100ºC) water bath for three hours. The coarse residues were filtered using cheesecloth. Then, the samples were centrifuged at 15, 000 x g for 30 minutes at 4 ± 2ºC. The supernatant was freeze-dried and the weights of the samples were determined once again. The freeze-dried samples were kept in -20 ºC for further use.

**Table 3.1: Temperatures at which fruiting bodies of the *P. sajor-caju* were processed**

| Processing method | Temperatures (ºC) |
|---|---|
| Blanching | 95 ± 2 |
| Freeze Drying | -48 ± 2 |
| Sun drying | 30 ± 2 |
| Oven drying | 45 ± 2, 50 ± 2, 55 ± 2 and 60 ± 2 |

## 3.2 Antioxidant potential and polyphenol content of *P. sajor-caju* extracts

### 3.2.1 Total phenolic content (TPC) of *P. sajor-caju* crude extracts

This assay was typically used to measure the total phenolic content of foods or plant extracts. Phenolic content of a compound contributes to free radical chain breaking properties and the ability to neutralize free radicals by donating hydroxyl group. Thus, in the presence of Folin Ciocalteu's phenol reagent, phenolic compounds react with the phenol reagent and yield a blue chromogen that absorbs at 750nm.

The assay was performed as reported by Singleton and Rossi (1965) but with slight modification. Folin Ciocalteu's phenol reagent (0.1v/v) (Appendix A) was prepared and 50µl of it was mixed with 50µl of processed *P. sajor-caju* extracts. A stock aqueous solution of 5mg/ml of processed *P. sajor-caju* extracts were prepared as mentioned in section 3.2.1.1. After 3 minutes incubation at ambient temperature, 100µl of 10 % sodium carbonate ($Na_2CO_3$) was added and incubated again in the dark for one hour.

The colour change was measured spectrophotometrically at 750 nm. Gallic acid was used as standard and the phenolic content was expressed as mg GAE / 100 g of mushroom extracts (Appendix B):-

$$\text{Total phenolic content (mg GAE / 100g sample)} = \frac{\mu g/ml\ GAE}{mg/ml\ sample}$$

$$= \frac{\mu g\ GAE}{mg\ sample}$$

$$= 100 \times \frac{mg\ GAE}{100g\ sample}$$

### 3.2.2 Antioxidant activity assay

#### 3.2.2.1 DPPH free radical scavenging activity assay

The DPPH assay is a fast method to determine the free radical scavenging activity of the mushroom extracts based on the reduction of 1, 1-diphenyl-2-picrylhydrazyl hydrate (DPPH) radical. In the presence of antioxidant, the DPPH free radical will donate its hydrogen which subsequently be reduced. The quenching of the DPPH radical was measured spectrophotometrically at 515 nm. This assay was carried out according to Gerhauser *et al.* (2003). Ascorbic acid was used as the positive reference standard in the DPPH assay (Appendix C). Reaction mixtures of ascorbic acid, DPPH and methanol for the assay were prepared freshly (Appendix A). A stock aqueous solution of 5mg/ml of processed *P. sajor-caju* extracts were prepared (the solubility of the processed *P. sajor - caju* was very low at concentration more than 5mg/ml). One hundred and ninety five microlitres of various concentrations of DPPH were added in a 96 well plate. Then, five

microlitre of various concentrations of ascorbic acid ranging from 0 – 1000 µg/ml and extracts of *P. sajor-caju* (5mg/ml) were added with the DPPH reagent. The final concentration of the diluents was 40x. The absorbance of the incubation mixture was measured every 20 minutes at a wavelength of 515nm.

The percentage of DPPH quenched of the test samples was calculated according to the following formula:

$$\text{DPPH quenched (\%)} = \frac{\text{OD blank} - \text{OD sample}}{\text{OD blank}} \times 100\%$$

### 3.2.2.2 Ferric reducing antioxidant power (FRAP) assay

FRAP assay is an inexpensive and simple assay whereby the reagents are simple to prepare; results are highly reproducible and rapid. Unlike other antioxidant assays which apply the inhibition principle, the FRAP assay is straightforward and measures 'antioxidant power' directly. In the presence of antioxidants at low pH, the ferric-tripyridyltriazine [Fe(III)-TPTZ] complex is reduced to a ferrous-tripyridyltriazine [Fe(II)-TPTZ] complex. This complex gives rise to an intense blue colour which can be detected spectrophotometrically at 593nm. The development of blue colour from the reduction reaction occurs due to the ability of the antioxidant to donate an electron to reduce Fe (III) to Fe (II). This reaction can be summarized as follow:

$$\text{Fe (III)-TPTZ} + \text{Antioxidant} \longrightarrow \text{Fe (II)-TPTZ} + \text{Antioxidant}^{+}$$

In this study, known concentrations of ferrous solutions in the range of 0-1000 µM were used as standard. The FRAP values are linearly related to the concentration of the antioxidant (Benzie and Strain, 1996).

A 96-well plate was used to carry out the FRAP assay. Freshly prepared FRAP reagent was warmed to 37°C (Appendix A). A series of standard (concentration range of 0 – 1000 µmol/ml) was prepared by adding 10µl of various concentrations of $FeSO_4.7H_2O$ and 300µl of FRAP reagent. As for the samples, 10µl of test samples were used to substitute the $FeSO_4.7H_2O$. A stock aqueous solution of 5mg/ml of processed *P. sajor-caju* extracts were prepared as mentioned in section 3.2.1.1. An ELISA microplate reader was then used to read the absorbance of the mixture at 593nm. The plate was monitored spectrophotometrically using the kinetic mode between 0 to 4 minutes. Absorbance was read against water as the reagent blank. All samples were analyzed in triplicates in a single run (Benzie and Strain, 1996).

A FRAP standard curve was plotted using the absorbance of various incubations containing $FeSO_4.7H_2O$ (Appendix D). The FRAP value for the samples tested were quantitated using the standard curve and were expressed as µmol/100g sample.

## 3.3 Cytotoxicity, cytoprotective, genotoxicity and genoprotective assays on processed *P. sajor-caju* extract

### 3.3.1 Isolation of peripheral blood mononuclear cells (PBMCs)

The lymphocytes were isolated according to the method of Boyum (1968). Fresh whole blood was collected by venipunture from healthy donors in sterile heparinized vacutainer tubes. The blood was centrifuged at 900 x g for 30 minutes and a sterile pipette was used to transfer the plasma into a Falcon tube. The packed cells were resuspended in an equal volume of phosphate buffer saline (PBS). This mixture was layered carefully on to an equal volume of Histopaque-1077 solution in a 50ml Falcon tube. The solution was centrifuged at 900 x g for 30 minutes.

After centrifugation, the upper layer of PBS was discarded to within 0.5cm of the opaque interface containing the lymphocytes. The lymphocytes were aspirated using sterile pipette and transferred into a sterile Falcon tube. Ten milliliters of PBS solution was added and centrifuged at 600 x g for ten minutes. The supernatant was discarded and the pellet was resuspended in 7ml of ammonium chloride solution to lyse the contaminating red cells for ten minutes. Then, the contents were centrifuged again for ten minutes at 600 x g. The supernatant was discarded and the pellet was resuspended again with PBS solution and centrifuged for another ten minutes. The packed lymphocytes obtained at the end of the centrifugation were resuspended with one to two milliliters of Roswell Park Memorial Institute -1640 (RPMI 1640) solution that contained 10% fetal bovine serum (FBS) and 1% L-Glutathione.

The haemocytometer grid system was used to estimate the number of lymphocytes. A sample of the PBMCs was diluted 1:1 with tryphan blue dye to a final volume of 20µl. Cell number was estimated according to the following formula:

$$\text{Cell concentration (cells/ml)} = \text{mean cell count} \times \text{dilution factor} \times 10^4$$
$$= \text{mean cell count} \times 2 \times 10^4$$

The cells were resuspended in RPMI 1640 medium to the required concentration and kept in the incubator with 5% $CO_2$ at 37ºC until further use. The maximum incubation period for the cells was two to three days.

### 3.3.2 Determination of cell viability of PBMCs

This assay can be carried out in the microtitre plates or microcentrifuge tubes. The number of surviving cells is dependent on the reduction 3-(4, 4-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) to form a purple-blue formazan crystal by the living cells. MTT was dissolved in PBS at 5mg/ml and filtered using 0.2µm pore size filters for sterilization purpose. MTT working solution (10µl per 100µl medium) was added into the wells containing the cells. The formazan crystals formed were then dissolved in dimethyl sulfoxide (DMSO). The optical density was measured at 570nm test wavelength and 690nm reference wavelength. Absorbance is directly proportional to the number of viable cells present (Mosmann, 1983; Denizot *et al.*, 1986)**.** In this study, 100µl of RPMI 1640 medium containing lymphocytes of different density (100,000 cells/ml, 250,000 cells/ml,

500,000 cells/ml and 1,000,000 cells/ml) were pipetted into microtitre wells in triplicates. This was done under sterile condition. Then, 10µl of MTT solution was added into each well and the plates were incubated at 37ºC, 5% $CO_2$ for four hours for the reaction to take place. Then, 100µl of 100% DMSO was added to solubilize the insoluble purple formazan product to form a colored solution. The absorbance of this colored solution was measured using Powerwave 340 Microtitre-Plate ELISA Reader at wavelength of 570nm. The plates have to be read within an hour after the addition of DMSO (Mosmann, 1983). Appendix E shows the curve for the absorbance versus the number of cells. Figure 3.1 shows the flow chart of MTT assay. The percentage of cell viability was calculated as follows:-

$$\% \text{ of Cell viability} = \frac{\text{Abs}_{\text{Test Sample}} - \text{Abs}_{\text{Test Control}}}{\text{Abs}_{\text{Test Control}}} \times 100\%$$

### 3.3.3 Cytotoxicity and cytoprotective activity of *P. sajor-caju* extracts on PBMCs

#### 3.3.3.1 Determination of hydrogen peroxide ($H_2O_2$) concentration as an oxidant

Hydrogen peroxide ($H_2O_2$) is poorly reactive, freely miscible with water and able to cross cell membranes readily (Halliwell *et al.*, 2000). It can act as a mild oxidizing or as a mild reducing agent, but it does not oxidize most biological molecules readily, including lipids, DNA and proteins. The LD50 values and the mode of cell death induced (apoptosis or necrosis) by $H_2O_2$ depends on the cell type used, its physiological state, length of exposure to $H_2O_2$, the $H_2O_2$ concentration used, and the cell culture media employed (Halliwell *et al.*, 2000). A number of reports have described high ($\geq$ 50 µM) levels of $H_2O_2$ as being cytotoxic to a wide range of animal, plant and bacterial cells in culture (Halliwell *et al.*,

2000). The PBMCs were incubated with various concentrations of $H_2O_2$ (0mM to 800mM) to determine the appropriate concentration to induce cytotoxicty (MTT).

### 3.3.3.2 Effects of *P. sajor-caju* extract on the viability of PBMCs

Hundred microlitre of freshly isolated cells were seeded into the 96-well plate at a density of 5 x $10^4$ cells/ well. Ten microlitre of various concentrations 0µg/ml to 200µg/ml (final concentration) of extracts or phytohemaglutinin (PHA) or $H_2O_2$ (final concentration of 300µM) were introduced to the cells and the cells were then incubated for two hours (Figure 3.2). Phytohemaglutinin (PHA) is known as a mutagen. Thus, in this experiment, three different concentrations of PHA were used to compare the viability of PBMCs mixed with mushroom extract alone. The concentration of PHA used was 10µg/ml (M1), 20µg/ml (M2) and 40µg/ml (M3). Water was used to blank the reading for spectrometer. Percentage of cell viability was calculated as shown in section 3.3.2. All the extracts and controls were diluted in water as the extraction of the processed mushroom was carried out using water. Phytohemaglutinin was used as positive control to maintain the cell viability whereas $H_2O_2$ was used as a negative control to induce toxicity to the PBMCs. The PBMC viability was assessed using the MTT assay (see section 3.3.2).

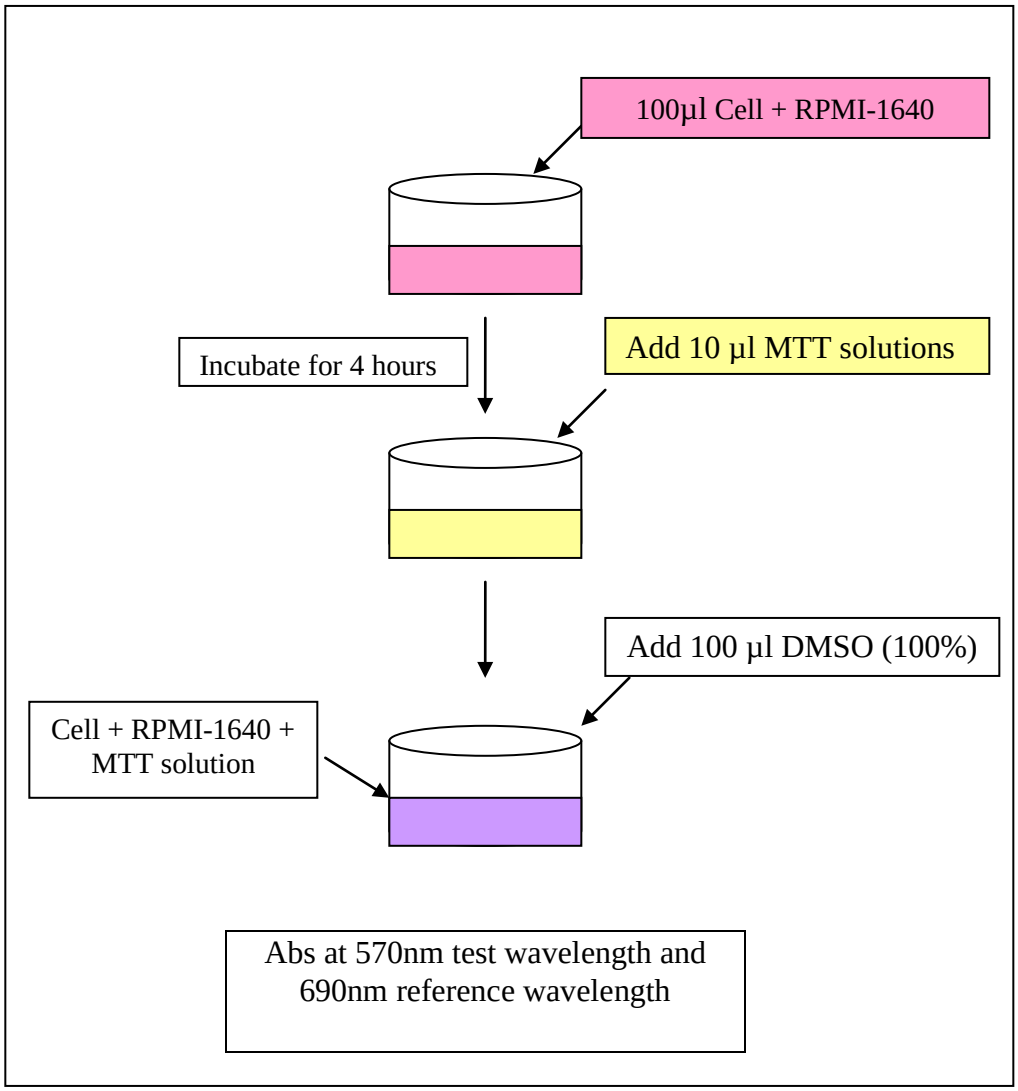



**Figure 3.1: Procedure for MTT assay.**

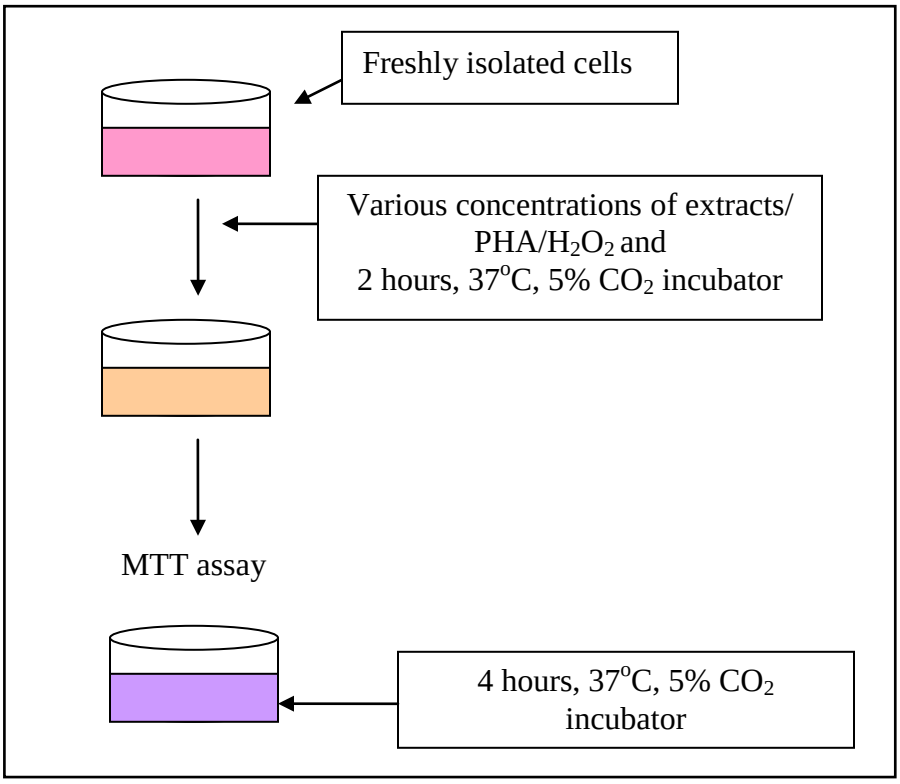



**Figure 3.2: Experimental protocol for the effects of mushroom extracts/PHA/$H_2O_2$ on the viability of PBMCs using MTT assay.**

#### 3.3.3.3 The cytoprotective effects of *P. sajor-caju* extract against $H_2O_2$ induced toxicity on PBMCs

Hundred microlitres of PBMCs in RPMI-1640 medium were seeded in 96 microtitre plate and were incubated with various concentrations of mushroom extracts for two hours at 37ºC, 5% $CO_2$. Subsequently, ten microlitres of $H_2O_2$ (final concentration of 300 µM) was added to the mixture of PBMCs and mushroom extracts (without washing off the mushroom extracts). Then, the mixtures were incubated for another two hours at 37ºC, 5% $CO_2$. Then, ten microlitre of MTT solution was added and the mixtures were incubated for another four hours under the same conditions (Figure 3.3). The viability of PBMCs was determined according to the procedure mentioned in section 3.3.2.

### 3.3.4 Genotoxicity and genoprotective activity of *P. sajor-caju* extracts on PBMCs

#### 3.3.4.1 Comet assay to access DNA damage on PBMCs

The study of DNA damage in individual cells popularly known as comet assay was first described by Ostling and Johanson (1984). In the comet assay, nucleated cells are embedded in low melting point agarose on a microscope slide, and the membrane and histones are removed by high salt solution (Tice *et al*., 2000). Generally, the DNA is organized in a tightly supercoiled form (Cook *et al*., 1976), and alkaline solution is used to unwind this supercoiled DNA for the migration during electrophoresis. The electrophoresis at neutral, mildly alkaline or strongly alkaline conditions is applied to continue the unwinding step (Angelis *et al*, 1999).

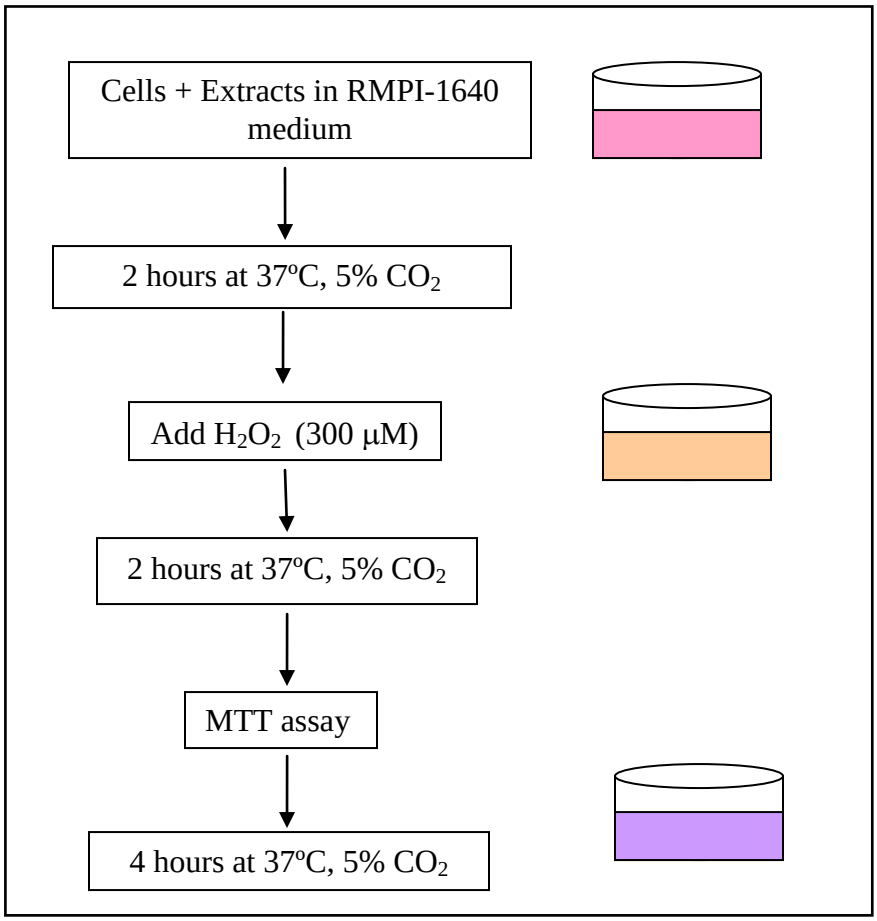



**Figure 3.3: Experimental protocol to study the effects of preincubated *P. sajor-caju* extracts on the viability of PBMCs which then challenged with 300 μM of $H_2O_2$.**

At alkaline pH the phosphate groups in DNA will be negatively charged and the relaxed loops of damaged DNA containing breaks will be pulled towards the anode during electrophoresis, forming a comet 'tail', while the DNA remaining coiled within the nucleoid forms the comet 'head' (Tice *et al*., 2000). The comet is visualized by a DNA staining fluorescent dye or silver stain (Clingen *et al*., 2000). The most frequently used stains are ethidium bromide (Tice *et al*., 2000), propidium iodide (Olive, 2002), 4,6-diamidino- 2-phenylindole (DAPI) (Panayiotidis and Collins, 1997) and YOYO-1 (Singh, 1996). The damaged DNA is scored using either visual or computerized image analysis.

**3.3.4.2 Preparation of slides for single cell gel electrophoresis (SCGE)**

Fully frosted slides and cover slip were placed on the bench. Fully frosted slides were selected because they increase gel bonding and thus increase the stability (Tice *et al*., 2000). Normal melting point (NMP) agarose, 1% (w/v) in PBS was liquefied. Then, 100 µl of the molten agarose was pipetted on one side of the slide and covered with microscope cover slip (Figure 3.4). This first layer is important to promote even and firm attachment of the second layer (Singh *et al*., 1988). The slides were transferred to a metal tray on ice for 5 minutes to allow solidification of the agarose. Then, 20µl of PBMCs, treated with different concentration of $H_2O_2$, *P. sajor-caju* extracts or both were mixed into 50µl of low melting point (LMP) agarose in an Eppendorf tube (Figure 3.4). The mixture was pipetted onto the solidified first layer and cover slips were replaced. The slides were placed on ice and allowed to solidify and the cover slips were removed gently and discarded. The slides were placed on a tray. The concentration of cells in agarose, as well as the concentration of agarose, is important parameters for ensuring a successful analysis (Tice *et al*., 2000).

Overlapping comets were obtained if the density of the cells were too high with high levels of DNA migration. The extent of DNA migration was also affected by higher concentrations of agarose.

After the slides were transferred into a tray, it was submerged in lysing solution containing high salts and detergents overnight at 4 ± 2ºC (Figure 3.4) to break the cells. When treated with alkaline solution the DNA unwinds and this enables the migration of the DNA during electrophoresis. The lysis solution contained EDTA, Tris Base, sodium chloride, sodium dodecyl sulphate (SDS) and Triton-X. The lysing solution was chilled 30 minutes prior to use, primarily to maintain the stability of the agarose gel (Tice *et al*., 2000). At the end of lysing period, the slides were rinsed carefully dipped thrice in TAE buffer in a Coplin jar to remove residual detergents and salts. The slides were drained on paper towel and were ready for electrophoresis.

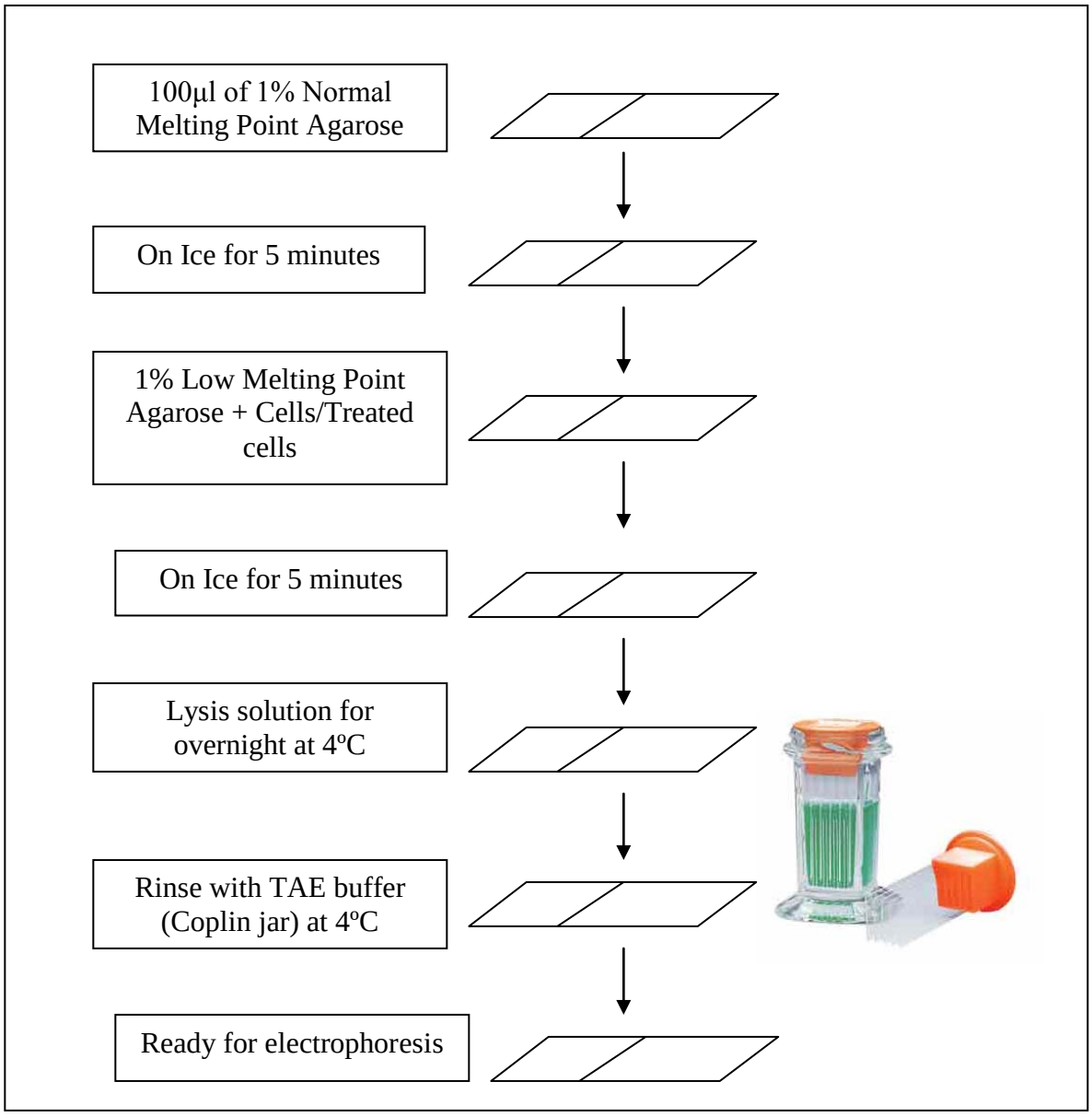



**Figure 3.4: Flow chart of the preparation of slides to be used before electrophoresis for Comet assay.**

### 3.3.4.3 Electrophoresis of microgel slides containing PBMCs in agarose gel

The slides were placed side by side on the horizontal electrophoresis tank near the anode end, sliding them as close together as possible. The buffer reservoir was filled with freshly prepared electrophoresis buffer (which was chilled at least 30 minutes prior to use) until the slides were submerged in the solution. Prior to electrophoresis, the slides were incubated for 40 minutes in alkaline (pH > 13) electrophoresis buffer at four degree Celsius to produce single stranded DNA (ssDNA) and to express the alkaline labile sites (ALS) as single strand breaks (SSB) (Tice *et al*., 2000). The power supply was turned on to 25V after 40 minutes and the current adjusted to 300mA. The duration of the electrophoresis was 20 minutes. Throughout the electrophoresis, the buffer temperature was maintained at four degree Celsius to prevent melting of the LMP layer of the microgel by placing the tank on bed of ice. After the electrophoresis, the power was switched off and the slides were lifted gently from the buffer. The slides were drained on paper towel.

After electrophoresis, the alkaline pH of the gels was neutralized by rinsing the slides with neutralization buffer pH 7.5. Singh *et al*. (1988) proposed three washes of trizma buffer for 5 minutes each. However, increased rinsing may be useful in situations where a high background was seen during scoring (Rojas *et al*., 1999). After, neutralization, the slides were fixed in absolute ethanol for two hours to dehydrate the agarose gel and this enabled longer storage period when compared with non-dehydrated agarose gel (Tice *et al*., 2000).

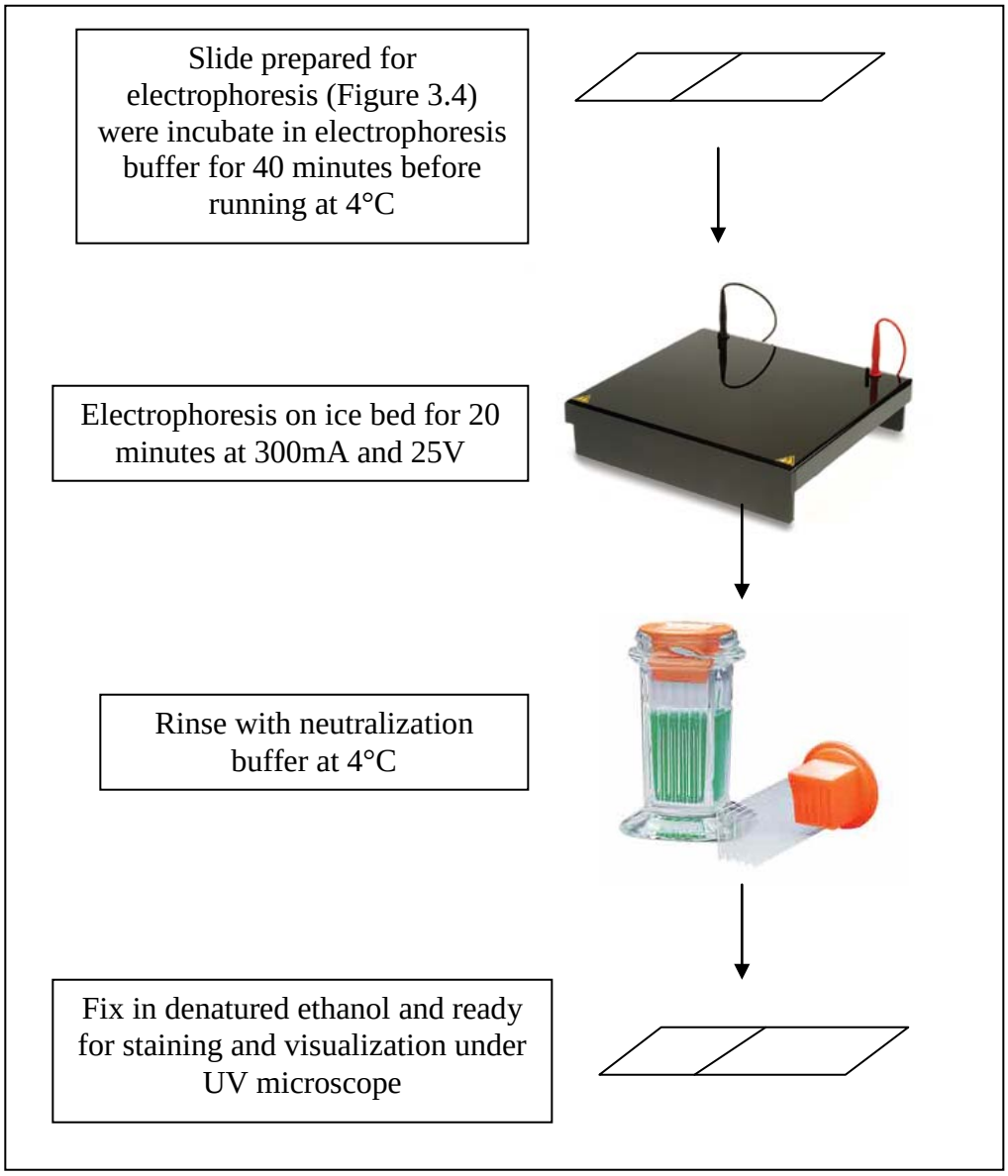



**Figure 3.5: Flow chart of electrophoresis process for comet assay after slides were prepared as shown in Figure 3.4.**

### 3.3.4.4 Effects of $H_2O_2$ on DNA damage of PBMC's

Peripheral blood mononuclear cells were prepared as described in section 3.3.1. Various concentrations of $H_2O_2$ (0mM to 0.3mM) were used to induce DNA damage. After the incubation of PBMCs at 37ºC and 5% $CO_2$ for 30 minutes, the cells were immediately placed in ice for 5 minutes and then slides were prepared and the electrophoresis was carried out as mentioned in section 3.3.4.2 and 3.3.4.3. The electrophoresed slides were stained with ethidium bromide as described in section 3.3.5. The stained slides were viewed under florescent microscope and the percentage of tail and head DNA were recorded. The concentration of $H_2O_2$ that caused 50% DNA damage was selected for the subsequent experiments that required DNA damage to be induced.

### 3.3.4.5 Effects of *P. sajor-caju* extract on DNA of PBMC's

Peripheral blood mononuclear cells (prepared as mentioned in 3.3.1) were incubated with various concentrations (0µg/ml to 200µg/ml) of different processed mushroom extracts for 30 minutes prior to electrophoresis. The concentration of $H_2O_2$ that caused 50% damage on DNA in PBMC's ($IC_{50}$) was used as positive control. After the incubation, the treated cells were incubated in ice for 5 minutes and then slides were prepared and the electrophoresis was carried out as described in sections 3.3.4.2 and 3.3.4.3. The electrophoresed slides were stained with ethidium bromide as described in section 3.3.5. The stained slides were viewed under florescent microscope and the percentage of tail and head DNA were recorded.

#### 3.3.4.6 Protective effects of *P. sajor-caju* extract on $H_2O_2$ induced DNA damage in PBMCs

Peripheral blood mononuclear cells were incubated with various concentrations of extracts ranging from 0µg/ml to 200µg/ml for 30 minutes. Subsequently, the concentration of $H_2O_2$ that caused 50% inhibition was added to the mixture and was incubated for another 30 minutes. After the incubation, the treated cells were incubated in ice for 5 minutes and then slides were prepared and the electrophoresis was carried out as described in sections 3.3.4.2 and 3.3.4.3. The electrophoresed slides were stained with ethidium bromide as described in section 3.3.5. The stained slides were viewed under florescent microscope and the percentage of tail and head DNA were recorded.

### 3.3.5 DNA Staining and Visualization

The slides were stained with 20µg/ml ethidium bromide (EtBr). The DNA specific dye and the magnification used for comet visualization depend largely on investigator-specific needs and presumably have little effect on assay sensitivity or reliability (Tice *et al*., 2000). Comet images were visualized and scored under 200x magnification on a fluorescent microscope. The selection of magnification is dependent on the type of cells, the range of migration to be measured and constraint of the microscope or imaging system (Tice *et al*., 2000).

All the slides, including the positive and negative controls were scored using an image analyzer which is fully automated comet analysis imaging system, where the extent of the migration of DNA was scored individually. The proportional level of SSB and ALS related directly to the DNA fragment size and generally the migration length (Tice *et al*., 2000). The average of head and tail DNA of a certain number of scored cells was calculated using SPSS software or Excel worksheet. Tables and bar graphs were generated from the data collected.

## 3.4 Statistical Analysis

Three replicates of each sample were used for statistical analysis. All the results obtained were expressed as mean ± standard error mean (S.E.M). The SPSS program version 13.0 and GraphPad Prism version 4.1 was used to calculate the significance of the results. Differences at $p < 0.05$ were considered to be significant.

# CHAPTER 4

# RESULTS & DISCUSSION

# CHAPTER 4

# RESULTS AND DISCUSSION

## 4.1 Preparation of aqueous extracts from fruiting bodies of *P. sajor-caju*

*Pleurotus sajor-caju* was processed and extracted using hot water. The fruiting bodies were divided into seven groups of one kilogram each that were processed at different temperatures based on the cooking or industrial processing methods of this edible oyster mushroom. The temperature for each processing method used is given in Table 3.1 (see section 3.1).

The weights of the various *P. sajor-caju* extracts were measured and the values are depicted in Table 4.1. The hot water extract for blanched *P. sajor-caju* contained high content of water (approximately 89%) and this was possibly due to the high water content in the freshly boiled mushroom. The moisture content of the mushroom observed in the present study was similar to that reported by Breene (1990). The dried *P. sajor-caju* lost approximately 90% (w/w) of its water content. The highest loss of water content was observed in freeze dried *P. sajor-caju.* Freeze-drying or lyophilisation is a usual method used in scientific work to remove water from any substance fast and efficiently.

The yield from the final extract of blanched *P. sajor-caju* was approximately eight grams. The total loss of water was more than 99% of the initial weight. This was probably due to the fact that blanching cause's water absorption and that contributed to the initial weight. The yield of the extracts was determined by comparing the mass of the final and initial weight of the *P. sajor-caju*. The highest percentage of yield was obtained from 45 ± 2ºC oven dried followed by sun dried, 50 ± 2ºC oven dried, 60 ± 2ºC oven dried, 55 ± 2ºC oven dried and freeze dried mushrooms. The crude hot water extracts of each processed *P. sajor-caju* were further tested for their cytotoxic and genotoxic activities using MTT assay and Comet assay respectively.

Mushroom polysaccharides exist as a structural component of fungal cell wall (Zhang *et al*., 2007). Their cell wall is composed of two major types of polysaccharides: - 1) A rigid fibrillar chitin (or cellulose) and 2) A matrix–like beta glucan, alpha glucan, and glycoproteins (Zhang *et al*., 2007). Selection of an extraction method depends on the cell wall structure of the mushroom. The hot water extraction has been a popular approach for mushroom extraction, especially to have a higher yield of polysaccharides' that are water soluble (Zhang *et al*., 2007). In addition, Lee *et al.* (2007) reported that the yields of water extracts of *P. citrinopileatus* were significantly higher than those of ethanolic extracts for three types of samples; fruiting bodies, mycelia and culture filtrate. The difference of the yield might be due to the fact that water extracts contained certain amount of soluble polysaccharides that could be precipitated from aqueous suspension of fruiting bodies or mycelia (Lee *et al*., 2007). Hence, in this study, the similar finding on the yield of aqueous extracts was expected.

**Table 4.1: The extraction yields after the processed samples were extracted in boiling water for three hours**

The *P. sajor-caju* obtained from the farmer was weight and processed accordingly. The weight before and after processing was recorded. Then, the different processed *P. sajor-caju* was extracted using aqueous extraction in 95ºC hot water bath. The weights of the lyophilized extracts were determined using weighing balance. The percentage of extraction (%) was calculated using this formula:-

(Lyophilized extract (g) ÷ Weight after processing (g) x 100%).

| ***Pleurotus sajor-caju*** | **Weight before (g)** | **Weight after processing (g)** | **Extracted with boiling water (L)** | **Lyophilized extract (g)** | **Percentage of Extraction (%)** |
|---|---|---|---|---|---|
| **Blanching (P1)** | 1000 | 882.5 | 2.5 | 8.4 | *1.0* |
| **Sun dry (P2)** | 1000 | 95.0 | 1.0 | 27.8 | *29.3* |
| **Freeze dry (P3)** | 1000 | 103.1 | 0.8 | 18.5 | *17.9* |
| **45º oven dry (P4)** | 1000 | 96.7 | 1.0 | 29.4 | *30.4* |
| **50º oven dry (P5)** | 1000 | 105.0 | 1.2 | 23.8 | *22.7* |
| **55º oven dry (P6** | 1000 | 104.4 | 1.1 | 22.7 | *21.7* |
| **60º oven dry (P7)** | 1000 | 103.6 | 1.1 | 23.0 | *22.2* |

## 4. 2 Antioxidant potential and polyphenol content of *P. sajor-caju* extracts

### 4.2.1 Antioxidant activity assay

#### 4.2.1.1 Total phenolic content (TPC) of aqueous extracts of *P. sajor-caju*

In this study, the phenol content of different processed *P. sajor-caju* was investigated. The total phenolic content was expressed as mg of GAEs/g of dry mushroom (Figure 4.1). The amount of phenolic compounds in extracts P2 (254.63±4.39 mg GAEs/g of extract) and P4 (257.56±3.62 mg GAEs/g of extract) were among the highest with $p<0.05$, followed by P5, P6 and P7 (approximately 230 mg GAEs/g of extract). The blanched (P1) mushroom extract contained the least amount of phenols (approximately 185 mg GAEs/g of extract). Based on the earlier results (Table 4.1), P2 and P4 had comparatively higher phenolic content could be due to their different processing methods. Meanwhile, the lowest extraction yield was in P1.

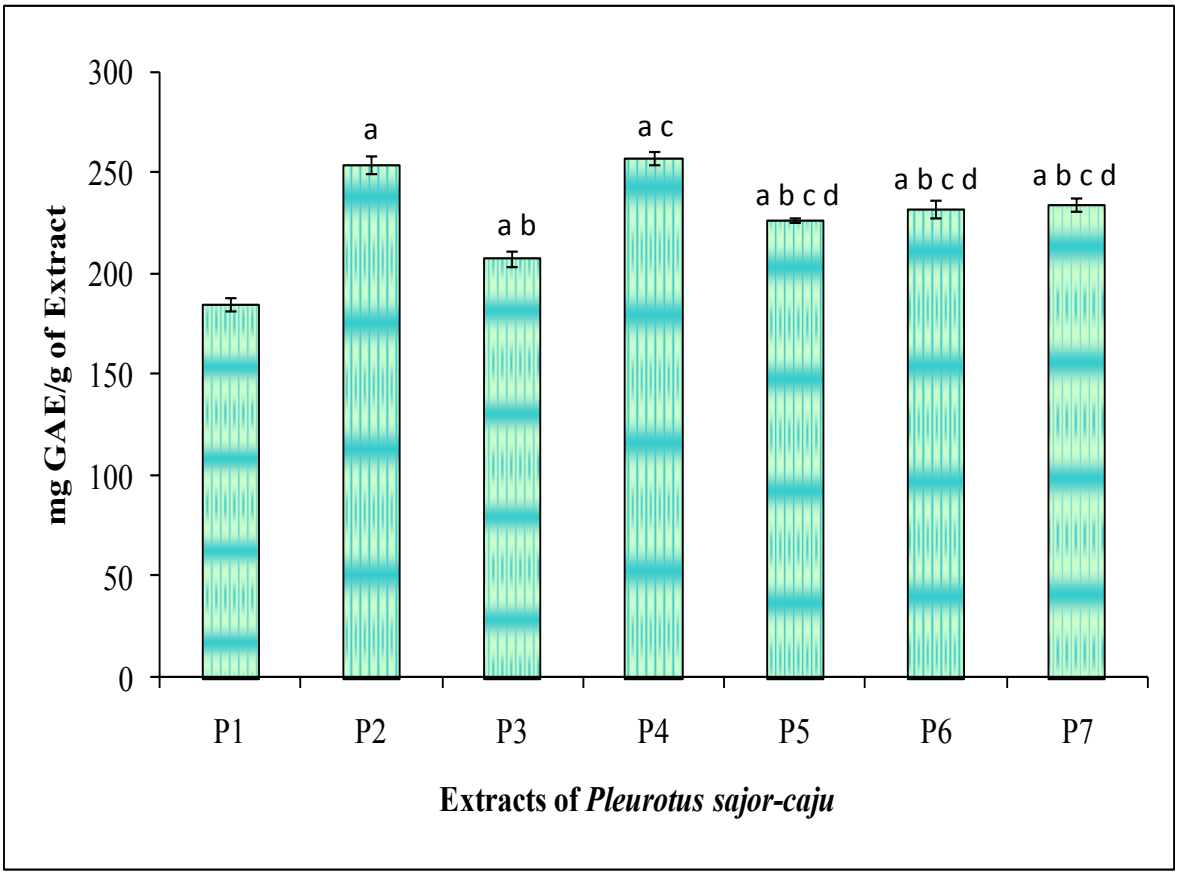



**Figure 4.1: Total phenolic contents of various aqueous extracts of different processed *P. sajor-caju*.**

The TPC levels for the extracts were obtained from standard curve (Appendix A). P1: blanched, P2: Sun dried, P3: Freeze dried, P4: 45ºC oven dried, P5: 50ºC oven dried, P6: 55ºC oven dried and P7: 60ºC ovens dried. The values were calculated and expressed as mg of Gallic Acid Equivalent (GAE) for every g of dry extracts (mg GAE/g). Results are represented as mean (n=3) ± S.E.M. of three separate experiments. One way ANOVA test with Bonferonni's post-test was used to compare the difference between each group; a: denotes $p < 0.05$ when compared to P1, b: denotes $p < 0.05$ when compared to P2, c: denotes $p < 0.05$ when compared to P3 and d: denotes $p < 0.05$ when compared to P4.

Phenolics are one of the major groups of non-essential dietary components that have been associated with the inhibition of atherosclerosis and cancer (Teissedre *et al*., 1996). The bioactivity of phenolics may be related to their ability to chelate metals, inhibit lipoxygenase activity and scavenge free radicals (Decker, 1997). Mushrooms accumulate a variety of secondary metabolites, including phenolic compounds, polyketides, terpenes and steroids. Velioglu *et al*., (1998) reported that there is a correlation between the antioxidant activities of plant extracts with the content of their phenolic compounds. In addition, Cheung *et al*. (2003) reported that the phenolic compound found in edible mushrooms such as *L. edodes* and *V. volvacea* could make a significant contribution to the antioxidant activity.

#### 4.2.1.2 DPPH free radical scavenging activity assay on processed mushroom extracts

Figure 4.2, depicts DPPH radical scavenging activity by different processed *P. sajor-caju* extracts. The aqueous extracts of *P. sajor-caju* were shown to scavenge the stable DPPH radical directly to different extents at a concentration of 5mg/ml (initial concentration) with the minimum percentage of inhibition exerted by extracts P1 and P3. In general, all the extracts depicted low scavenging activity. However, from this result it can be assumed that the extracts are a free radical inhibitor or scavenger, acting possibly as primary antioxidants.

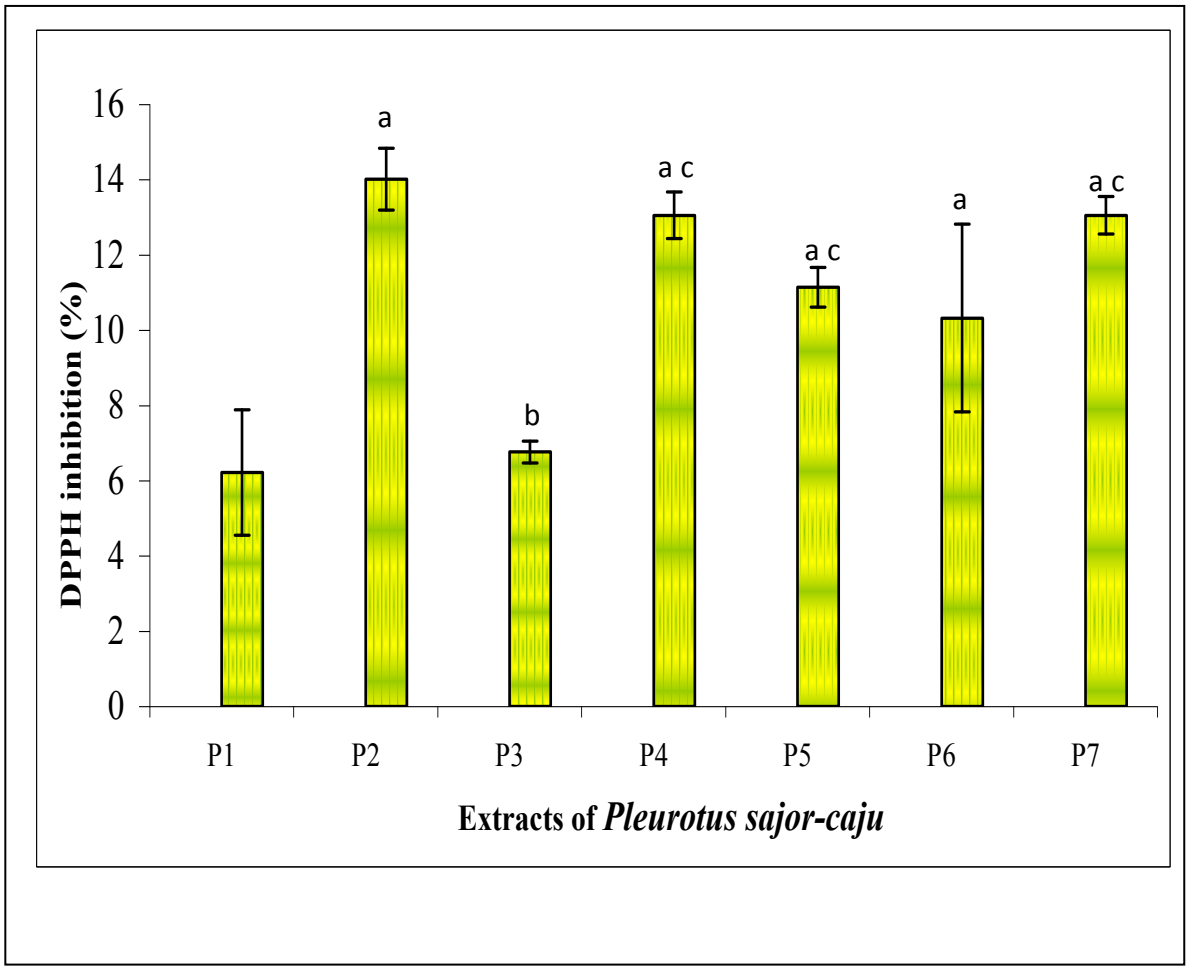



**Figure 4.2: Scavenging ability of various processing methods of *P. sajor-caju* aqueous extracts on 1,1-diphenyl-2-picrylhydrazyl radicals (DPPH).**

The DPPH levels for the extracts were obtained from standard curve (Appendix B). P1: blanched, P2: Sun dried, P3: Freeze dried, P4: 45ºC oven dried, P5: 50ºC oven dried, P6: 55ºC oven dried and P7: 60ºC ovens dried. The values were calculated and expressed as percentage of DPPH inhibition (%). Results are represented as mean (n=3) ± S.E.M. of three separate experiments. One way ANOVA test with Bonferonni's post-test was used to compare the difference between each group; a: denotes $p < 0.05$ when compared to P1, b: denotes $p < 0.05$ when compared to P2, c: denotes $p < 0.05$ when compared to P3, d: denotes $p < 0.05$ when compared to P4 and e: denotes $p < 0.05$ when compared to P6.

Free radical scavenging is one of the known mechanisms by which antioxidants inhibit lipid oxidation. The method of scavenging DPPH free radicals can be used to evaluate the antioxidant activity of specific compounds or extracts in a short time. The scavenging abilities of hot water extracts of *G. tsugae, A. cylendracea*, *H. marmorues* and *P. citrinopleatus* fruiting bodies at 20 mg/ml were 73.8-80.1%, 66.2%, 77.2% and 52.3% respectively (Lee *et al*., 2007). Apparently, the scavenging ability of *P. sajor-caju* hot water extracts at 5mg/ml was less effective (Figure 4.2) than those mentioned above. This could be caused by the concentration of the extract or the extraction method might play an important role in the free radical scavenging activity. Lee *et al*. (2008) reported that ethanolic extracts showed higher scavenging activities on DPPH radical than hot water extracts. The scavenging activities of the fruiting bodies of the white mutant of *H. marmoreus* at 5mg/ml were 75.5% for ethanolic extracts and 36.8% for hot water extracts, respectively (Lee *et al*., 2008). At 10 mg/ml, scavenging abilities were 94.8% for ethanolic extracts and 40.3% for hot water extracts from fruiting bodies of this mushroom, respectively. This shows that at two different concentrations the mushrooms extracts conferred different level of scavenging activities regardless of the method of extraction employed.

Generally, the aqueous/solvent extracts contained antioxidant components regardless of their processing method, which could react rapidly with DPPH radicals, and reduce most DPPH radical molecules (Brand-Williams *et al*., 1995). Hence, this result discloses that the *P. sajor-caju* extracts are free radical inhibitors or scavengers, acting possibly as primary antioxidants. The scavenging activity of the extracts might be due to the presence of hydrogen-donating components in the mushroom extracts (Mau *et al*.,

2002). Although the different processed *P. sajor-caju* extracts showed lower than 20% scavenging activity compared to other reported mushrooms as mentioned above, one should not rule out the possibility that the *P. sajor-caju* aqueous extracts might react with free radicals, particularly peroxy radicals, which are the major propagator of autooxidation chain of fat, thereby terminating the chain reaction (Mau *et al*., 2002). Antioxidant activity of natural antioxidants has been shown to be involved in termination of free radical reaction (Shimada *et al*., 1992; Tanaka *et al*., 1988).

#### 4.2.1.3 Ferric reducing antioxidant power (FRAP) assay of *P. sajor-caju* crude extracts

In this experiment the reducing power of seven different processed mushrooms was assessed. Figure 4.3 depicted that P2 and P7 had the highest reducing power with 49.66±0.94 µmol FRAP/ g extract and 48.33 µmol FRAP/ g extract, respectively. P1 and P3 showed almost the same amount of reducing power (38 µmol FRAP/ g extracts). Tsai *et al*., (2007) reported that *P. citrinopileatus* showed a high reducing power of 1.03 at 5 mg/ml.

The reducing capacity of various extracts might be due to its hydrogen-donating ability, as described by Shimada *et al.* (1992). Therefore, the extracts might contain reductones (strong reducing agents commonly derived from saccharides by oxidation at the carbon atom alpha to the carbonyl function), which could react with free radicals to stabilize and terminate radical chain reactions.

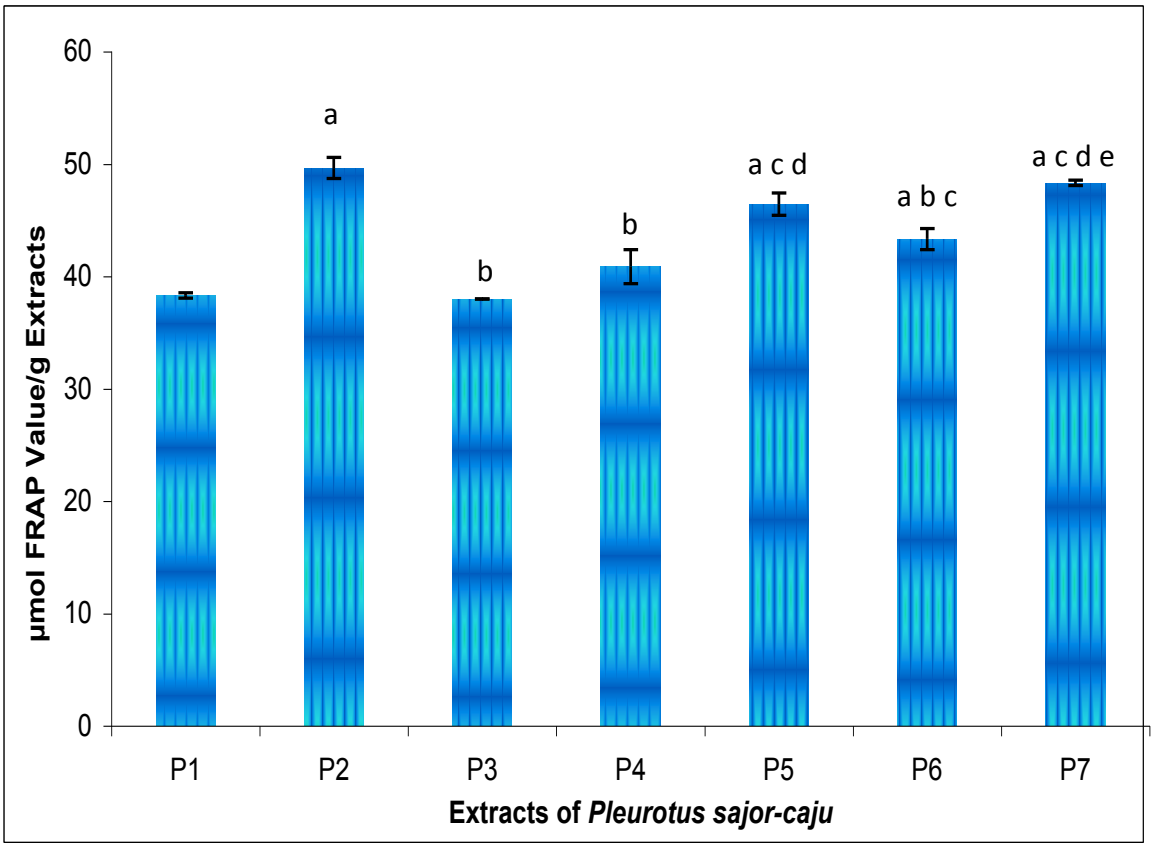



**Figure 4.3: Ferric reducing antioxidant power (FRAP) level of various aqueous extracts of different processed *P. sajor-caju*.**

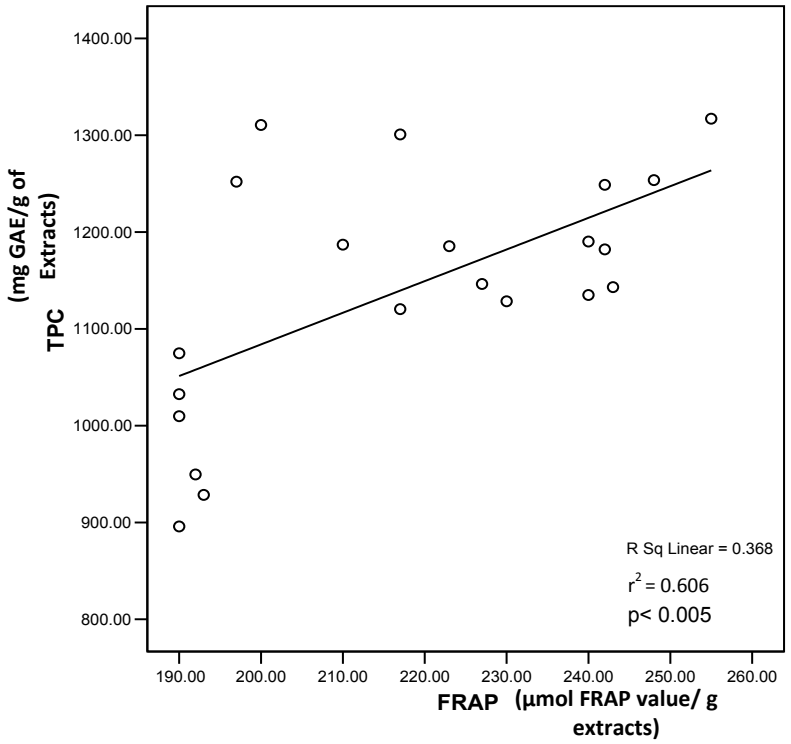
The FRAP levels for the extracts were obtained from standard curve (Appendix C). P1: blanched, P2: Sun dried, P3: Freeze dried, P4: 45ºC oven dried, P5: 50 ºC oven dried, P6: 55ºC oven dried and P7: 60ºC ovens dried. The values were calculated and expressed as µmol FRAP Value for every g of dry extracts (µmol FRAP Value/g). Results are represented as mean (n=3) ± S.E.M. of three separate experiments. One way ANOVA test with Bonferonni's post-test was used to compare the difference between each group; a: denotes $p < 0.05$ when compared to P1, b: denotes $p < 0.05$ when compared to P2, c: denotes $p < 0.05$ when compared to P3, d: denotes $p < 0.05$ when compared to P4 and e: denotes $p < 0.05$ when compared to P6.

### 4.2.3 Correlation

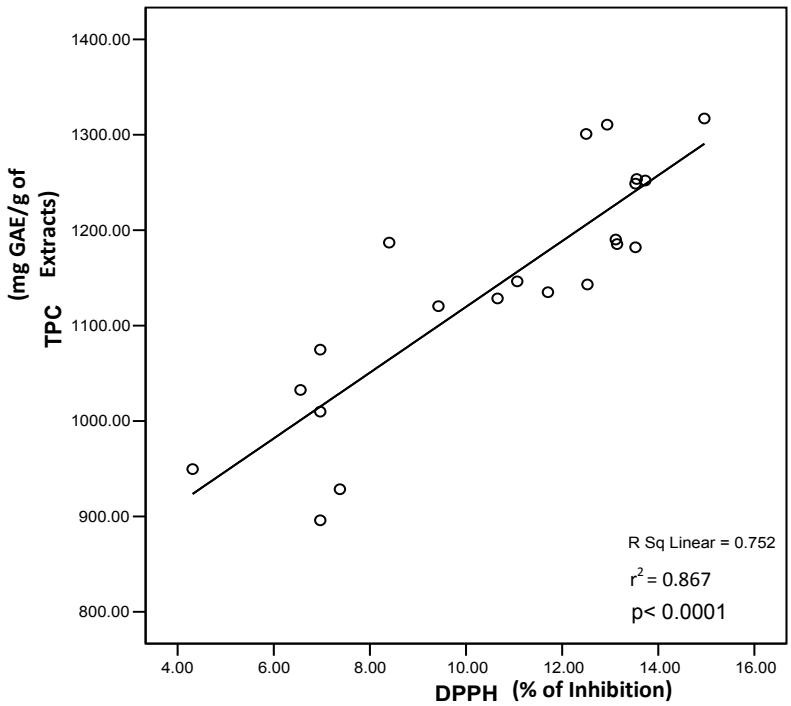
The correlation between total phenol content, FRAP level and DPPH scavenging activity was carried out. . Figure 4.4a-c showed the correlation (linear regression curve) for TPC and FRAP, TPC and DPPH as well as FRAP and DPPH respectively. The strongest correlation coefficient and regression was observed between scavenging activity of DPPH and phenolic content in processed mushroom ($r^2$ = 0.867, $R^2$ = 0.752 with $p<0.0001$) (Figure 4.4b). The lowest correlation coefficient and regression ($r^2$ = 0.606, $R^2$ = 0.368 with $p<0.005$) was present between phenolic content and FRAP in the processed mushroom (Figure 4.4a). Thus, the phenolic compounds present in the *P. sajor-caju* extracts contribute to free radical scavenging activity and less of the reducing activity.

Effective antioxidants can be found in compound with phenol group such as BHT and gallate (Madhavi *et al*., 1996). Phenolic compounds are well known for their ability to scavenge free radicals and chelating ferrous ions which make them the best candidate as antioxidant, antimutagen and anticancer (Ahmad and Mukhtar, 1999). Thus, the higher content of phenols in the fruiting bodies of *P. sajor-caju* extracts was responsible for their better antioxidant properties. In general, all the different extract of processed *P. sajor-caju*, contained antioxidant properties which were moderate to high at 5mg/ml. The P2 and P4 had the highest content of phenols; FRAP level and free radical scavenging activity, where as the P1 showed the lowest antioxidant and phenolic content amongst the extracts tested.

(a)


1400.00
1300.00
1200.00
1100.00
1000.00
900.00
800.00
(mg GAE/g of Extracts)
TPC
190.00
200.00
210.00
220.00
230.00
240.00
250.00
260.00
FRAP (µmol FRAP value/ g extracts)
R Sq Linear = 0.368
r2 = 0.606
p< 0.005

(b)


1400.00
1300.00
1200.00
1100.00
1000.00
900.00
800.00
(mg GAE/g of Extracts)
TPC
4.00
6.00
8.00
10.00
12.00
14.00
16.00
DPPH (% of Inhibition)
R Sq Linear = 0.752
r2 = 0.867
p< 0.0001

(c)

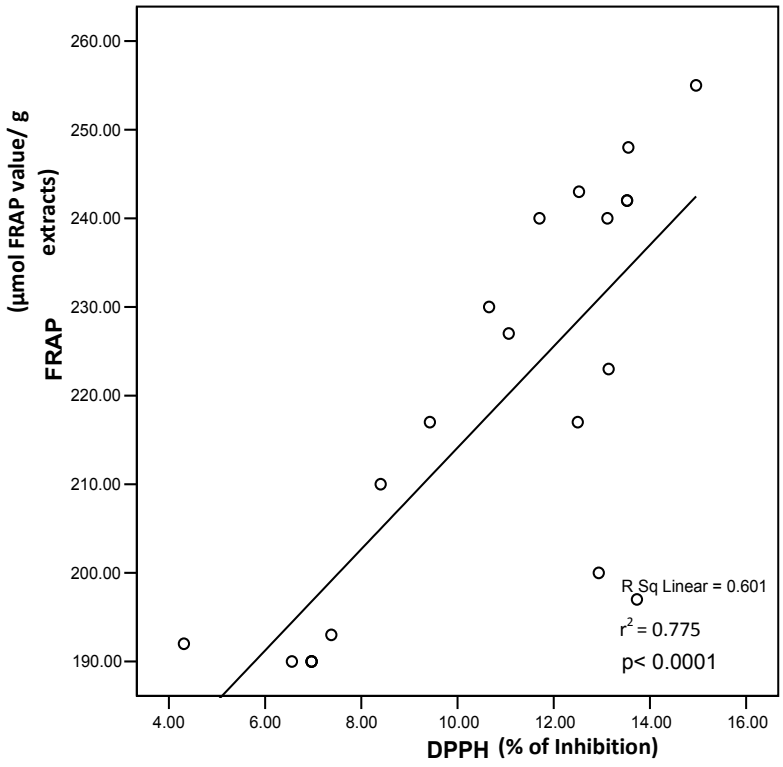



**Figure 4.4: Correlation between TPC, DPPH and FRAP of different processed aqueous extracts of *P. sajor-caju*.**

Correlation of TPC levels, DPPH scavenging activities and FRAP levels of seven processed *P. sajor-caju* extracts were assessed using Pearson correlation analysis. Panels (a) depicts the correlation between TPC and FRAP levels, panel (b) depicts correlation between TPC levels and DPPH scavenging activities and panel (c) shows the correlation between FRAP levels and DPPH scavenging activities.

Generally, the *P. sajor-caju* extracts showed moderate polyphenolic content, RAP value and DPPH scavenging activities regardless of their processing methods. Lee *et al*. (2007) reported the correlation of TPC– FRAP, TPC-DPPH and FRAP-DPPH with the correlation coefficients ($r^2$) of 0.527, 0.556 and 0.425, respectively. Alternatively, in this study the correlation coefficient ($r^2$) that was established was higher (Figure 4.4a-c) compared to correlation coefficients reported by Lee *et al*. (2007). Hence, it shows that processed *P. sajor-caju* extracts can still be a reliable source of antioxidants and phenols for health benefits.

During food processing, considerable changes in level and nature of antioxidant activity can frequently occur as a result of various reactions such as hydrolysis, oxidation and the Maillard reaction (Konecny *et al*., 2009). In future, a detailed study of antioxidant properties of the different processed *P. sajor-caju* must be carried out to identify the mechanism and the specific phenolic component/s that is/are present in the extracts. The fractionation of these processed mushrooms will help us to discover the specific phenolic component mentioned above.

## 4.3 Determination of viable cells

### 4.3.1 Determination of hydrogen peroxide ($H_2O_2$) concentration as an oxidant

The cells were incubated with $H_2O_2$ concentrations ranging from zero to 800mM (final concentration). However, at concentrations above 10mM, $H_2O_2$ showed an apparent increase in cell viability (Figure 4.5). In order to rule out the possible interference of the RPMI medium in the MTT assay, the assay was carried out using cell-free medium. Figure 4.6 shows an increase in absorbance with increasing concentration of $H_2O_2$ (5mM to 800mM). It is possible that the media, MTT solution or the $H_2O_2$ concentrations have reactions between each other that showed false positive reactions. Hence, the identification of $IC_{50}$ was not possible and the range of $H_2O_2$ that was used in this experiment was limited to a maximum of 1mM. The cell viability of the PBMCs exposed to $H_2O_2$ concentrations ranging from 0 – 1mM is depicted in Figure 4.7. Hydrogen peroxide concentration of 0.3mM was used as the baseline or control to induce a mild cytotoxic effect (25% inhibition of cell viability). Kuppusamy *et al.* (2009) reported that 5µM of $H_2O_2$ can cause 50% inhibition on PBMCs; however, some other reports have shown a higher concentration of $H_2O_2$ can be employed to induce required toxicity on PBMCs as there are variations in susceptibility of PBMC from different individual to $H_2O_2$ (Kuppusamy *et al*., 2009).

Hydrogen peroxide appears to be a ubiquitous molecule and poorly reactive. It is relatively stable and is known for its capacity to diffuse across cellular membrane (Knaapen, 2006). They can be a mild oxidizing or reducing agent but it does not oxidize most biological molecules such as lipids, DNA and proteins unless the latter have hyper-

reactive thiol groups or methionine residues (Halliwell *et al*., 2000). Various report has been published on the levels of $H_2O_2$ as being cytotoxic (usually > 50μM) to a wide range of animal, plant and bacterial cells in culture (Halliwell *et al*., 2000). However, the LD50 values and the mode of cell death (either apoptosis or necrosis) induced by $H_2O_2$ depend on the cell type used, its physiological state, length of exposure to $H_2O_2$, the $H_2O_2$ concentration used, and the cell culture media in use (Halliwell and Gutridge, 2003).

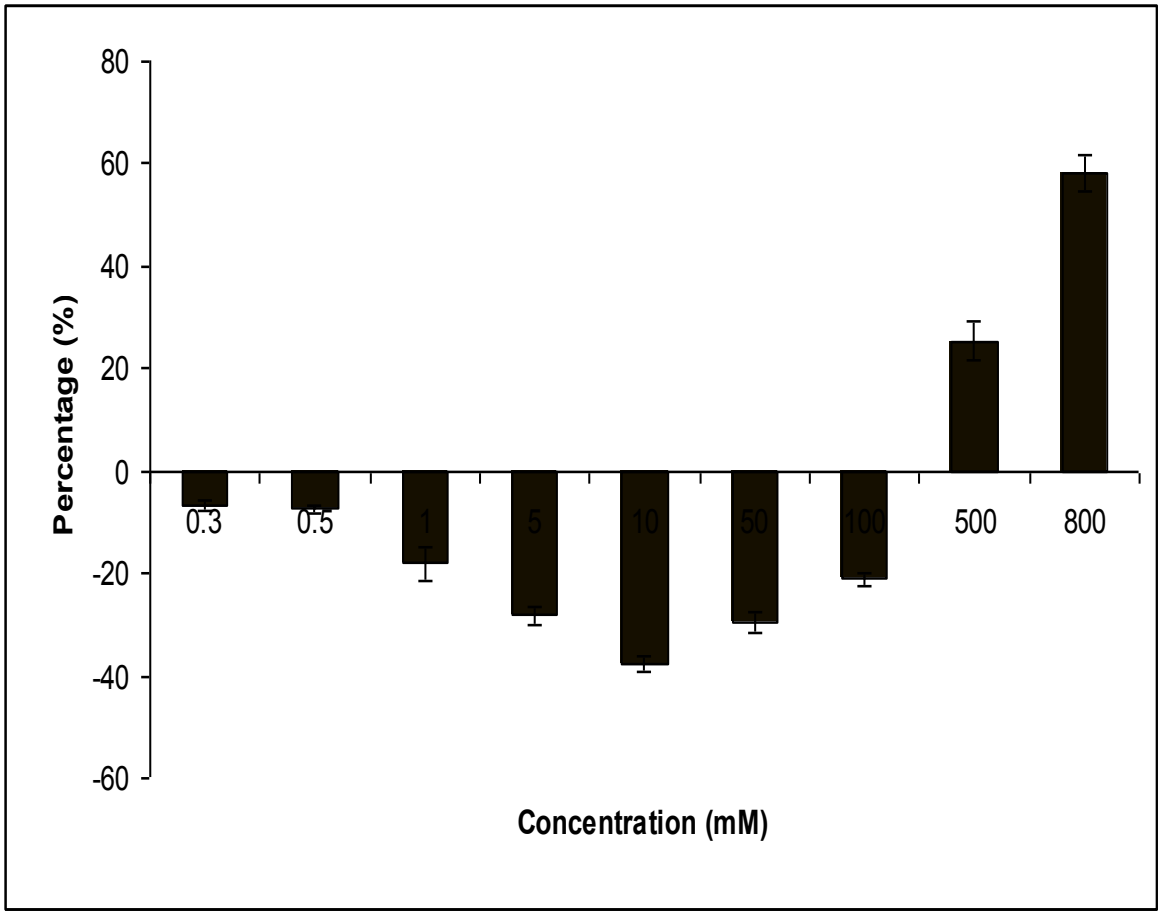



**Figure 4.5: Determination of the cell viability of PBMC in the presence of $H_2O_2$ in RPMI-1640 medium.**

Hydrogen peroxide concentrations ranging from 0 – 800mM were incubated for two hours with the concentration of 50,000 cells/well. The absorbance of the mixture was read after 4 hours of incubation (with 10µl MTT solution) at 37ºC and 5% of carbon dioxide ($CO_2$). The data presented as mean (n=3) ± S.E.M as a single representation of three separate experiments.

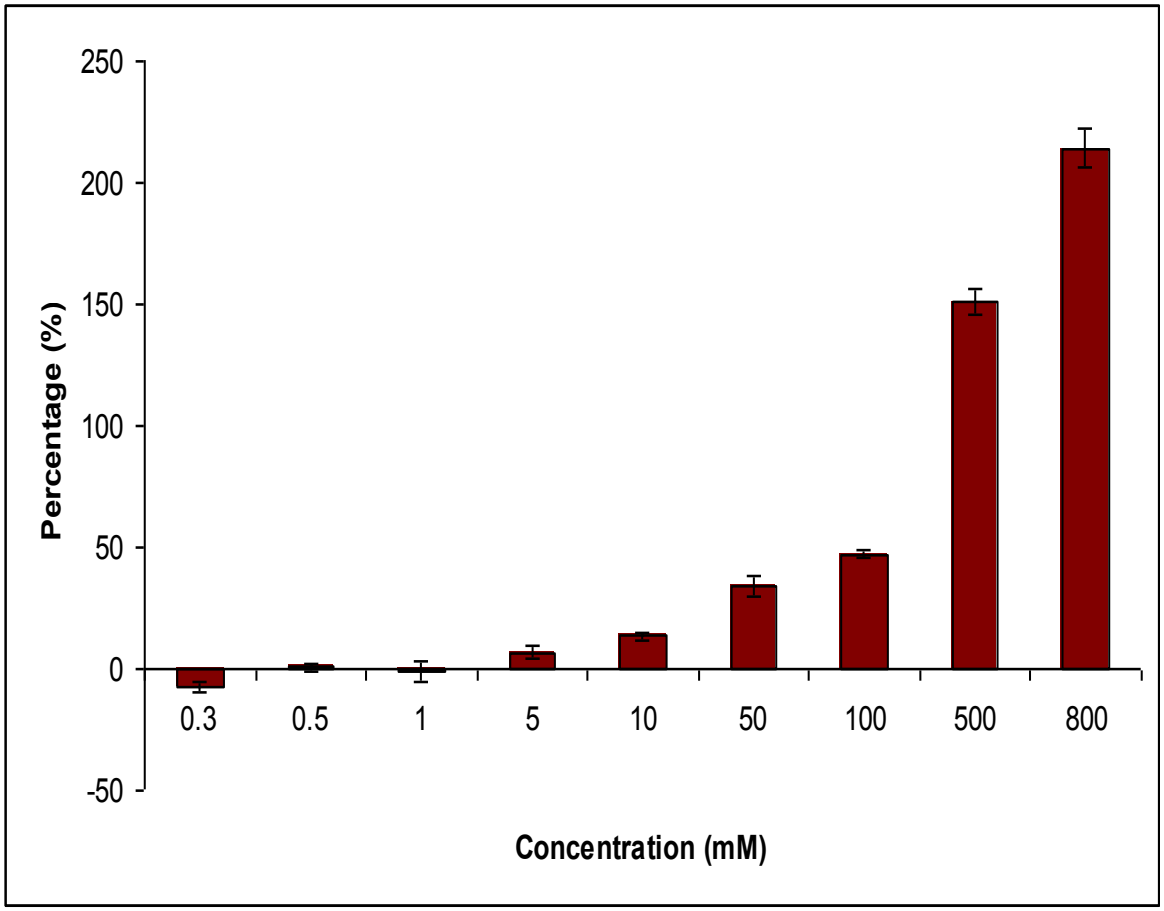



**Figure 4.6: Determination of the cell viability of PBMC in the absence of $H_2O_2$ in RPMI-1640 medium.**

Hydrogen peroxide concentrations ranging from 0 – 800mM were incubated for two hours with the concentration of 50,000 cells/well. The absorbance of the mixture was read after 4 hours of incubation (with 10µl MTT solution) at 37ºC and 5% of carbon dioxide ($CO_2$). The data presented as mean (n=3) ± S.E.M as a single representation of three separate experiments.

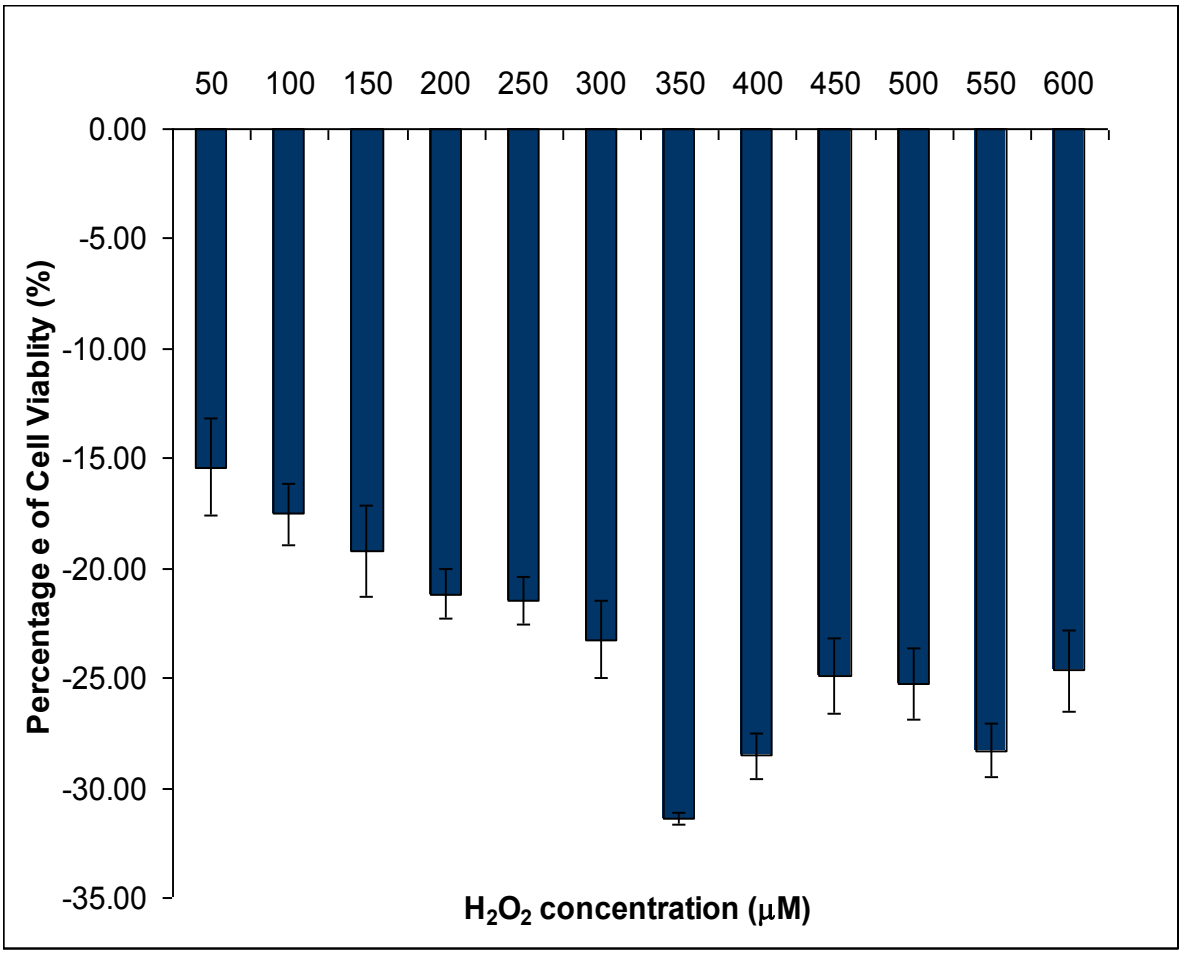



**Figure 4.7: Dose response effects of $H_2O_2$ on PBMC viability.**

Hydrogen peroxide concentrations ranging from 50µM to 600µM were incubated for 2 hours with the concentration of 50,000 cells/well. The absorbance of the mixture was read after 4 hours of incubation (with 10µl MTT solution) at 37ºC and 5% of carbon dioxide $CO_2$). The data presented as mean (n=3) ± S.E.M as a single representation of three separate experiments.

Higher concentration of $H_2O_2$ will be more susceptible to diffuse into the cell membranes. Hydrogen peroxide is hazardous to human tissues when it is either exposed to ultraviolet light or interacts with a range of transition metal ions which will convert them into reactive hydroxyl radical ($^{\bullet}OH$) (Ueda *et al*., 1996). On the other hand, $H_2O_2$ can contribute to Fenton chemistry not only by being one of the substrates but also by liberating iron from heme proteins (Halliwell *et al*., 2000). Addition of $H_2O_2$ to cells in culture can lead to transition metal ion-dependent $^{\bullet}OH$ mediated oxidative DNA damage, although this damage appears to be rapidly repaired provided that the cells have not turned non-viable by an excess of $H_2O_2$ (Spencer *et al*., 1996).

In this study, $H_2O_2$ showed a significant interference or false positive cell viability ($p<0.05$) at concentrations higher than 1mM (Figure 4.5 and 4.6). Thus, the results were considered acceptable for the concentration of $H_2O_2$ used in this experiment (0.3mM) which showed no significant interference at lower concentration of $H_2O_2$ (Figure 4.5 and 4.6). Halliwell (2003) have reported that resistance of cells to damage by ROS varies widely, depending on the extent to which the cell has adapted to the oxidative stress of the cell culture environment. Low level of $H_2O_2$ can have the paradoxical effects of accelerating proliferation in some cell type as depicted in Figure 4.5 and 4.6 (Halliwell, 2003). Most culture media maintained the survival of cells in the culture with the addition of foetal bovine serum which contains some antioxidant enzymes including low level of catalase; superoxide dismutase and glutathione peroxidase (Halliwell, 1990). Hence, the presence of these enzymes could be the possible effect of protection on the PBMCs against high concentration of $H_2O_2$.

However, in this study, the cytotoxic effects of $H_2O_2$ are the main concern in understanding the *in vitro* mechanism of *P. sajor-caju* extracts as potential antioxidant that could protect the normal cells from known oxidant ($H_2O_2$). Therefore, the 25% inhibition shown by $H_2O_2$ on PBMCs is considered acceptable to be employed throughout this study. The percentage of inhibition showed by $H_2O_2$ are significant compared to the cell with no $H_2O_2$ treatment i.e. blank (Figure 4.9). Hence, MTT assay was used to determine the cell survival of PBMCs with exposure to various extracts (processed *P. sajor-caju*) and $H_2O_2$.

### 4.3.2 Effects of *P. sajor-caju* extract on the viability of PBMCs

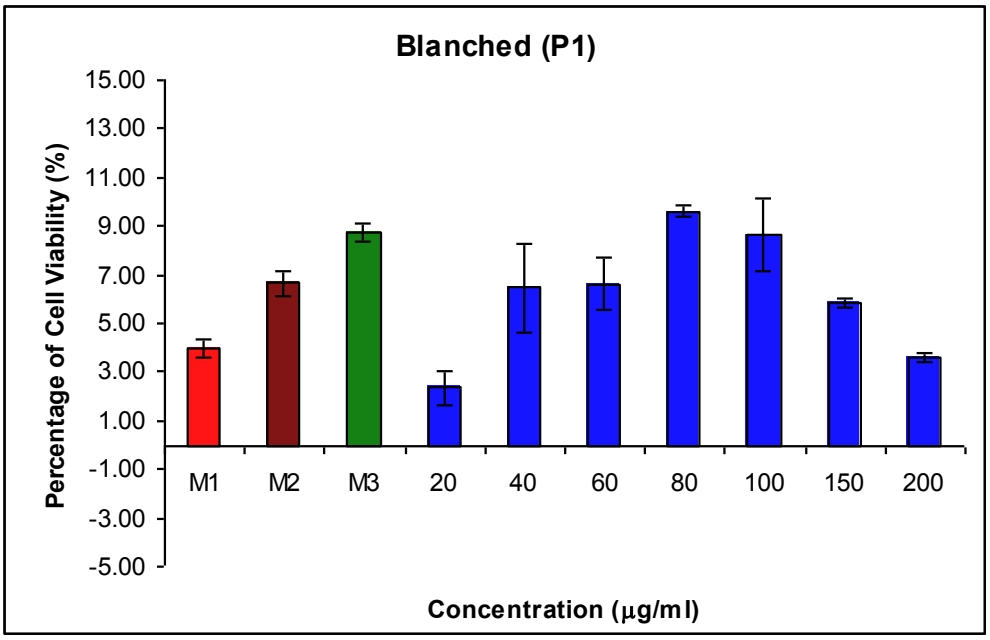
In the present study, a simple colorimetric assay (MTT assay) was used to estimate the cell viability in PBMC in the presence of $H_2O_2$ and or seven different processed *P. sajor-caju* (Section 3.3.3). Figures 4.10a to Figure 4.10g show the effects of crude water extracts of seven processed *P. sajor-caju* on PBMC viability. None of the processed *P. sajor-caju* extracts showed any cytotoxic effect (Figure 4.8a – g). Thus it is pertinent to suggest that the mushroom (*P. sajor-caju*) is non toxic to human PBMC regardless of the temperature at which the mushroom was processed.

All the *P. sajor-caju* extracts showed higher cell viability (10%) compared to positive control cells incubated with PHA for two hours of incubation with PBMCs. The incubation period of two hours was used to have a quick look at the effect of *P. sajor-caju* extracts on the viability of PBMCs. This could give an idea of rapid effect of *P. sajor-caju* extracts on cells. In addition, in order to perform comet assay, a cytotoxicity test should be

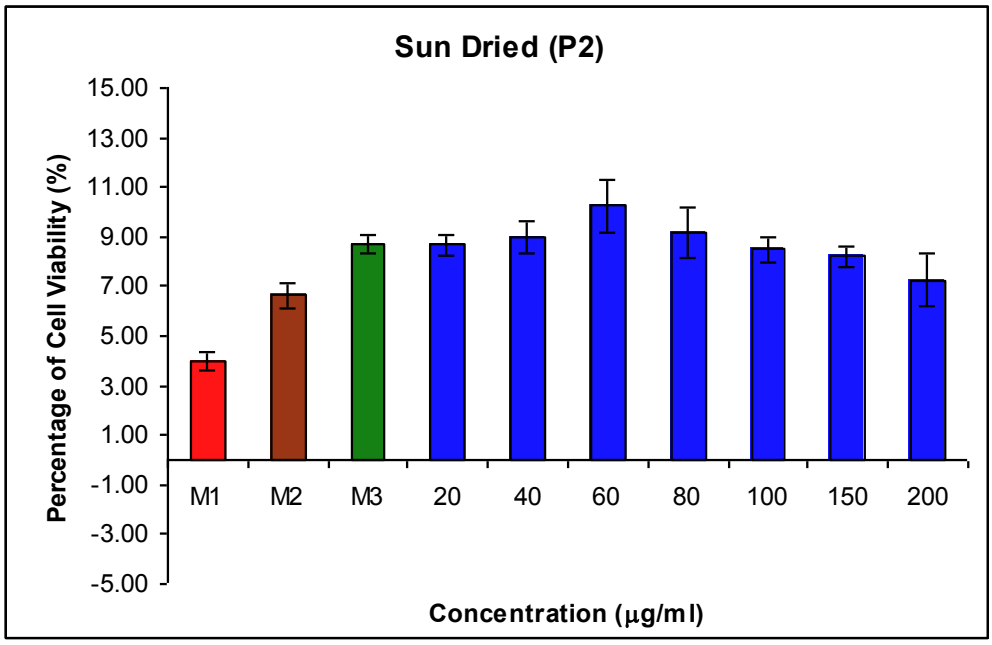
performed earlier to determine the non-cytotoxic concentration of the extracts to better evaluate cytoprotective effects and induced DNA damage (Hartmann and Speit, 1997). Hence, a quick cytotoxic assay with the extracts indicates that the extracts are non toxic to the normal cells.

Drug development programs for the identification of new cancer chemotherapeutic agent involve extensive pre-clinical evaluation of vast number of chemicals (Wilson, 1986). Since secondary metabolites from natural sources have been elaborated within living systems, they are often perceived as showing more "drug-likeness and biological friendliness than totally synthetic molecules," (Koehn and Carter, 2005) making them good candidates for further drug development (Balunas and Kinghorn, 2005; Jones *et al*., 2006). The secondary metabolites from plants can be toxic to human cells, especially in killing the cancer cells. Thus, they are referred to as cytotoxic agents.

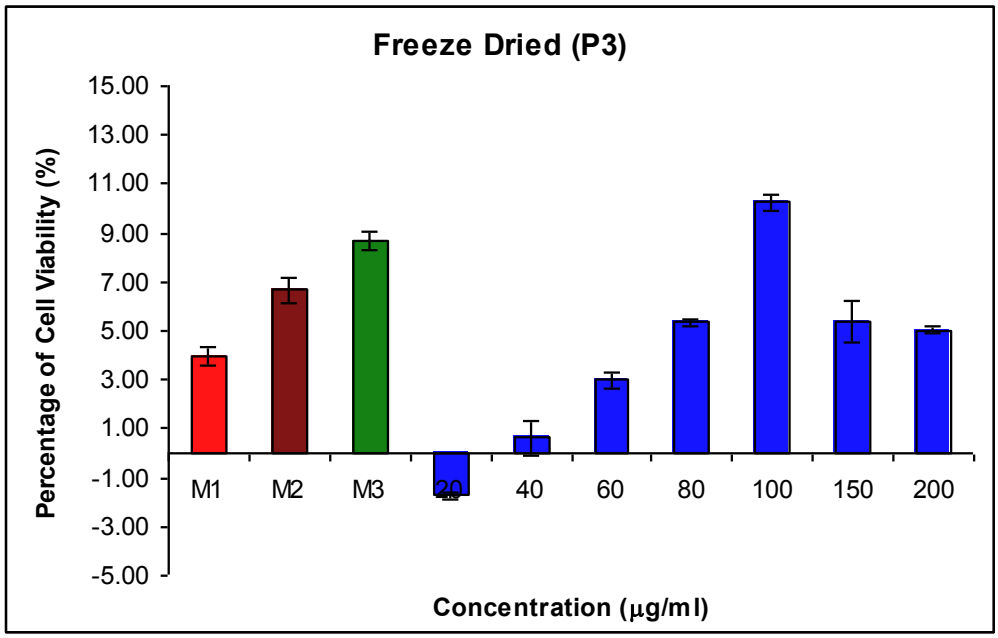
(a)


Blanched (P1)
Percentage of Cell Viability (%)
15.00
13.00
11.00
9.00
7.00
5.00
3.00
1.00
-1.00
-3.00
-5.00
M1
M2
M3
20
40
60
80
100
150
200
Concentration (µg/ml)

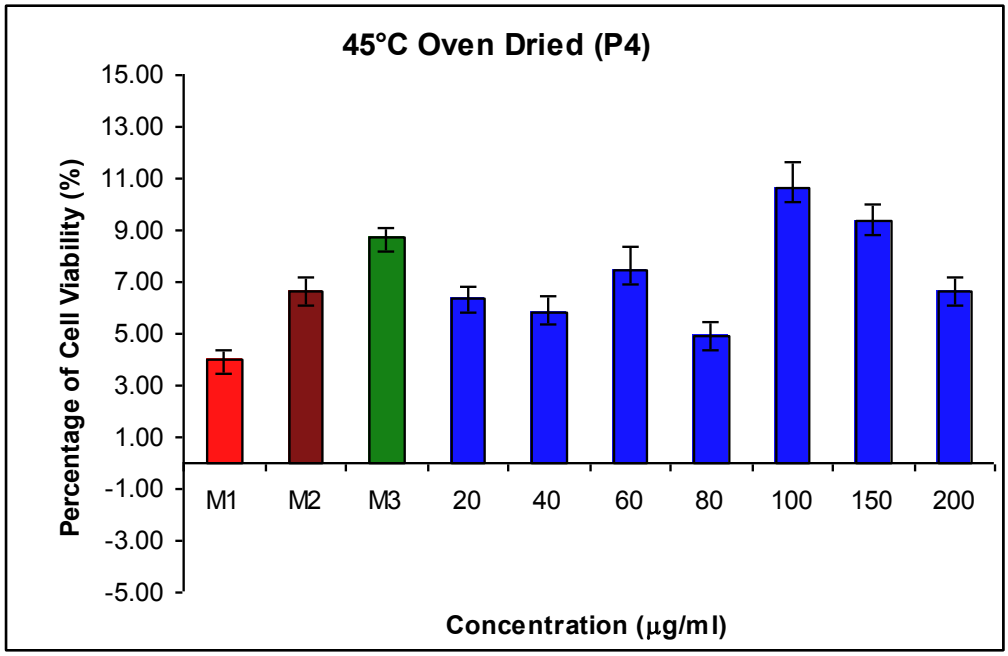
b)


Sun Dried (P2)
Percentage of Cell Viability (%)
15.00
13.00
11.00
9.00
7.00
5.00
3.00
1.00
-1.00
-3.00
-5.00
M1
M2
M3
20
40
60
80
100
150
200
Concentration (µg/ml)

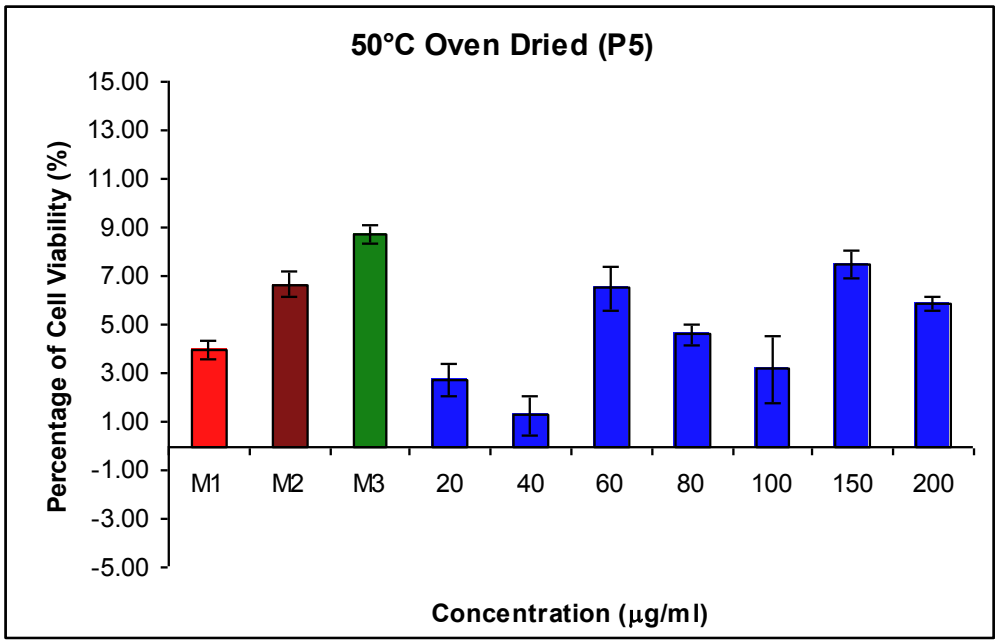
(c)


Freeze Dried (P3)
Percentage of Cell Viability (%)
15.00
13.00
11.00
9.00
7.00
5.00
3.00
1.00
-1.00
-3.00
-5.00
M1
M2
M3
20
40
60
80
100
150
200
Concentration (µg/ml)

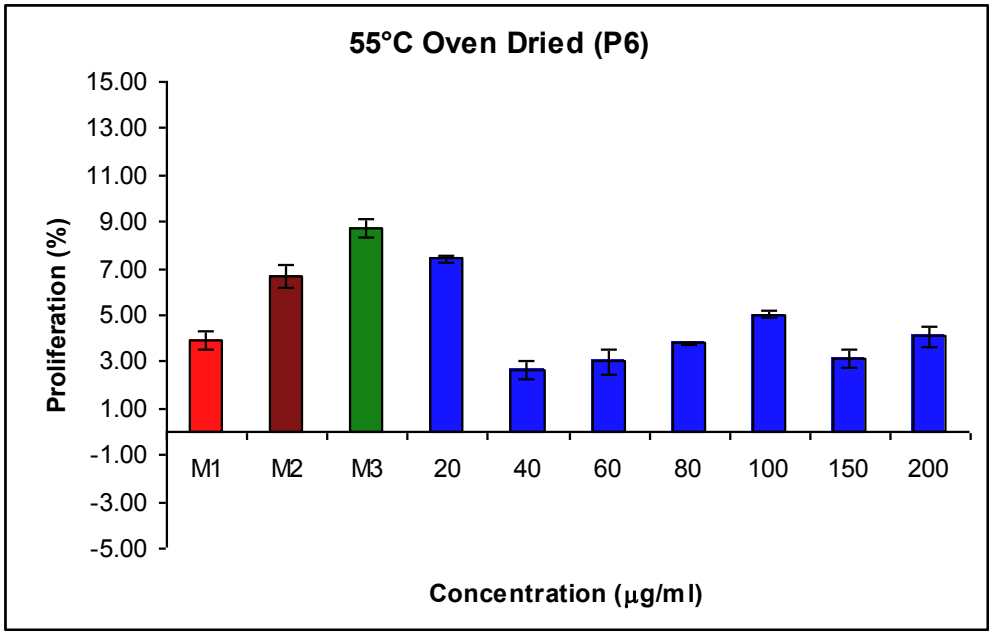
(d)


45°C Oven Dried (P4)
Percentage of Cell Viability (%)
15.00
13.00
11.00
9.00
7.00
5.00
3.00
1.00
-1.00
-3.00
-5.00
M1
M2
M3
20
40
60
80
100
150
200
Concentration (µg/ml)

(e)


50°C Oven Dried (P5)
Percentage of Cell Viability (%)
15.00
13.00
11.00
9.00
7.00
5.00
3.00
1.00
-1.00
-3.00
-5.00
M1
M2
M3
20
40
60
80
100
150
200
Concentration (µg/ml)

(f)


55°C Oven Dried (P6)
Proliferation (%)
15.00
13.00
11.00
9.00
7.00
5.00
3.00
1.00
-1.00
-3.00
-5.00
M1
M2
M3
20
40
60
80
100
150
200
Concentration (µg/ml)

(g)

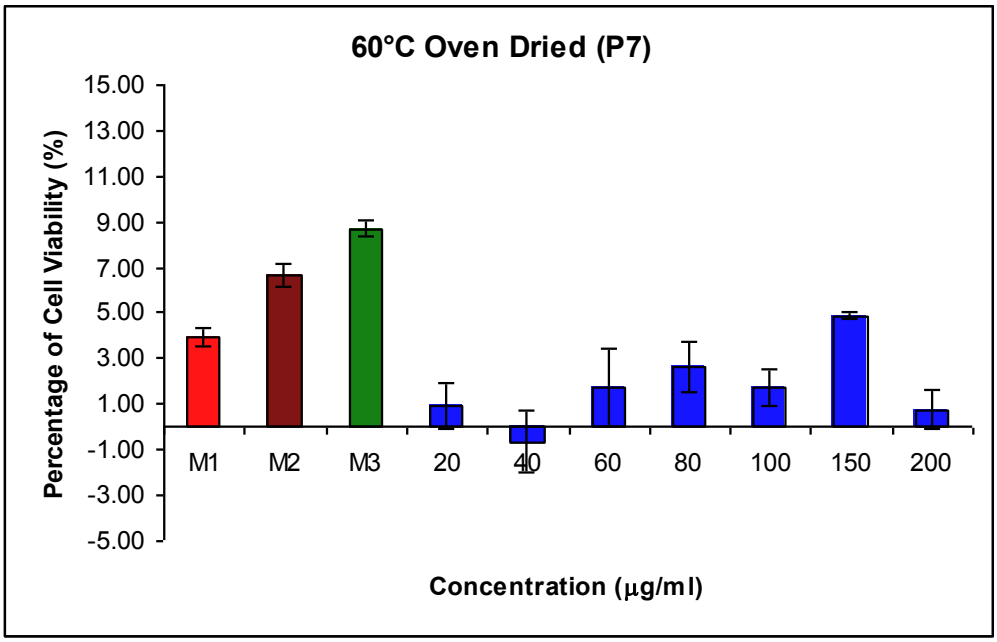



**Figure 4.8: Cytotoxicity of different processed aqueous extracts of *P. sajor-caju* on PBMCs.**

The aqueous extracts of different processed *P. sajor-caju* were assed for their cytotoxic effects on PBMCs. The cells were incubated with various concentrations (20 – 200 µg/ml) of the extracts as described in Materials and Methods (Section 3.3.3) followed by MTT assay as described in Section 3.3.1 to determine the cell viability of the PBMCs. The values were calculated against control which contained reverse osmosis (RO) water instead of extracts. PHA (M1 – 10µg/ml, M2 - 20µg/ml and M3 - 40µg/ml) were used as positive control which promotes the cells proliferation Results are single representation of mean (n=3) ± S.E.M. of three separate experiments.

Cytotoxicity assays are widely used in *in vitro* toxicology studies and serve as one of the chemotherapeutic targets of antitumor activity (Suffness and Pezzuto, 1991). Most of the clinically used antitumor agents possess significant cytotoxic activity in cell culture systems (Ajith and Janardhanan, 2003). It is a rapid standardized, sensitive and inexpensive method to measure drug-induced alterations in metabolic pathways or structural integrity which may or may not be related directly to cell death. The methyl tetrazolium (MTT) assay, the neutral red (NR) assay, the lactate dehydrogenase leakage (LDH) assay and a protein assay are the most common methods employed for the determination of cytotoxicity or cell viability following the exposure to toxic substances. Weyermann *et al*. (2005) have previously reported that different cytotoxic assays can give variable results depending on the test agent. The NR assay and MTT assay are the most sensitive cytotoxic assays that show statistically significant difference between the treated cells and the controls (Fotakis and Timbrell, 2005).

On the other hand, in the development of drugs from natural products, the potential chemopreventive agents selected for testing in people at high risk of developing cancer must have low toxicity as compared to the drugs used to treat existing cancer (Greenwald, 2002). Most cancer chemotherapeutants severely affect the host normal cells (Mascarenhas, 1994). The discovery of new protective substances with chemopreventive characteristics has been the target of various studies (Machado *et al*., 2005). Polysaccharides from mushrooms are among the various naturally occurring substances that may prove to be one of the useful candidates in the search for non-toxic, effective substances with antioxidant activity (Tsai *et al*., 2007).

Hence, the application of natural product especially from macrofungi which mainly represented by mushrooms has been contemplated to have exceptional value in the control of cancer and its eradication program (Ajith and Janardhanan, 2003). The studies should embrace the way extracts obtained as important factor in evaluating parameters such as cytotoxicity, biotransformation and genotoxicity. For an example, the desert mushroom (*Tirmania pinoyi*) which was extracted sequentially with boiling water, chloroform and ethanol showed various effects; chloroform extracts exerted mutagenic activity but the ethanol extract was able to prevent the mutagenic activity of some carcinogens (Machado *et al*., 2005). In addition, the active substances of mushroom exhibits neither cytotoxicity nor side effects that are commonly seen with the use of conventional anticancer agents such as, decrease in the number of leucocytes, anemia, atrophy of the spleen, loss of body weight and loss of appetite (Ajith and Janardhanan, 2003). Wasser and Weis (1999) described those polysaccharides from mushrooms specifically known as beta glucans are the active substances responsible in exerting the antitumor action mostly via activation of the immune response of the host organism.

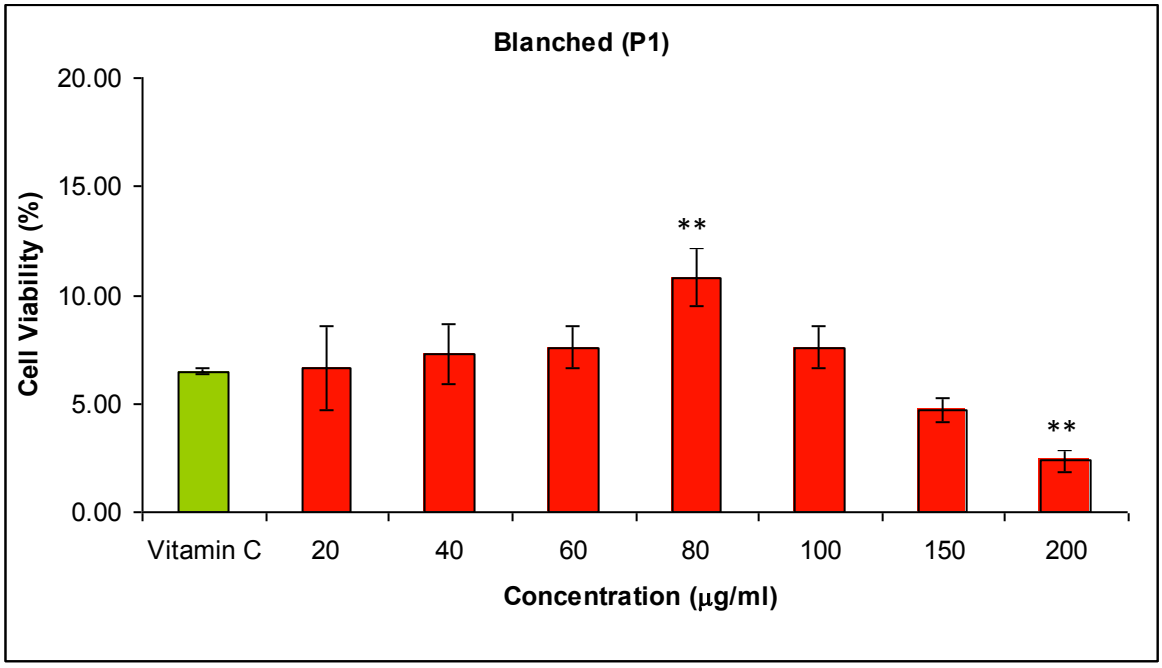
Thus, mushroom extracts are generally harmless and place no additional stress on the human body, help the body to adapt to various environmental and biological stresses and exert a nonspecific action on the body (Brekhman, 1980). Concisely, the compound should be toxic to the cancer cell but not to normal cell. Therefore, it is evident that the *P. sajor-caju* aqueous extracts are not cytotoxic to the PBMC and in a brief incubation period with PBMCs (two hours); they maintained the cell viability or slightly increase the percentage of cell viability of PBMCs compared to PHA.

### 4.3.3 Protective effects of *P. sajor-caju* extract on the viability PBMCs

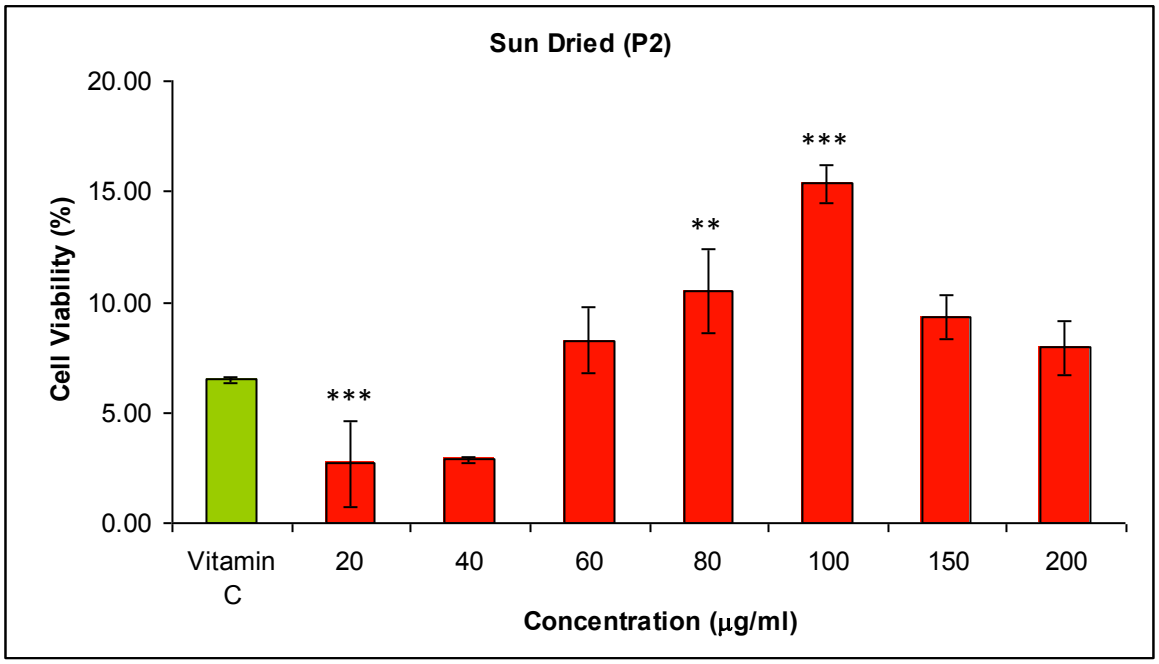
The mushroom extracts obtained via different processing methods were not toxic to human peripheral blood mononuclear cells. The cells were preincubated with the mushroom extracts at various concentrations for two hours. The cells were then challenged with known concentrations of $H_2O_2$ (0.3mM) for two hours. The concentration of $H_2O_2$ (0.3mM) was previously shown to cause approximately 25% inhibition of cell viability (Figure 4.7). The MTT assay to estimate cell viability was carried out according to the procedure described in Material and Methods (Section 3.3.2). Figures 4.9a-g illustrate the protective effects of the seven different processed (P1-P7) crude water extracts of *P. sajor-caju*. Vitamin C was used as the positive control for this test as it is a well known water-soluble antioxidant.

All the mushroom extracts demonstrated a mild protective effect against $H_2O_2$-induced toxicity. The protection level in PBMCs for 10µg/ml of vitamin C was 6.50±1.94% cell viability. In brief, the processed mushroom extract (P1 to P7) showed cell viability in the range between 2%-15%. The *P. sajor-caju* extracts, regardless of their concentration showed a mild protective effect against $H_2O_2$ which was comparable to vitamin C (10µg/ml).

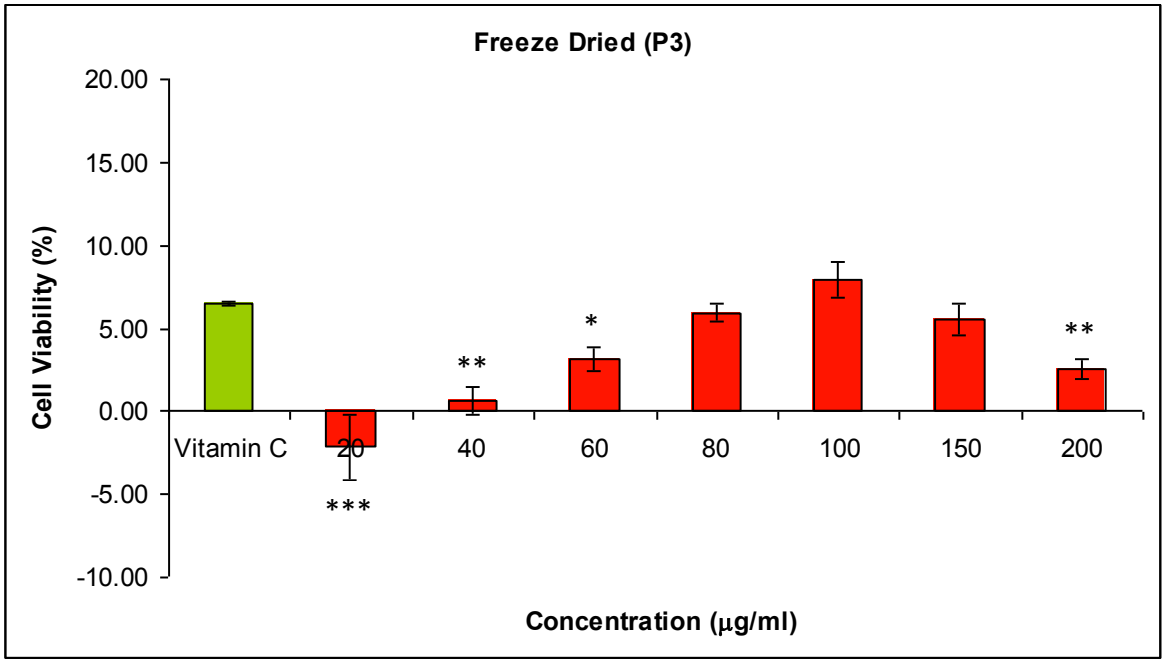
(a)


Blanched (P1)
Cell Viability (%)
20.00
15.00
10.00
5.00
0.00
**
**
Vitamin C
20
40
60
80
100
150
200
Concentration (µg/ml)

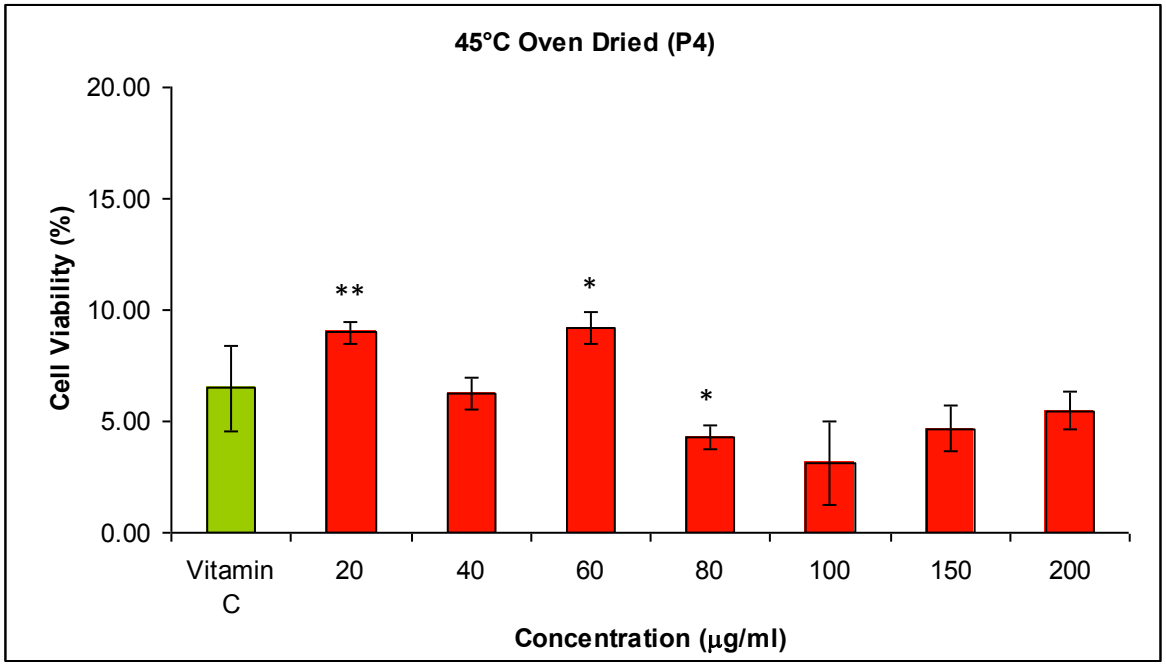
(b)


Sun Dried (P2)
Cell Viability (%)
20.00
15.00
10.00
5.00
0.00
***
**
***
Vitamin C
20
40
60
80
100
150
200
Concentration (µg/ml)

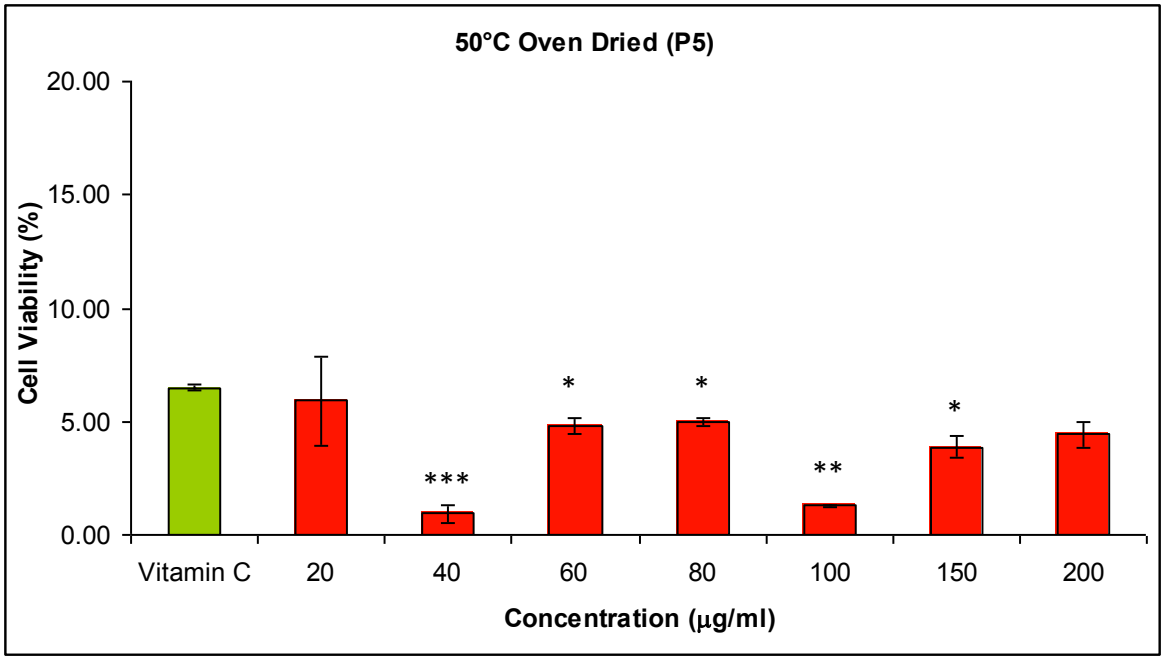
(c)


Freeze Dried (P3)
Cell Viability (%)
20.00
15.00
10.00
5.00
0.00
-5.00
-10.00
Vitamin C
20
40
60
80
100
150
200
**
*
**
***
Concentration (µg/ml)

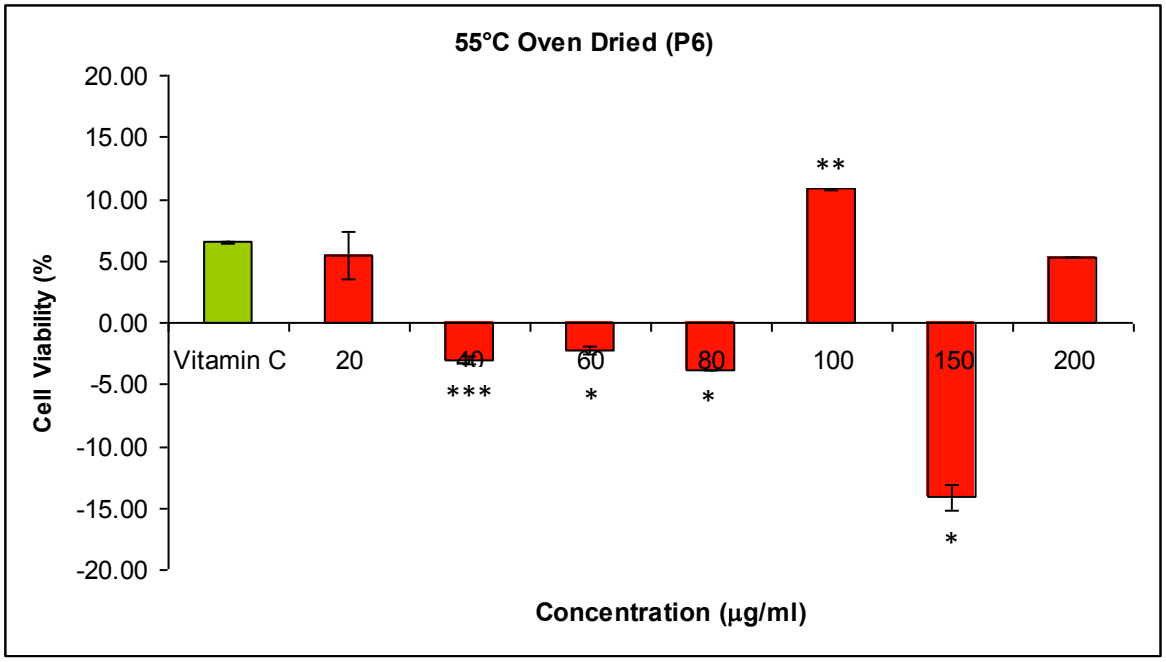
(d)


45°C Oven Dried (P4)
Cell Viability (%)
20.00
15.00
10.00
5.00
0.00
Vitamin C
20
40
60
80
100
150
200
**
*
*
Concentration (µg/ml)

(e)


50°C Oven Dried (P5)
Cell Viability (%)
20.00
15.00
10.00
5.00
0.00
***
*
*
**
*
Vitamin C
20
40
60
80
100
150
200
Concentration (µg/ml)

(f)


55°C Oven Dried (P6)
Cell Viability (%
20.00
15.00
10.00
5.00
0.00
-5.00
-10.00
-15.00
-20.00
**
***
*
*
*
Vitamin C
20
40
60
80
100
150
200
Concentration (µg/ml)

(g)

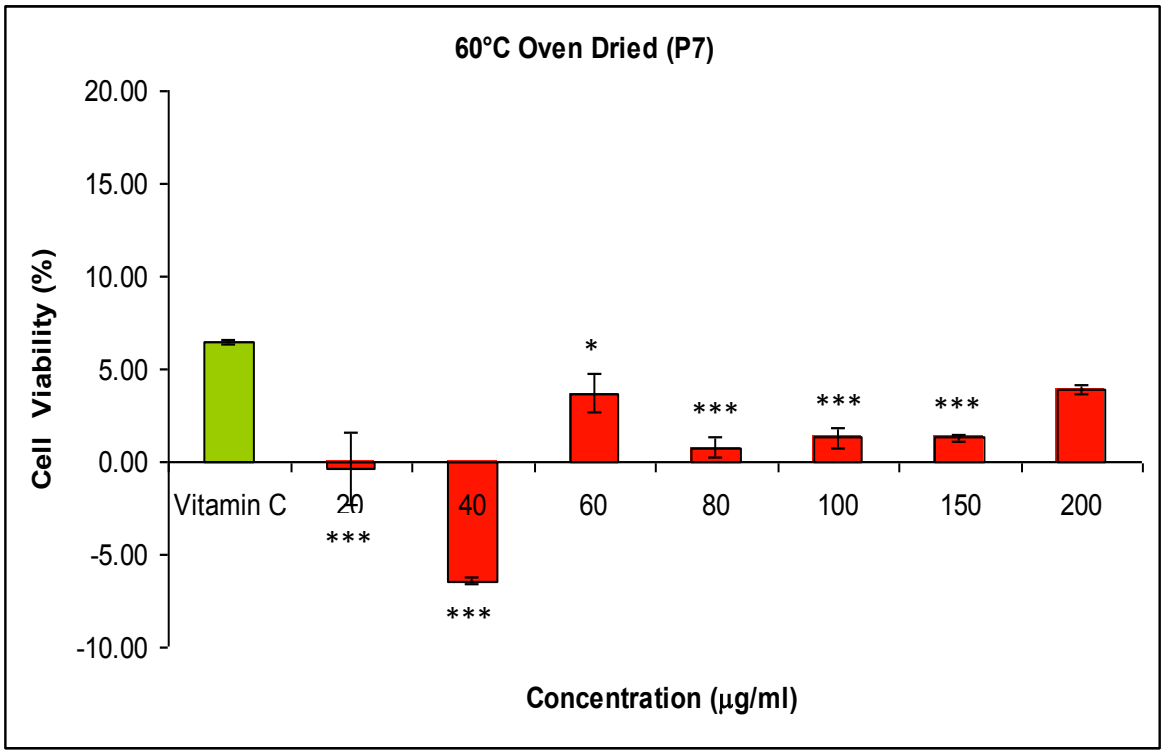



**Figure 4.9: Cytoprotective effect of different processed aqueous extracts of *P. sajor-caju* against $H_2O_2$-induced (300µM) damage on PBMC**

The aqueous extracts of different processed *P. sajor-caju* were assed for their cytoprotective effects on PBMCs. The cells were incubated with various concentrations (20 – 200 µg/ml) of the extracts together with 300µM of $H_2O_2$ as described in Materials and Methods (Section 3.3.3.3) followed by MTT assay as described in Section 3.3.2 to determine the cell viability of the PBMCs. The values were calculated against control which contained reverse osmosis (RO) water instead of extracts. Vitamin C (10µg/ml) were used as positive control which known to have the ability to protect the cells from toxicity. The mean (n=3) ± S.E.M. were plotted in single representation of three separate experiments. Student t-test with was used to compare the difference between each group and Vitamin C (10µg/ml); *** denotes $p < 0.0001$; ** denotes $p < 0.001$; * denotes $p < 0.05$. Percentage of cell viability was calculated using the formula below:-

$$\% \text{ Cell Viability} = \frac{\text{Absorbance of mushroom extracts} - \text{Absorbance of blank}}{\text{Absorbance of blank}}$$

In terms of processing methods, the sun dried (P2) mushroom demonstrated the highest level of protection (Figure 4.9b) (9% higher at 100µg/ml compared to vitamin C). This could be possibly caused by the elevation of vitamin D or the transformation of ergosterol peroxidase into vitamin D (Imtiaj and Rahman, 2008). They reported that exposure of shiitake to direct sunlight for 3 hour/ day increases the vitamin D content up to 5 times. The peroxide of the ergosterol, 5α, 8α-epidioxy-22E-ergosta-6, 22-dien-3β-ol (ergosterol peroxide) is common in mushrooms such as *Hypsizigus marmoreus, Pleurotus eryngii,* and *Ganoderma lipsiense* (Bok *et al*., 1999 and Takei *et al*., 2005). However, the real mechanism/s involving ergosterol or vitamin D with the protective activity is still not clear and further investigation should be carried out in future.

Biologically active compounds found in mushroom have been known for their medicinal and nutritional benefits to human health. Many studies have reported that the various types of polysaccharides isolated from mushrooms possess bioactive properties such as anti-tumor, immunological, anti-inflammatory, anti-coagulant, and hypoglycemic activities (Kim *et al.*, 2007). Mushroom polysaccharide extracts were also reported to have scavenging effects on superoxides and hydroxyl radicals produced by reactive oxygen species (Liu *et al.*, 1997). Mushroom polysaccharides are known to stimulate natural killer cells, T-cells, B-cells and macrophage-dependent immune system responses (Wasser, 2002). Various effects of precursor T cells and macrophages on the response to cytokines were produced by lymphocytes after specific recognition of tumour cells (Hamuro and Chihara, 1985).

As mushroom could be useful candidates as effective, non-toxic substances with free radical scavenging activities as mentioned in section 4.2, the presence of antioxidant and polyphenol activity in processed mushroom extracts shows that the processing method does not affect the mushroom properties. In addition, the non-toxic properties of this mushroom extracts enabled them to become a good candidate for protective activity on human cells. Although, several biological activities of the mushroom polysaccharides extracts have been reported, studies on their cytoprotective effects against $H_2O_2$ induced damage on human mononuclear cells is limited. Therefore this study shed some light on the protective effects of *P. sajor-caju* extracts which are grown extensively in Malaysia. In addition, a time course study (24 hours or 48 hours) on the effect of $H_2O_2$ induced damage on PBMC and other cell lines with processed *P. sajor-caju* extracts should be carried out in future.

The experiments were continued to study the effects of $H_2O_2$ induced damage at the DNA level of PBMCs. Extracts P1, P2, P3 and P4 which were processed at different temperatures were further tested for the genotoxicity and genoprotective activity. From the oven dried group of sample, 45°C oven dried (P4) were chosen for the genotoxicity and genoprotective assays. In the food processing industry, a variety of oven drying temperature ranging from 40 - 80°C was applied to achieve better products depending on the type of food or fruits or mushrooms. All the oven dried extracts showed approximately similar antioxidant activity as mentioned in Section 4.2 and 4.2.3. However, when the cytoprotective activity was taken into consideration the extract P4 (Figure 4.9d) showed a mild protection which is comparably better than the other oven dried extracts.

## 4.4 The effect *P. sajor-caju* aqueous extracts on DNA damage of PBMCs

### 4.4.1 DNA staining and visualization (Comet assay)

Scoring of DNA damage was performed using a fluorescent microscope and automated software scoring system with an image capture device attached to the microscope. A grade was given to each comet image according to the intensity and length of the comet tail visualized. Figure 4.12 illustrates the different grades of DNA damages. Generally two or three gels per treatment were scored, and the mean and SEM of the gels are presented as graph in Sections 4.4.2, 4.4.3 and 4.4.4.

The DNA-specific dye and the magnification used for comet visualization depend largely on investigator-specific needs (Tice *et al*., 2000). There are many types of florescent dyes that can be used for visualizing the DNA such as ethidium bromide, propidium iodide, 4,6- diamidino-2-phenylindole (DAPI), SYBR Green and YOYO-1 (benzoxazolium- 4-quinolinum oxazole yellow homodimer) (Tice *et al*., 2000). The most common florescent dye employed in DNA visualizing is the ethidium bromide. Kazilian *et al* (1999) reported the use of non fluorescent technique for visualizing comets which are based on staining with silver nitrate that is less harmful than the ethidium bromide (carcinogenic). Comet image magnification can vary between 160X to 600X. However, the most common magnification used varies between 200X to 400X (Tice *et al*., 2000).

DNA migration in the comet assay is mainly induced by strand breaks and alkali labile sites (Speit and Hartmann, 1999). Based on the migration of cell which contains the DNA, the percentage of head and tail of comet like structure formed by the PBMCs was calculated.

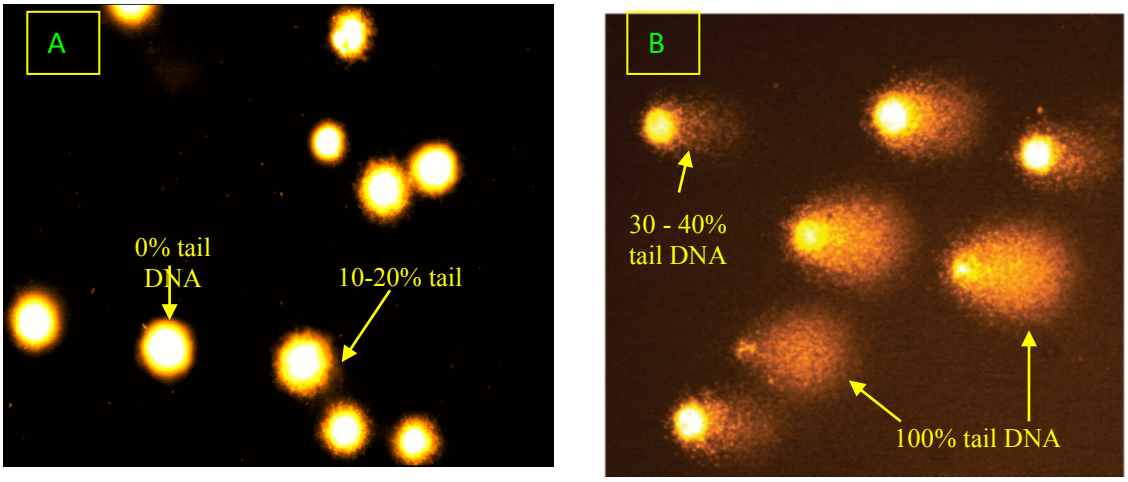



**Figure 4.10: Representative comet images showing different levels of damage in visual scoring**

A) Score 0-20% tail DNA (undamaged DNA); B) various degrees of damage from minor (30%) to severe (100%) tail DNA.

### 4.4.2 Effects of $H_2O_2$ on PBMC's DNA

In the present study, the genotoxicity activity was carried out with the cell embedded onto the frosted slides using agarose gel. The cells were treated with different concentration of $H_2O_2$ to determine the 50% damage caused by the oxidant on the cells. Figure 4.11 depicts the percentage of head and tail formed by the cells after the electrophoresis. The result indicate that the control group (water) only showed very slight DNA damage, with the percentage of tail DNA distributed between 0-10%. Significant DNA damage was induced

by $H_2O_2$ concentrations greater than 200µM. At 300 µM $H_2O_2$, most of the cells exhibited tailed DNA, indicating extensive damage (Figure 4.11). The higher head DNA represents minimum DNA strand breaks. The toxicity of oxidant i.e. $H_2O_2$ was considered significant when the tail DNA is more than 30% and the head DNA is less than 70%. The tail DNA exceeded 30% in PBMC incubated with $H_2O_2$ concentrations higher than 0.15mM whereas 50% tail DNA ($IC_{50}$) was achieved at 0.20mM. Thus, this concentration was selected for further investigation on the protective effect of mushroom extracts on $H_2O_2$ induced genotoxicity.

The realization that the DNA damage and mutation arise from endogenous products of cellular metabolism has been a major concern for the past two decades (Ames, 1989) The generation of damaged bases and strand breaks are the consequence of oxygen radicals that are produced during the reduction of $O_2$ which attack DNA bases or deoxyribose residues (Ward, 1987). Replication of this damaged strands leads to mutation or apoptosis (Johnson, 1996). Ames *et al*. (1993) reported that low levels of reactive oxygen species (ROS) have useful roles in signal transduction and in the modulation of gene expression but are extremely harmful to DNA and other biological macromolecules at higher concentrations. Injury to DNA is caused by the accumulation of ROS resulting from overproduction and/or inefficient intracellular antioxidant defense systems (Halliwell, 1999). The ROS are important as direct and indirect initiators as well as promoters of mutagenesis and carcinogenesis. They also increase the lipid peroxidation, which in turn alter the integrity of membrane bound enzymes (Halliwell and Gutteridge, 2003). The free radical scavenging efficiency of the extract thus might play an important role in the antimutagenic activity.

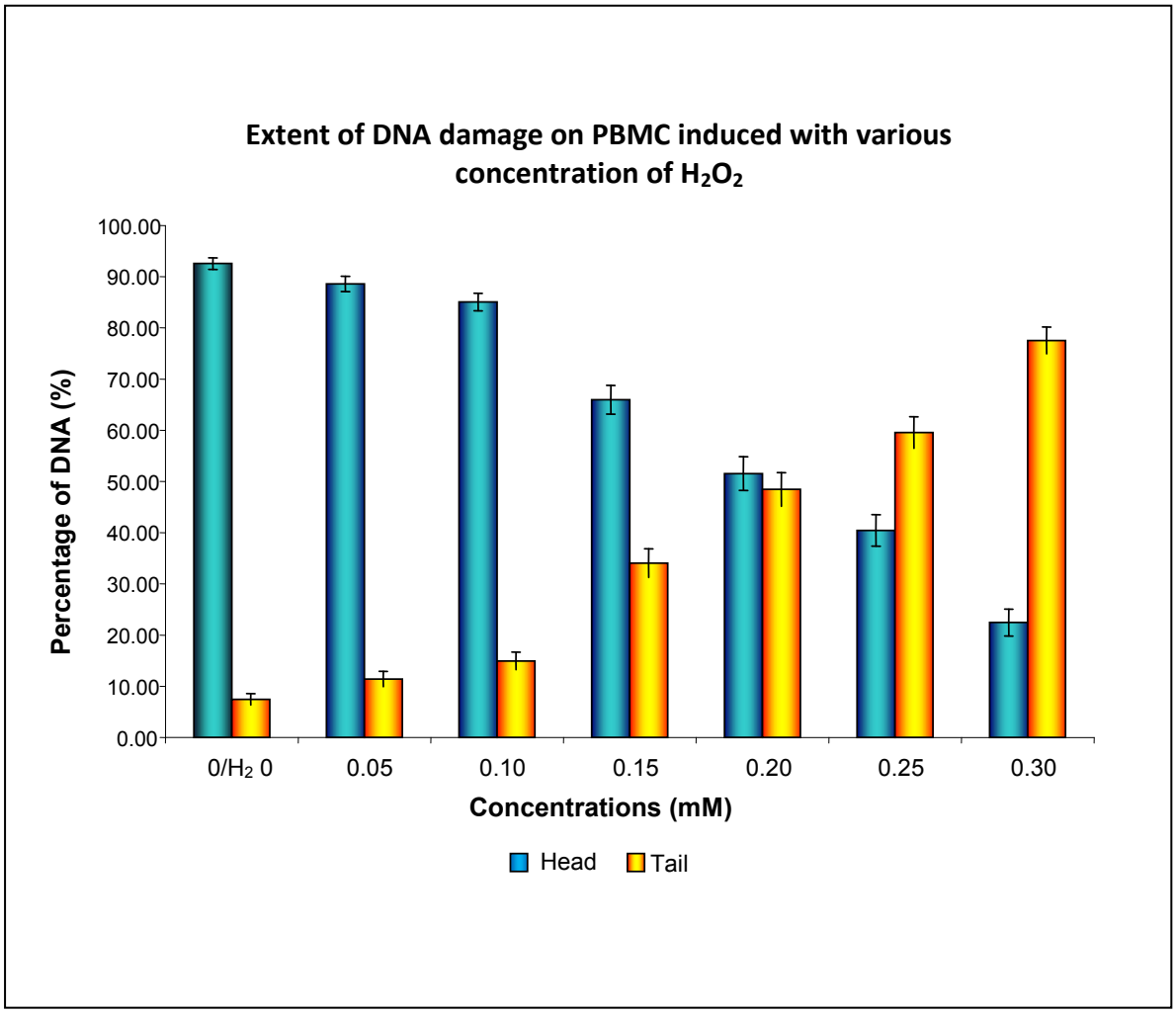



**Figure 4.11: DNA damage in PBMC exposed to $H_2O_2$**

Peripheral blood mononuclear cells (PBMC) (1,000,000 cells/ml) were incubated with various concentrations of $H_2O_2$ (0.05mM – 0.30mM) for 30 minutes at 37ºC and 5% of $CO_2$. The treated PBMCs were electrophoresed using single cell gel electrophoresis (SCGE) technique and stained with EtBr (Section 3.3.4.3 and 3.3.5). The normal control (0 mM) comprised cells with RO water in media. The mean (n=150) ± S.E.M. were plotted in single representation of three separate experiments.

The damage caused by these free radicals can be detected using comet assay. Theoretically, the comet assay can be applied to any eukaryotic cell; however, the human white cells mainly lymphocyte cells are most frequently employed (Vijayalaxmi *et al*., 1993). White cells are easily harvested from venous or capillary blood, whereas it is generally difficult to obtain other types of nucleated cells from human subjects. Besides human lymphocytes, other cell types such as spermatocytes, fibroblasts, epithelial cells from the buccal (oral) cavity, stomach, tear duct, nose and bladder have been used, although not always successfully because of their resistance to lysis buffer, high level of background and DNA damage (Wong *et al*., 2005). Various white cell lines also can be used, such as RAJI and TK6 from B-cell lines and HUT-78 from T-cell lines (Wong *et al*., 2005).

The advantages of the comet assay are its speed, simplicity, low cost, the small number of cells required (<10,000 cells), its sensitivity, and its widespread applicability with eukaryotic cells, whether proliferating or non-proliferating (Tice *et al*., 2000). Furthermore, effects on different cell types can be explored. This is an advantage because genotoxic and genoprotective effects can be tissue or cell type specific. Tice *et al.* (2000) also reported that another advantages of the comet assay is its flexibility; different combinations of unwinding and electrophoresis conditions and lesion-specific enzymes can be used to detect different types and levels of DNA damage (Tice *et al*., 2000).

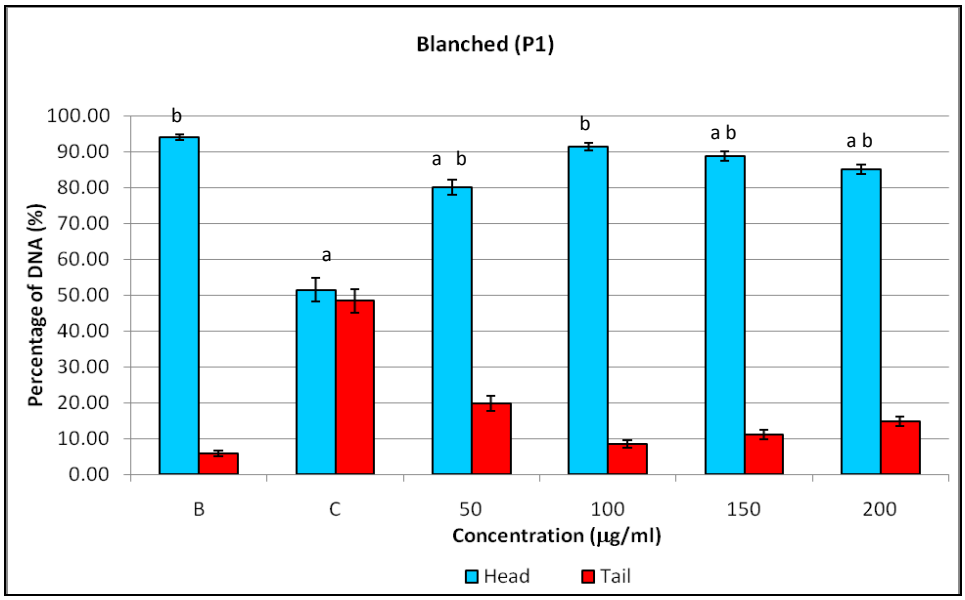
### 4.4.3 Effects of *P. sajor-caju* on PBMC's DNA

In the present study, the genotoxic activity of the crude water extracts of *P. sajor-caju* was established using the single cell gel electrophoresis (Comet assay). The genotoxicity assessment of the processed *P. sajor-caju* extracts were carried out using the methods described Materials and Methods (Section 3.3.4.5). Figures 4.12a-d show the result obtained from the comet assay of different processed *P. sajor-caju*. None of the processed *P. sajor-caju* extracts were genotoxic. As the result shown in Figure 4.12a-d, the processed *P. sajor-caju* extracts caused a slight DNA damage at very low and very high concentrations. The tail DNA was distributed between 0-20% at concentration of 50 μg/ml to 200 μg/ml, this was significantly ($p < 0.05$) less that the damage induced by $H_2O_2$ (0.2mM).

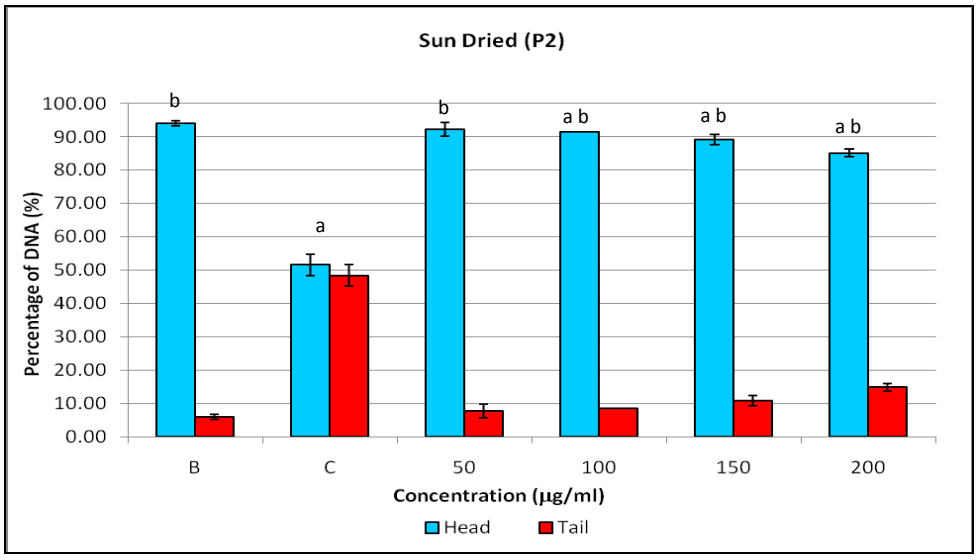
Efforts to develop dietary supplements and preventive treatments for offsetting the detrimental effects of ROS have focused on identifying antioxidants from a variety of natural products (Shi *et al*., 2002b). More recently, mushrooms have been identified as a potential source of natural antioxidants. This special group of fungi has long been acknowledged in Eastern cultures as possessing a wide range of medicinal properties (Chang and Buswell, 1996), and modern techniques have identified numerous bioactive mushroom components which have been reported to exhibit anti-cancer, anti-tumour, anti-viral, immunomodulatory, hypocholesterolaemic and hepatoprotective activities.

Commonly, it was possible to see a relationship between cytotoxicity and genotoxicity determined in the comet assay (Machado *et al*., 2005). The cytoprotective and genoprotective effects of *A. bisporus* extracts showed a positive dose-dependent correlation. However, other non cytotoxic concentration showed genotoxicity. It is important to perform comet assay at non cytotoxic concentrations to better evaluate the DNA damage (Hartmann and Speit, 1997). Therefore, it is evident that a cytotoxicity test should be performed concomitantly with genotoxicity tests (Machado *et al*., 2005). Nevertheless, the results obtained for the genotoxic activity of *P. sajor-caju* as shown in Figure 4.12a-d correspond/ correlate to the pattern of cytotoxic activity in section 4.3.2.

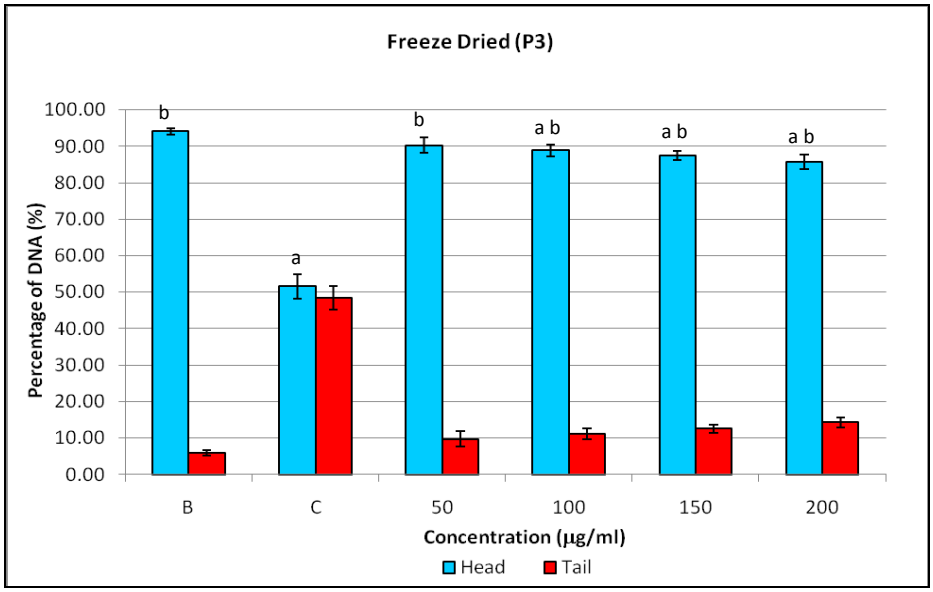
(a)


Blanched (P1)
Percentage of DNA (%)
100.00
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
b
a
a b
b
a b
a b
B
C
50
100
150
200
Concentration (µg/ml)
Head
Tail

(b)


Sun Dried (P2)
Percentage of DNA (%)
100.00
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
b
a
b
a b
a b
a b
B
C
50
100
150
200
Concentration (µg/ml)
Head
Tail

(c)




(d)

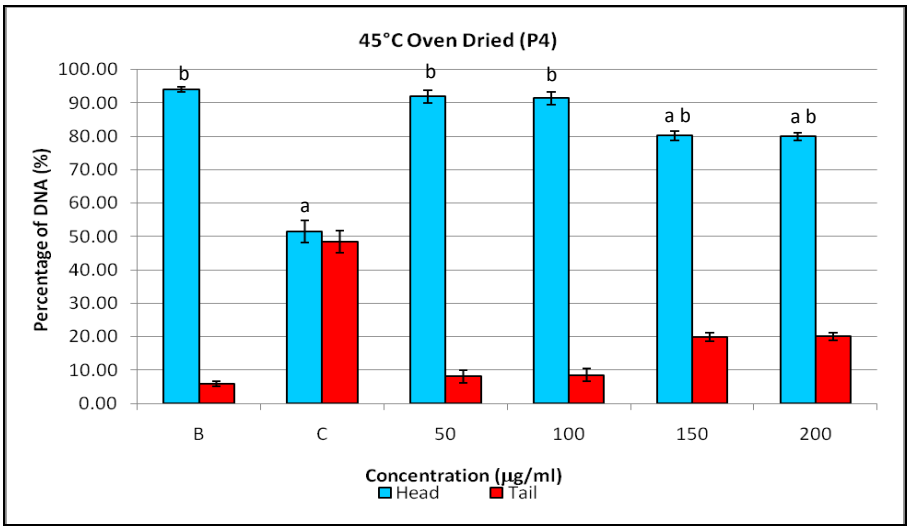



**Figure 4.12: Genotoxicity effects of aqueous extracts of different processed *P. sajor-caju* on DNA damage in PBMCs**

The aqueous extracts of different processed *P. sajor-caju* were assessed for their genotoxic effects on PBMCs. The cells were incubated with various concentrations of extracts (50µg/ml - 200µg/ml) as described in Material and Methods (Section 3.3.4.5) followed with comet assay as described in Section 3.3.4.2, 3.3.4.3 and 3.3.5 to determine the level of DNA damage in the cells. B: The normal control comprised cells with RO water in media, and C: Cells treated with 0.2mM $H_2O_2$. The mean (n=150) ± S.E.M. were plotted in single representation of three separate experiments. Student t-test with was used to compare the difference between groups; a: denotes $p < 0.0001$ compared with B; b: denotes $p < 0.0001$ compared with C.

In addition, the mild damage in DNA could be caused by the natural antioxidant such as vitamin C that is present in the mushroom extracts. Anderson *et al*. (1994) reported that ascorbic acid can induce DNA damage in human lymphocytes under certain concentrations in SCGE assay. It had an inhibitory effect on DNA damage of human lymphocytes inducted by $H_2O_2$ under incubation with lymphocytes and $H_2O_2$ at the same time. In addition, Duthie *et al.* (1997) also reported that some flavonoids induced genotoxicity and cytotoxicity in cells under higher concentrations. Therefore, in the present study, processed *P. sajor-caju* may have caused mild DNA damage (not significant) at concentrations as mentioned above because it contained significant amounts of phenolic compounds (Figure 4.1) and ascorbic acid (as mentioned in section 2.5.1).

*Pleurotus* species a well known basidiomycetes species is consumed as food especially in oriental region for centuries. Thus, it can be concluded here that the extracts obtained via different processing method (temperatures) of *P. sajor-caju* did not exert any significant genotoxicity to normal human cell.

### 4.4.4 Protective effects of mushroom extracts on PBMC's DNA damage by $H_2O_2$

The genoprotective activity of *P. sajor-caju* was carried out only for extract P1, P2, P3 and P4. Figure 4.13a-d depicts the genoprotective effect of extracts P1, P2, P3 and P4 respectively. The cells were incubated with extracts and were compared with cells incubated with water. As the concentration of extract increases the protection level of the mushroom against $H_2O_2$ induced genotoxicity also increased with highest protection level

(approximately 85% protection) observed in the presence of 80µg/ml extract P1 (Figure 4.13a). The extracts P2 and P3 also showed significant protection against $H_2O_2$, at 80µg/ml (80.82±8.08% DNA protection) and 100µg/ml (82.26±8.26% DNA protection) (Figure 4.13b and 4.13c respectively).

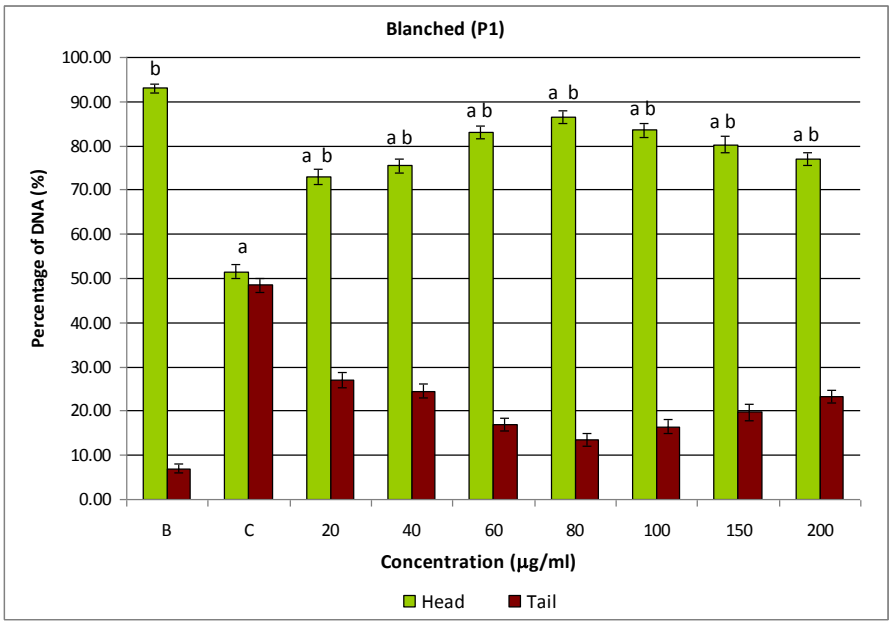
The percentage of protection over the DNA damage caused by $H_2O_2$ was calculated and the results are depicted in Figure 4.14. From the figures, it can be assumed that all the extracts showed almost a similar pattern of DNA protection. The protection level increased as the concentration of extracts increased and decreased at concentrations greater than 80µg/ml. The extract P3 showed a plateau trend after 20µg/ml to 200µg/ml. A nonlinear regression curve was determined to estimate the EC50 (50% effective concentration) of the extracts (Figure 4.14). The aqueous extracts of P1, P3 and P4 of *P. sajor-caju* showed 50% protection at approximately at 15µg/ml, 9µg/ml and 10µg/ml compared to extract P2 which showed 50% protection level at 42µg/ml.

Nutraceuticals that protect DNA from oxidant challenge or that promote DNA repair may have potential health benefits and help lower risk of age-related disease. To date, most investigations are revolving around cells that has been exposed to known micronutrients, such as vitamin C (Anderson *et al*., 1994) or vitamin E (Sierens *et al*., 2001). Effects of supplementation of these two well known micronutrients on DNA protection have also been reported (Choi *et al*., 2004). Some non-nutrients, such as quercetin (Duthie and Dobson, 1999) and non-foods, e.g. the traditional *Ganoderma lucidum* (Shi *et al*., 2002b) and other herbal medicines have also been used. Hence, from

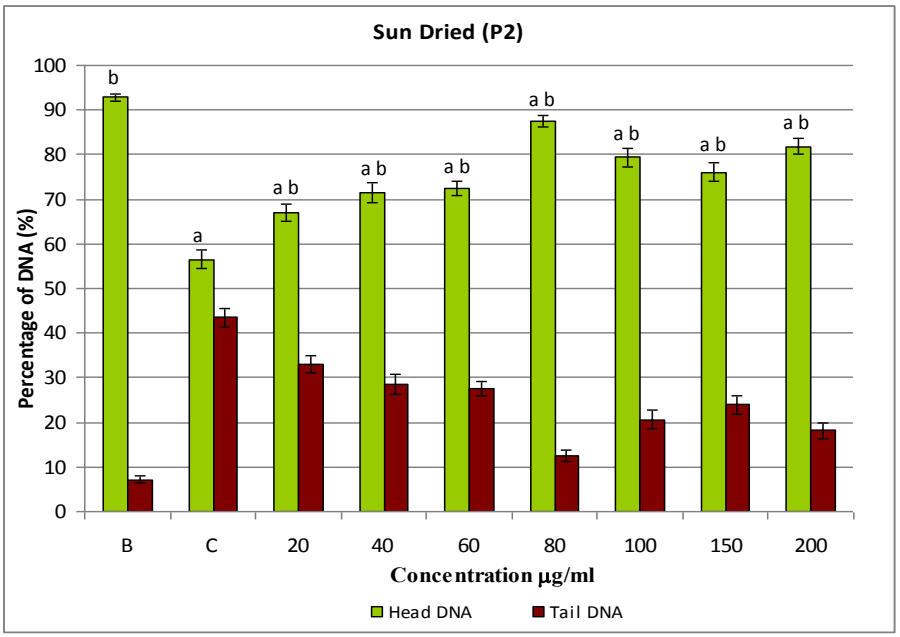
this study we could see the potential effects of *P. sajor-caju* as a good genoprotective agent that are available cheaply compared to other micronutrients.

The potential genoprotective or genotoxic effect of whole foods, simple mixtures and purified components in *in vitro*, animal and human supplementation trials can be investigated quickly with simple assays. The comet assay provides this simple and relatively quick way of investigation (Wachtel-Galor *et al*., 2004). It is well known that reactive free radicals attack cellular DNA and nucleotide pools to form various types of oxidized bases; e.g., 8-OHdG (Kasai *et al*., 1986), 5-hydroxydeoxycytidine (Feig *et al*., 1994) and 2 hydroxydeoxyadenosine (Kamiya and Kasai, 1995). Among these oxidized bases, 8-OHdG has especially received much attention because of the accumulated data indicating that it may play crucial roles in a chain of events from reactive oxygen and/or nitrogen oxide species–induced stress (RONOSS) to carcinogenesis (Kasai, 1997). 8-OHdG pairs with adenine as well as cytosine, subsequently yielding GC-to-TA transversions upon replication by DNA polymerases (Cheng *et al*., 1992; Shibutani *et al*., 1991). This mutation is considered to contribute to the activation of oncogenes and/ or the inactivation of tumor suppressor genes, leading to carcinogenesis (LePage *et al*., 1995).

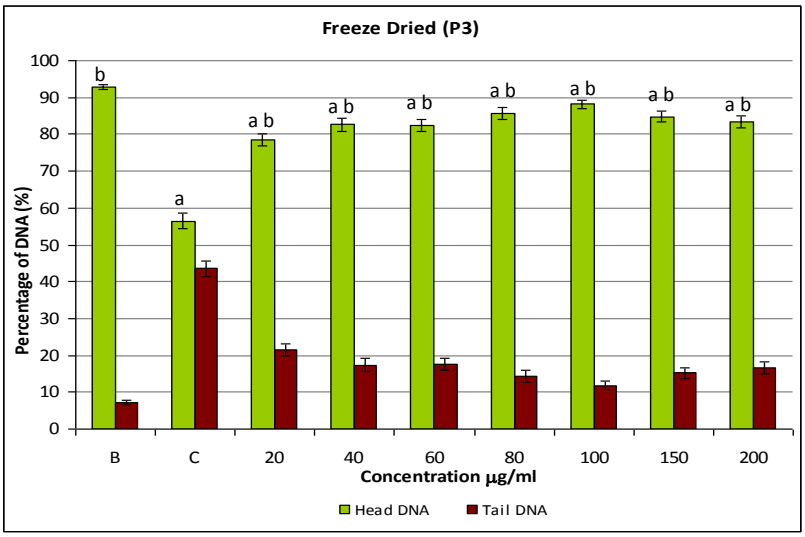
(a)


Blanched (P1)
Percentage of DNA (%)
100.00
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
b
a
a b
a b
a b
a b
a b
a b
B
C
20
40
60
80
100
150
200
Concentration (µg/ml)
Head
Tail

(b)


Sun Dried (P2)
Percentage of DNA (%)
100
90
80
70
60
50
40
30
20
10
0
b
a
a b
a b
a b
a b
a b
a b
B
C
20
40
60
80
100
150
200
Concentration µg/ml
Head DNA
Tail DNA

(c)




(d)

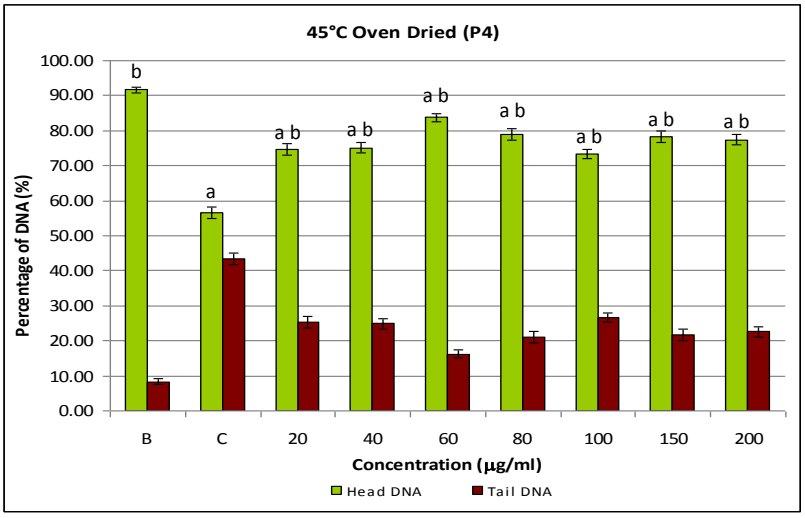



**Figure 4.13: Genoprotective effects of aqueous extracts of *P. sajor-caju* on PBMC against $H_2O_2$-induced DNA damage**

The aqueous extracts of different processed *P. sajor-caju* were assessed for their genoprotective effects against $H_2O_2$-induced DNA damage (0.2mM) on PBMCs. The cells were incubated with various concentrations of extracts (20µg/ml - 200µg/ml) as described in Material and Methods (Section 3.3.4.6) followed with comet assay as described in Section 3.3.4.2, 3.3.4.3 and 3.3.5 to determine the level of DNA damage in the cells. B: The normal control comprised cells with RO water in media, and C: Cells treated with 0.2mM $H_2O_2$. The mean (n=150) ± S.E.M. were plotted in single representation of three separate experiments. Student t-test with was used to compare the difference between groups; a: denotes $p < 0.0001$ compared with B; b: denotes $p < 0.0001$ compared with C.

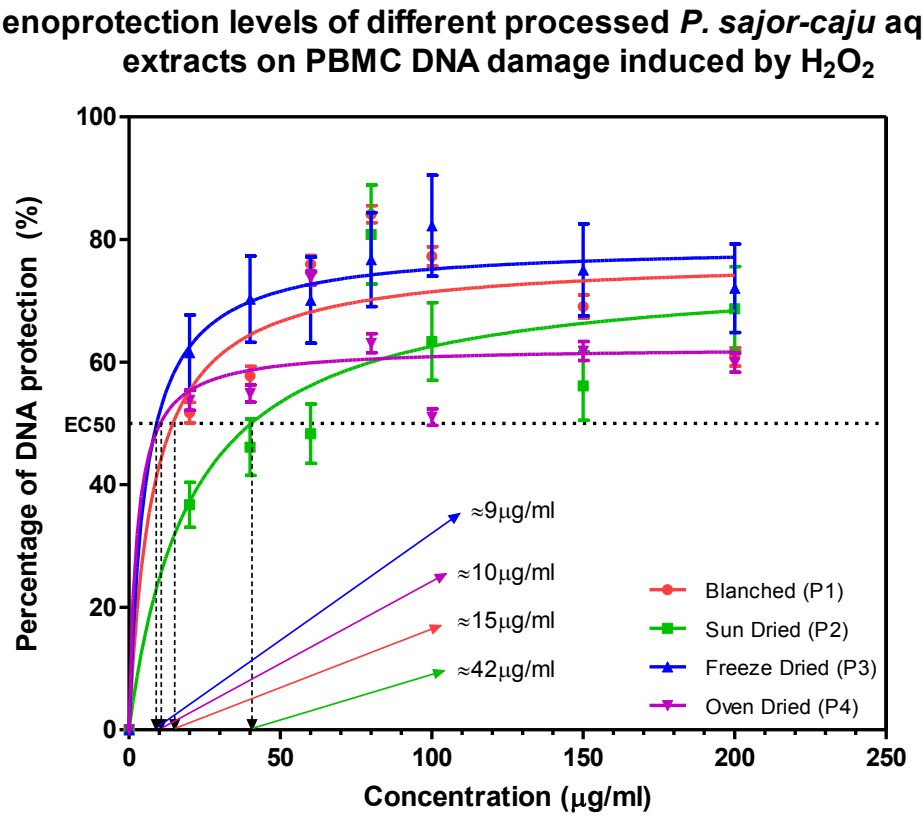



**Figure 4.14: Fifty percentage (50%) effective concentration (EC50) on genoprotection of different processed *P. sajor-caju* extracts against $H_2O_2$-induced DNA damage.**

The aqueous extracts of different processed *P. sajor-caju* (0-200 µg/ml concentrations) were incubated together with PBMCs and then were induced with 0.2mM $H_2O_2$. The non – linear regression curve was plotted and the EC50 for the different processed aqueous extracts of *P. sajor-caju* were calculated from the graph. The percentage was calculated as the formula below:-

$$\text{Percentage of DNA protection (\%)} = \frac{\text{(Head DNA sample – Tail DNA sample)}}{\text{Head DNA of blank}} \times 100\%$$

Shi *et al*. (2002a) has reported that tyrosinase plays an important role in genoprotection activity/effect of button mushroom. The nature of the genoprotective activity of tyrosinase is dependent upon the two associated catalytic activities of the enzyme, namely hydroxylation of tyrosine to L-DOPA and the subsequent oxidation of L-DOPA to dopaquinone (Shi *et al*., 2002a). The basis of the observed genoprotective effects of tyrosinase-generated L-DOPA oxidation products is not known (Shi *et al*., 2002a). However, in addition to its pro-oxidant properties, L-DOPA also stimulates cellular antioxidant defense mechanisms under ambiguous conditions. Although tyrosinase activity in *Pleurotus sp.* (Ratcliffe *et al*., 1994) is lower than in *Agaricus sp*., it is possible to speculate that tyrosinase-generated L-DOPA oxidation products could contribute to the genoprotective effects observed in this study. However, further investigation to elucidate the underlying mechanisms would have to be carried out.

The phenolic compounds in *P. sajor-caju* might play an important role in modulating $H_2O_2$-mediated DNA damage in PBMCs. However, the absorption and bioavailability of *P. sajor-caju* need further evaluation and investigation. As result shown in Figure 4.13 and Figure 4.15, *P. sajor-caju* had a protective effect against $H_2O_2$ induced damage in PBMCs. *Pleurotus sajor-caju* had antioxidant activity, scavenging activity on free radicals or ROS (Figure 4.1 - 4.3). Thus, the antioxidant and scavenging activity of *P. sajor-caju* on free radicals induced by $H_2O_2$ might be mainly related to its protective effect on DNA damage on PBMCs.

The potential inhibition of *P. sajor-caju* on $H_2O_2$ induced oxidative DNA damage, suggests that *P. sajor-caju* has prospective role in the chemopreventive function.

## 4.5 Future Investigation

The following studies are needed to validate the findings of this study:

I. A time dependent study had to be carried out to determine the appropriate time of incubation for the extracts to show a percentage of viability of cell more than 30%.

II. The polysaccharides, specifically beta glucans are well known for their ability to protect the cells. Hence, in future study, if the polysaccharides from this mushroom could be extracted and the antioxidant properties, cytoprotective and genoprotective ability studied. It could give an idea of the actual process involved in the protective effect of the *P. sajor-caju* extracts.

III. In comet assay, it was shown that P. sajor-caju extracts had genoprotective activity. The potential of *P. sajor-caju* extracts to repair the DNA, if any, need further investigation.

IV. Fractionation of the processed mushroom extract using column chromatography or HPLC/ anion exchange chromatography could be carried out and the active compound/s that give protective effect against $H_2O_2$ induced toxicity need to identified.

V. The laboratory rats that were treated with oxidant or tumor induced can be given processed *P. sajor-caju* in their diet. The lymphocytes can then be isolated and Comet assay and MTT assay can be carried out to study if there are *in vivo* protective effects of *P. sajor-caju* extracts.

# CHAPTER 5

# CONCLUSION

# CHAPTER 5

# CONCLUSION

## 5.1 Conclusion

In this study, antioxidant, cytotoxicity, cytoprotective, genotoxicity and genoprotective effect of different processed *P. sajor-caju* were analyzed. Studies of antioxidant activity with *P. sajor-caju* extracts of blanched (P1), sun dried (P2), freeze dried (P3), 45°C oven dried (P4), 50°C oven dried (P5), 55°C oven dried (P6) and 60°C oven dried (P7), showed that processing methods did not affect the content of antioxidant in the fruiting bodies of *P. sajor-caju*. The low percentage of DPPH scavenging activity showed by the processed extracts in this study compared to other reports which depicted a higher percentage of DPPH scavenging activity could be caused by the limiting factor such as solubility of the extracts and also the method of extraction employed in this study. The concentrations of the extracts above 5mg/ml were not soluble. This problem could be overcome in future by diluting the samples with different solvents or by using different type of extraction methods i.e. solvent extractions. The correlation between FRAP, DPPH scavenging abilities and total phenols were low to high respectively. The highest correlation was shown by DPPH scavenging abilities of mushrooms extracts and total phenol content. Thus, it can be concluded that the higher content of total phenols in seven different fruit bodies of *P. sajor-caju* extracts were responsible for their better antioxidant level.

Aqueous extracts of *P. sajor-caju* did not show toxicity when it was incubated together with PBMCs. These effects were compared to PBMCs treated with $H_2O_2$ (known oxidant) that could cause damage to the DNA of the cells. Cytotoxicity is one of the chemotherapeutic targets of antitumor activity on tumor cells but in some cases these agents turn out to be toxic to normal adjacent cells. The *in vitro* study on the non toxic effect of *P. sajor-caju* extracts despite of their processing methods shed some light on the ability of them to be implicated in chemopreventive treatment without harming normal cells. In addition, all the processed extracts showed mild protection against $H_2O_2$ induced toxicity. In this brief study on the protective effect of processed fruiting bodies of *P. sajor-caju*, the extracts actually maintained the viability of PBMCs from further destruction by the $H_2O_2$ (300μM) which showed percentage of cell inhibition approximately 25%. The protective effect could be caused by the presence of significant antioxidant level in the processed mushroom.

In the *in vitro* comet assay for genotoxicity in PBMCs, control (water) and processed *P. sajor-caju* were comparable. Obvious DNA damage in PBMCs after exposure to $H_2O_2$ (200μM) were observed. The DNA damage cause by this concentration of $H_2O_2$ was approximately 50%. This concentration was used to study the genoprotective activity of processed *P. sajor-caju* extracts. The extract P1 and P2 showed a protection of approximately 80% at 80μg/ml, where as extract P3 showed about the same level of protection at 100μg/ml. However, the freeze dried *P. sajor-caju* extract (P3) showed 50% effective concentration (EC50) at approximately 9μg/ml, in contrast to P1 and P2 which illustrated EC50 at 10μg/ml and 42μg/ml respectively. Hence, the processed mushroom showed different protective level on DNA damage induced with $H_2O_2$.

Total phenols are reported to be the major antioxidant component found in mushrooms. According to the results of this study, it is clearly indicated that the aqueous extracts of processed *P. sajor-caju* has significant antioxidant activity against various *in vitro* antioxidant system. Furthermore, the mushroom species can be used as an easily accessible source of natural antioxidant and to add on, they have become more popular in oriental and western cuisine. Processing or cooking the mushroom at different temperature showed that the antioxidant activities of *P. sajor-caju* were refrained although they showed a slight different in their properties. The preserved antioxidants were able to protect not only the integrity of plasma membrane of the normal cells but also the DNA of the cells.

The results underline the interest of further investigations of *P. sajor-caju* into possible chemopreventive activities and properties such as the isolation of possible polysaccharides or proteins with medicinal properties. The intake of more local edible mushroom especially oyster mushrooms in our daily diet could be an effective or one of the early strategies to reduce the risk of cancer. *Pleurotus sajor-caju* has a high potential to be produced economically on large scale in Malaysia. The nutritive and nutraceutical value or fact of this fungus should be emphasized to increase the mushroom intake in daily diet of Malaysian (2kg/year). By doing so, the popularity of *P. sajor-caju* can wide spread to standards of other known medicinal or edible mushrooms such as *L. edodes* (shitake), *H. erinaceus* (lion's mane), *G. lucidum* (ling-zhi), *Tuber sp.* (truffles) and *C. sinensis* (cordyseps).

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# APPENDICES

## Appendix A: Preparation of standard reagents and solutions

1. DPPH Solution
   0.00197 g of powder DPPH (Sigma) was dissolved in 50 ml ethanol and was used for the antioxidant test. It should be prepared freshly prior to experiments.

2. FRAP Reagent
   0.0155 g of powder sodium acetate (Merck) was dissolved in 40 ml of distilled water and 800 µl of acetic acid. The mixture was topped up to 50 ml. Then, 0.0156 g of TPTZ powder (Sigma) was dissolved in 0.2 ml of hydrochloric acid (1M) and topped up with distilled water to 5 ml. Both prepared solution were added together with 5 ml of 20mM of $FeCl_3.6H_2O$ (Merck). It should be prepared freshly prior to experiments.

3. 10% Folin Ciocaltue
   One mililitre ml of Folin Ciocaltue (Sigma) was topped up with 9 ml of distilled water. It should be prepared fresh prior to polyphenol test.

4. 10% Sodium carbonate
   Three gram of powder sodium carbonate (Sigma) was dissolved in 300 ml of distilled water. It should be prepared fresh prior to polyphenol test.

5. RPMI (10%)
   90 ml of basic medium RPMI 1640 (Sigma), 10 ml of FBS, 1ml of Penicillin/ Streptomycin (P/S) and L-Glutathione were added in a flask. The medium was kept at 4ºC prior to use.

6. Phosphate Buffered Saline (PBS)
   One tablet PBS (ICN) was dissolved in 100ml of distilled water. The solution was sterilized at 121ºC for 20 minutes if it is used for cell culture purposes.

7. MTT solution
   50 mg of powder MTT (Sigma) was dissolved in 10 ml of PBS solution. The prepared solution was filter sterilized and kept in aliquots of 1 ml Eppendorf tube in -20ºC. The solution was used for MTT assay.

8. Lysing solution
   A stock solution containing 146.4 g of sodium chloride (Sigma), 37.2 g of sodium EDTA (Merck), 1.2 g tris base (Sigma) and 8 g of sodium hydroxide (Merck) were prepared by dissolving to 890 ml distilled water. The pH should be adjusted to 10 with either HCl or NAOH. The stock solution was kept at room temperature for further use. The working solution of lysing solution was prepared by adding 10ml of triton-X 100 (Merck) and 10 g of SDS powder (Merck) into the stock and stir. The working solution should be kept at 4°C, 30 minutes before use.

9. 1% agarose
   One gram of agarose powder (Sigma) was dissolved in 100 ml of PBS solution. Heat required for the agarose dissolving properly in the buffer.

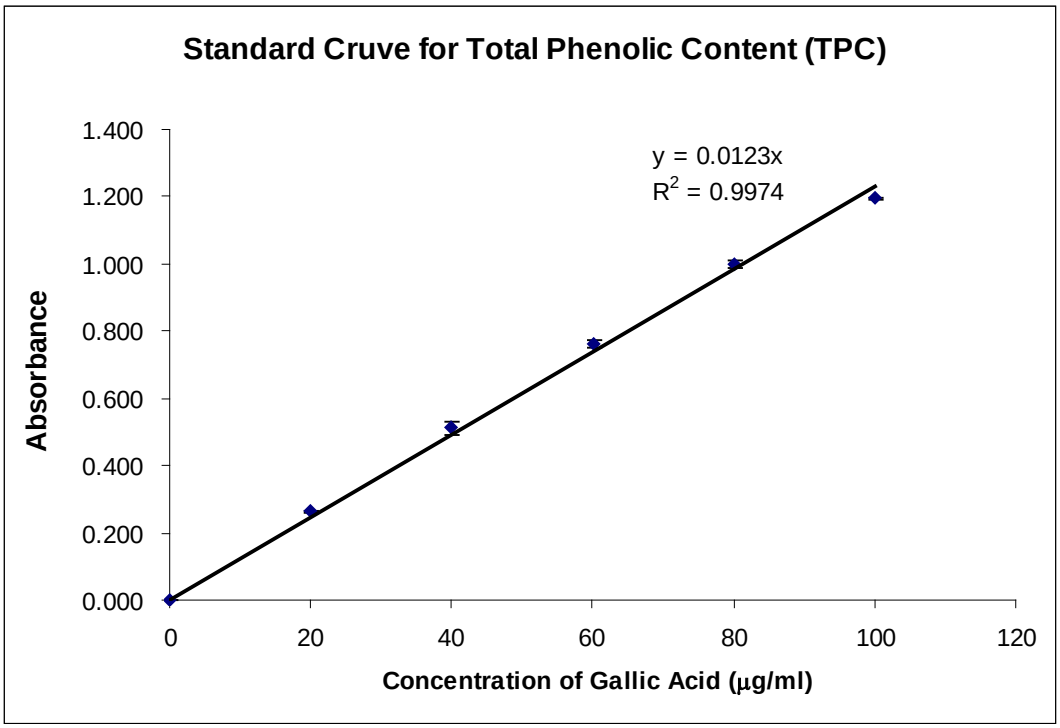
10. Electrophoresis buffer
    Stock solution of 10N of NAOH (Merck) and 200mM of EDTA (Sigma) were prepared. The working solution was prepared by adding 30 ml of NAOH and 5 ml of EDTA together and were topped up to 1 litre with distilled water. . The working solution should be kept at 4°C, 30 minutes before use.

11. Neutralization buffer
    48.5 g of tris base powder (Sigma) was dissolved in with 700 ml of distilled water. The pH was adjusted to 7.5 with HCL. Later, the solution was topped up to 1 litre.

12. TAE buffer
    4.85 g of tris powder (Sigma) was added together with 0.37g of sodium EDTA (Merck) into 700 ml of distilled water. The pH was adjusted to 8. Later, the solution was topped up to 1 litre.

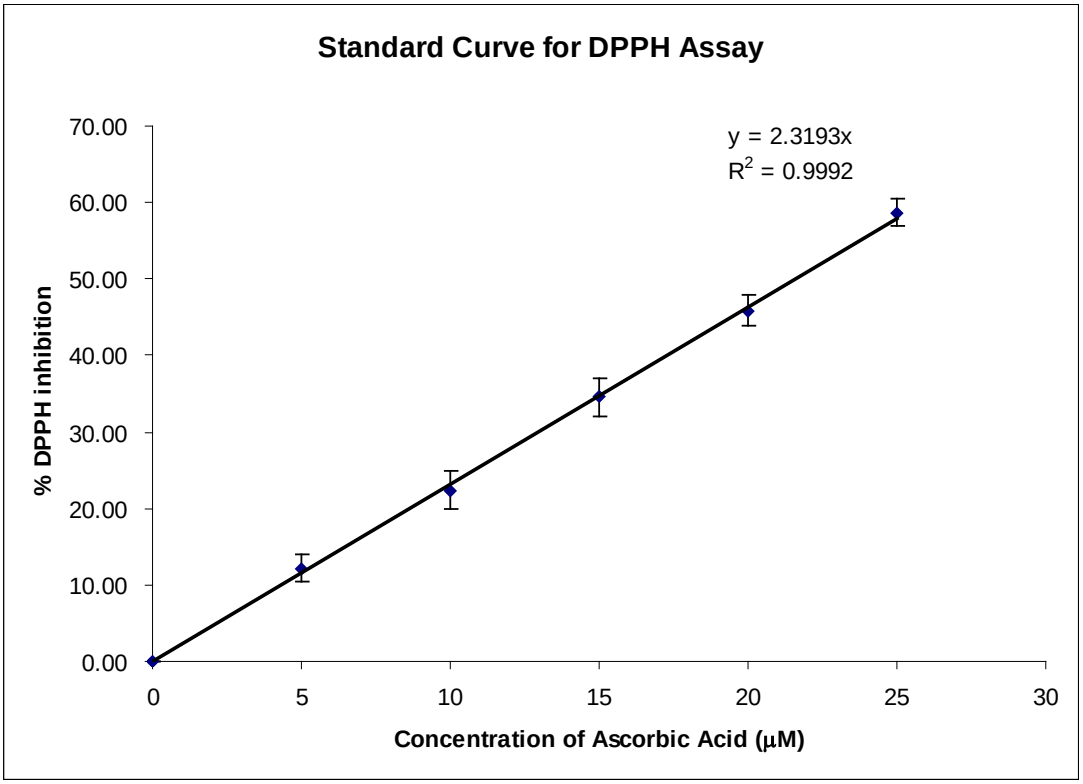
13. Ethidium bromide solution
    Two microlitre of ethidium bromide (10mg/ml) (Sigma) was diluted in 998 µl of distilled water. The Effendorf tube was covered with aluminium foil.

**Appendix B: Standard curve for TPC assay**



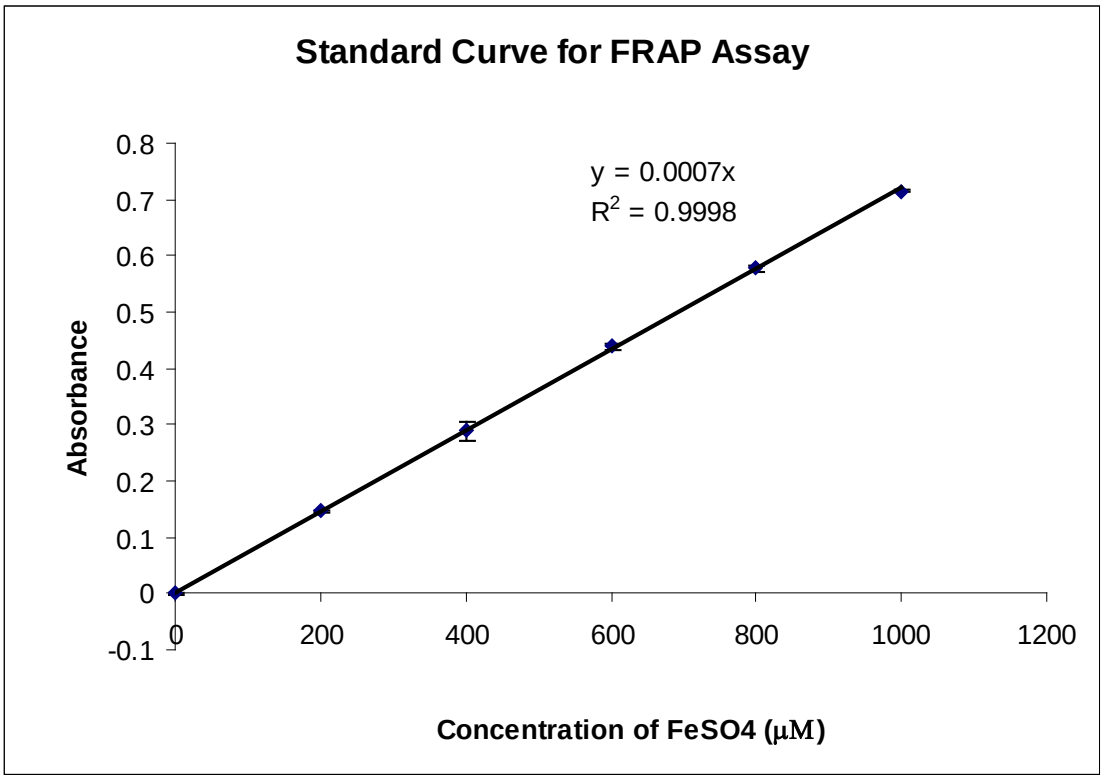

The results were expressed as absorbance ± S.E.M of triplicate assays.

**Appendix C: Standard curve for DPPH assay**



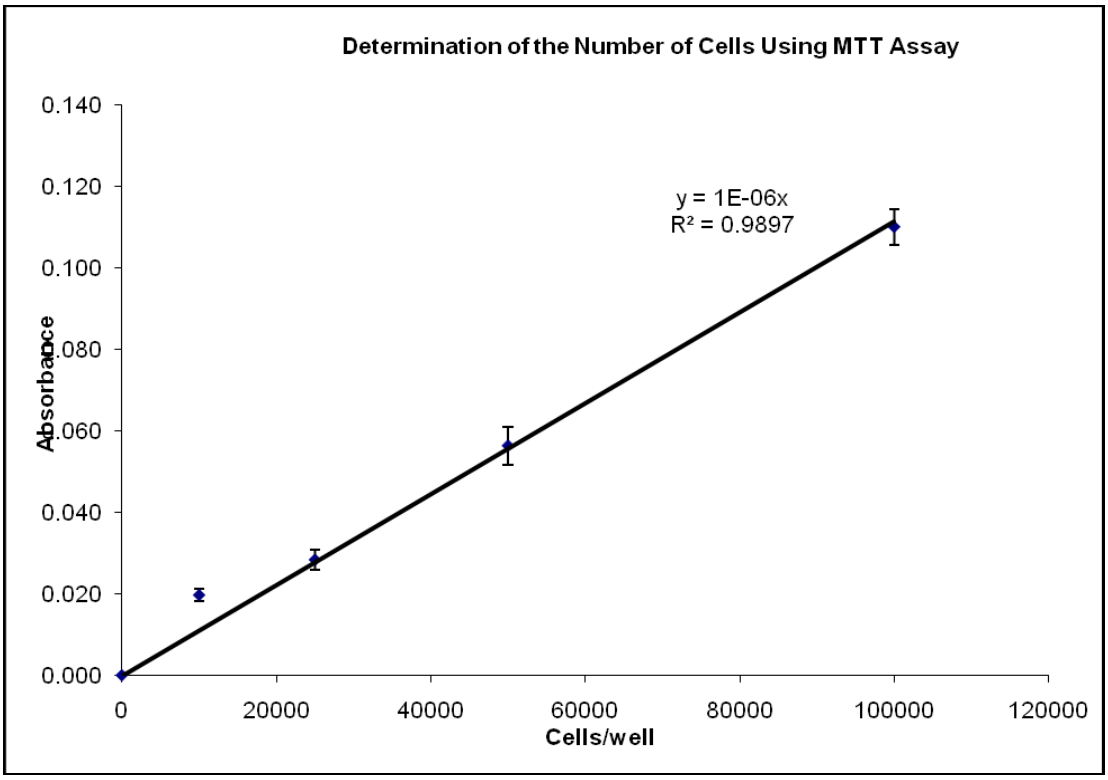

The results were expressed as % of inhibition ± S.E.M of triplicate assays.

**Appendix D: Standard curve for FRAP assay**




The results were expressed as absorbance ± S.E.M of triplicate assays.

**Appendix E: Determination of the number of cell using MTT assay**




The results were expressed as absorbance ± S.E.M of triplicate assays