Studies on the antineoplastic properties of polysaccharides isolated from a wood rotting macrofungus *Phellinus rimosus* (BERK) Pilat

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in

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Under the **Faculty of Science**

Under the Guidance of

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Certificate

I hereby certify that the thesis entitled **"Studies on the antineoplastic properties of polysaccharides isolated from a wood rotting macrofungus *Phellinus rimosus* (BERK) Pilat"** submitted by Meera C.R, Amala Cancer Research Centre, Thrissur, is an authentic record of research carried out by her under my supervision and guidance, in partial fulfilment of the requirement for the Ph.D degree of the Mahatma Gandhi University in Microbiology and that no part of this has previously formed the basis for the award of any degree, diploma or associateship in any university.

Thrissur-53
10/09/2009

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Declaration

I hereby declare that the thesis entitled **"Studies on the antineoplastic properties of polysaccharides isolated from a wood rotting macrofungus *Phellinus rimosus* (BERK) Pilat"** is an authentic record of research carried out by me, under the supervision and guidance of Dr. K.K. Janardhanan, Ph.D, FNABS, Professor, Amala Cancer Research Centre, Thrissur-53, in partial fulfilment of the requirement for the Ph.D degree of the Mahatma Gandhi University in Microbiology and that no part of this has been presented before for any other degree, diploma or associateship in any university.

Meera C.R.

Thrissur-53
10/09/2009

Dedicated to my loving parents…
my dear teachers…
my beloved husband & son…

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Preface

Natural products have been the major molecular and structural resources for drug discovery. In modern medicine, natural products for the prevention or treatment of tumors are attractive because of their very low clinical toxicity compared to synthetic chemical anti-tumor drugs. In the long pursuit for developing new anti-tumor compounds with low toxic potential, several polysaccharides from different biological origins, e.g. yeast, algae, bacteria, higher plants and fungi, have been investigated. Mushrooms have a great potential for the production of useful bioactive metabolites and they are a prolific resource for drugs. The spectrum of pharmacological activities of mushrooms is fascinating. Discovery and evaluation of new polysaccharides from the various medicinal mushrooms as new safe compounds for cancer treatment has become a hot spot of research.

Mushrooms are a taxonomic group that include well over 12,000 species which have macroscopic fruit-bodies large enough to be seen by the naked eye. Some of the most recently isolated and identified compounds originating from the medicinal mushrooms have shown promising immunomodulatory, antitumor, cardiovascular, antiviral, antibacterial, antiparasitic, hepatoprotective and antidiabetic properties. Mushroom-derived polysaccharides are now considered as compounds which are able to modulate animal and human immune responses and to inhibit growth of certain tumors. While mushroom glucans are mostly non-cytotoxic, the same is not true for glucan-protein complexes. All of these compounds, have been shown to potentiate the host's innate (non-specific) and acquired (specific) immune responses and activate many kinds of immune cells that are important for the maintenance of homeostasis. However, for most of the mushroom derived anti-cancer compounds, a detailed understanding of their exact mode of action has not yet been elucidated.

Many mushroom polysaccharides have been shown to have considerable antitumor activity in several xenographs, but only a limited number has undergone clinical trials. In many cases there have been significant improvements in quality

of life and survival. Increasingly, several of these compounds are now used extensively in Japan, Korea and China, as adjuvant therapy to standard radio- and chemotherapy. Perhaps the most encouraging observations is the ability of the mushroom-derived polysaccharides when taken prior to and during radiotherapy and chemotherapy to significantly reduce the side-effects of these treatments. The safety criteria for the mushroom polysaccharides have been exhaustively studied with little evidence of any toxicity.

Clinical efficacy of mushroom polysaccharides will depend on understanding their precise scope of activity verifiable through *in vitro* and *in vivo* animal and tissue culture tests. *Phellinus rimosus* is a less extensively studied species of the genus *Phellinus.* The present study aims at the isolation and characterization of polysaccharides from *P. rimosus* and to elucidate its anti-neoplastic properties using *in vitro* and animal experimental models.

The thesis is divided in to eleven chapters. Chapter 1 and 2 covers Introduction and Review of literature. In Chapter 3, the materials and methods of the present study are described. Chapter 4 describes the methodology for the isolation and characterization of polysaccharide-protein complex from the fruiting bodies of *P.rimosus* (PPC-*Pr*) and Chapter 5 describes the demonstration of the anti-tumor activity of the compound. In Chapter 6, anti-oxidant as well as free radical scavenging properties of PPC-*Pr* is described in detail. The anti inflammatory activity as well as the *in vivo* anti-oxidant activity of PPC-*Pr* is presented in Chapter 7. In Chapter 8, studies on the cytotoxic, anti-proliferative and apoptotic activities of PPC-*Pr* are described. In Chapter 9, the isolation of neutral polysaccharide from the PPC-*Pr* and its chemical characterization are presented. Possible structure of neutral polysaccharide was elucidated using advanced techniques. Antineoplastic properties like anti-tumor, anti-inflammatory and anti-oxidant activities of the neutral polysaccharide are described in Chapter 10. The experimental findings are summarized and the projected significant conclusions are presented in Chapter 11. This chapter is followed by profuse literature citations.

List of Abbreviations

AHCC	Active hexose correlated compounds
AIF	Apoptosis inducing factor
ALA	A-Lipoic acid
ANOVA	Analysis of variance
AO	Acridine orange
AP-1	Activator protein-1
$ASc^{\cdot -}$	Semidehydroascorbate radical
BP	Benzo [*a*] pyrene
BRM	Biological Response Modulators
BSA	Bovine serum albumin
CCl_4	Carbon tetrachloride
CR3	Complement receptor type 3
CTA-OH	Cetyl trimethyl ammonium hydroxide
Da	Dalton
DAPI	4', 6-Diamidino-2-phenylindole dihydrochloride hydrate
DC	Dentritic cells
DEAE	Diethyl aminoethyl
DHLA	Dihydrolipoic acid
DLA	Dalton's lymphoma ascites
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Di methyl sulphoxide
DNA	Deoxyribo Nucleic Acid
DPPH	1,1-diphenyl 2-picryl hydrazyl
DTNB	5,5-diothiobis-(2-nitrobenzoic acid)
EAC	Ehrlich's ascites carcinoma
EB	Ethidium bromide
EBV	Epstein-Barr virus
EGFR	Epidermal growth factor receptor

FBS	Fetal Bovine Serum
FCA	Freund's complete adjuvant
FRAP	Ferric reducing antioxidant power
FT-IR	Fourier transform-infra red spectrometry
GPC	Gel permeation chromatography
GPx	Glutathione peroxidases
GSH	Reduced glutathione
GST	Glutathione-*S*-transferase
H_2O_2	Hydrogen peroxide
HBV	Hepatitis B virus
HCC	Hepatocellular carcinomas
HIV	Human Immunodeficiency Viruses
HPLC	High performance liquid chromatography
HPV	Human papilloma viruses
HTLV	Human T-Cell Leukemia Viruses
IC_{50}	50 % inhibiting concentration
IU	International units
LMPA	Low melting point agarose
MALDI-TOF	Matrix Assisted Laser Desorption / Ionization-Time of Flight
MDA	Malondialdehyde
MNU	*N*-methyl-*N*-nitrosourea
MTT	3-(4-5 dimethylthiozol-2-yl) 2-5 diphenyl tetrazolium bromide
NADP	nicotinamide adenine dinucleotide phosphate (disodium)
NaN_3	Sodium azide
NBT	Nitroblue tetrazolium
NF-kB	Nuclear factor kappa B
NK	Natural killer
NMA	Normal melting agarose
NMR	Nuclear magnetic resonance
$NO^{\cdot}$	Nitric oxide
NOSs	Nitric oxide synthases

NPC	Nasopharyngeal carcinoma
$O_2^{\cdot -}$	Superoxide anion
$^{\cdot}OH$	Hydroxyl radical
$ONOO^-$	Peroxynitrite anion
PAHs	Polycyclic aromatic hydrocarbons
PC	Paper chromatography
PKC	Protein kinase C
PL	Polysaccharide of *P. linteus*
PM	Peritoneal macrophages
PPC-*Pr*	Polysaccharide-protein complex from *P. rimosus*
PSP	Polysaccharopeptides
PTEN	Phosphatase and Tensin Homolog Deleted on Chromosome 10
PTK	Protein tyrosine kinase
QOL	Quality of life
Rb	Retinoblastoma
RNS	Reactive nitrogen species
$ROO^{\cdot}$	Peroxyl radicals
ROS	Reactive oxygen species
SCGE	Single cell gel electrophoresis
SOD	Superoxide dismutase
TBARS	Thiobarbituric acid reacting substance
TCA	Trichloroacetic acid
TFA	Trifluro acetic acid
TLC	Thin layer chromatography
TPP	Triphenyl phosphene
TPTZ	2,4,6-tripyridyl-s-triazine
TPTZ	2,4,6-tripyridyl-s-triazine
UVR	Ultra violet radiation

CONTENTS

Chapter-1

Introduction

Synopsis

This chapter briefly describes cancer and available methods of cancer treatment. Medicinal mushrooms have an established history of use in traditional oriental therapies. Biological properties of mushroom components with special emphasis to the mushroom polysaccharides are beiefly outlined. This chapter also examines the relationship of free radicals, inflammation and apoptosis to the development of neoplasia. A brief account of the medicinal mushroom, *P.rimosus*, used in the present study is given at the end of this chapter.

The global awareness of cancer as the second largest cause of death in people of various ages and racial background has lead to so much research efforts and clinical studies in the fight against the disease (Daba and Ezeronye, 2003). Cancer is a group of diseases characterized by uncontrolled growth and spread of abnormal cells. If the spread is not controlled, it can result in death. Cancer is caused by both external factors (tobacco, chemicals, radiation, and infectious organisms) and internal factors (inherited mutations, hormones, immune conditions, and mutations that occur from metabolism). These causal factors may act together or in sequence to initiate or promote carcinogenesis. Ten or more years often pass between exposure to external factors and detectable cancer (American Cancer Society, 2008).

Cancer is treated with surgery, radiation and chemotherapy. It has been generally recognized that in the treatment of cancer surgery with or without radiotherapy remains the modus operandi for most cancer cures. Radiotherapy is used quite successfully for many forms of cancers while chemotherapy has become an integral part of a multi-disciplinary treatment of cancers and has served also as a palliative measure in cases of advanced cancer. However, cancer treatment by conventional therapies is known to have adverse effects. The adverse effects are greatest on hematopoietic tissue, gastrointestinal mucosa, gonads and

skin. Injury to hematopoietic tissue causes severe immunosuppression and negatively affects therapy by leaving the host susceptible to infection by opportunistic and pathogenic microorganisms. It is well recognized that both radiotherapy and chemotherapy invariably damage or weaken the patient's immunological defences which may also have been damaged by the cancer itself. Furthermore, within the holistic approach of clinical cancer therapy there is now increasing emphasis being given to patient quality of life (QOL) following these above classical treatments. Survival should not be the sole criterion for assessing the treatment results.

Recent investigations have been channeled on the development of immunotherapy to target and remove cancer cells as well as on substances such as immunopotentiators, immunoinitiators and biological response modulators (BRM) that act to prevent carcinogenesis and induce carcinostasis (Wasser and Weis, 1999a; 1999b). Biological Response Modifiers has now evolved as the fourth method of cancer treatment in addition to surgery, radiotherapy and chemotherapy. Immunostimulating agents would possibly be useful adjuncts to conventional treatments of cancer if they do not interfere with the ability of the conventional treatment to kill tumor cells.

Medicinal mushrooms have an established history of use in traditional oriental therapies. Historically, hot water-soluble fractions (decoctions and essences) from medicinal mushrooms were used as medicine in the Far East, where knowledge and practice of mushroom use primarily originated (Hobbs, 2000; Wasser, 2002). The use of medicinal mushrooms in the fight against cancer is known for a very long time in Korea, China, Japan, Russia, USA and Canada. An old Japanese legend reports that wild monkeys rarely experience cancer, high blood pressure or diabetes. The legend suggests that perhaps it is due to some extent their consumption of wild mushrooms. Mushrooms belong to the group of immunoceuticals by their mode of action. Nowadays macrofungi are distinguished as important natural resources of immunomodulating and anticancer agents and with regard to the increase in diseases involving immune dysfunction, cancer, autoimmune conditions in recent years, applying such immunomodulator agents

especially with the natural origin is vital. The physiological constitution of host defence mechanisms are improved by the intake of mushroom compounds which restore homeostasis and enhance resistance to disease. A central premise in Oriental medicine is to regulate homeostasis of the whole body and to return the diseased individual to the normal state. Several major substances with immunomodulatory and/or anti-tumor activity have been isolated from mushrooms. These include mainly polysaccharides (in particular β-D-glucans), polysaccharopeptides (PSP), polysaccharide proteins and proteins. Furthermore, other bioactive substances, including triterpenes, lipids and phenols, have been identified and characterized in mushrooms with proven medicinal properties.

Polysaccharides are the best known and most potent mushroom derived substances with anti-tumor and immunomodulating properties. Several purified mushroom polysaccharides have been in clinical use in Japan, China, and US for several years with no reports of any significant short-term or long-term adverse effects. Five mushroom preparations have shown clinically significant efficacy against human cancers and are used as BRMs: lentinan from *Lentinus edodes*, D-fraction from *Grifola frondosa*, schizophyllan from *Schizophyllum commune*, PSK (krestin) and PSP (polysaccharide peptide) from *Trametes versicolor*. All of these preparations are chemically β-D-glucans in nature or β-D-glucans linked to proteins. Mushroom polysaccharides offer a lot of hope for cancer patients and sufferers of many devastating diseases (Chihara, 1992). These compounds have been shown to be safe when taken over long periods of treatment; and appear to reduce the adverse effects of radiotherapy and chemotherapy. Indeed, there are also many examples where the use of these compounds allows the reduction in dose level of the toxic chemotherapeutic compound without reduced efficacy.

Mushroom polysaccharides display a wide array of biopharmacological activities. While much attention has been drawn to various immunological and anti-cancer properties of the mushroom compounds, they also offer other potentially important therapeutic properties including antioxidants, anti-hypertensive, cholesterol-lowering, liver protection, anti-fibrotic, anti-inflammatory, anti-diabetic, anti-viral and anti-microbial. Unlike proteins and

nucleic acids, polysaccharides contain repetitive structural features which are polymers of monosaccharide residues jointed to each other by glycosidic linkages. Among these macromolecules, polysaccharides offer the highest capacity for carrying biological information because they have the greatest potential for structural variability. For example, the number of possible permutations for four different sugar monomers can be up to 35,560 unique tetrasaccharides, whereas four aminoacids can form only 24 different permutations (Ooi and Liu, 2000). Therefore, this enormous potential variability in polysaccharide structure gives the necessary flexibility for the precise regulatory mechanisms of various cell-cell interactions in higher organisms.

Polysaccharides, which are the major constituents of mushrooms, have been demonstrated to play an important role as dietary free-radical scavenger for the prevention of oxidative damage. Free radicals and active oxygen can induce oxidant damage. There are increasing evidence indicating that reactive oxygen species (ROS) produced by sunlight, ultraviolet light, ionizing radiation, chemical reactions and metabolic processes have a wide variety of pathological effects, such as causing DNA damage, carcinogenesis and cellular degeneration related to aging (Blander et al., 2003; Harman, 1993; Liu et al., 1997). The oxyradicals attack DNA causing change in genomic sequences leading to mutation, deletion, gene-amplification or rearrangement. In this manner, oxidative DNA damage was found important in the etiology of many human cancers (Irshad and Chaudhuri, 2002).

Free radicals have long been implicated in damage of connective tissues in inflammation. It is becoming more evident that many aspects of tumor promotion arise from persistent and unresolving inflammation. Chronic inflammation may be one of the driving forces of transformation that together with other determinants, including the intrinsic properties of pre-malignant cells support the initiation of cancer. Obviously, if inflammatory conditions prevail at the tumor site, they may further support the progression of tumor into more advanced stages and also promote metastasis. Acceleration of neoplastic processes by chronic inflammation

was clearly observed in many malignant diseases (Coussens and Werb , 2002; Schwartsburd ,2003).

In normal tissues, a homeostasis is maintained by a balance between cell proliferation and apoptosis. Apoptosis or programmed cell death is a regulated process involving activation of a series of molecular events leading to cell death. Recent scientific advances indicate that reduced apoptosis and/or increased cell proliferation play a central role in carcinogenesis. Therefore, treatments of human neoplasia have been directed to promote apoptosis of tumor cells. Agents that specifically induce apoptosis in tumor cells without affecting the corresponding normal cells could serve as key factors in successful treatment of human cancers. Mushroom polysaccharides are widely being used as nonspecific immunostimulants for cancer patients. Now it has been suggested that the anti-cancer effects of mushroom polysaccharides are not only immunomodulatory, but may result from cell cycle arrest and/or induction of apoptosis. It is generally believed that polysaccharides do not induce direct toxicity in cancer cells. Recently, many mushroom polysaccharides have been shown to exert a direct cytotoxic effect on cancer cells in vitro.

Phellinus is a large and widely distributed genus belonging to the *Hymenochaetaceae basidiomycetes.* There are approximately 220 known species of *Phellinus* mushrooms in the world, and they were found mainly in tropical areas of America and Africa (Kim et al., 2003d). Many kinds of *Phellinus* species including *P. igniarius, P. hartigii, P. gilvus, P. pini,* etc. are known to have different medicinal effects such as anti-tumor and immuno-stimulating activities (Ayer et al., 1996; Jung et al., 1997; Rew et al., 2000; Shibata et al., 1968; Shon and Nam, 2001). Among them, *P.linteus* is well known as one of the most popular medicinal mushrooms due to its high anti-tumor (Han et al., 1999) and immunostimulating (Lee et al., 1996b; Kim et al., 1996) activities. About 18 species of *Phellinus* are found to occur in Kerala, most of them are wood inhabiting (Leelavathy and Ganesh, 2000). *Phellinus rimosus* (Berk) Pilat, commonly called 'Bracket fungus' or 'Cracked cap polypore', referred to as 'Plamanjal' or 'Plachanam' in Malayalam, is a polyporus macrofungus, parasitic

mostly on jack fruit tree trunks in Kerala (Figure 1.1). It has reddish-brown cork-like texture and resembles a shelf, growing on the trunk of living trees. The dorsal side has woody appearance, whereas the ventral side has velvet like appearance (Figure 1.2). It lacks stalk and grows up to 25 cm. It is a kind of wood rotting species, which causes so-called "whitening", that is, decaying a host tree to the point where it shows a white color due to its strong lignin decomposition ability. It causes white pocket rot initially but later the heartwood is transformed into a white spongy mass. The present study was focused on the isolation and characterization of the total and neutral polysaccharides from the fruiting bodies of *P.rimosus* and intensive investigation on the anti-tumor, antioxidant, anti-inflammatory, cytotoxic, anti-proliferative and apoptotic, activities of the isolated polysaccharides was carried out. The findings are presented in this thesis.

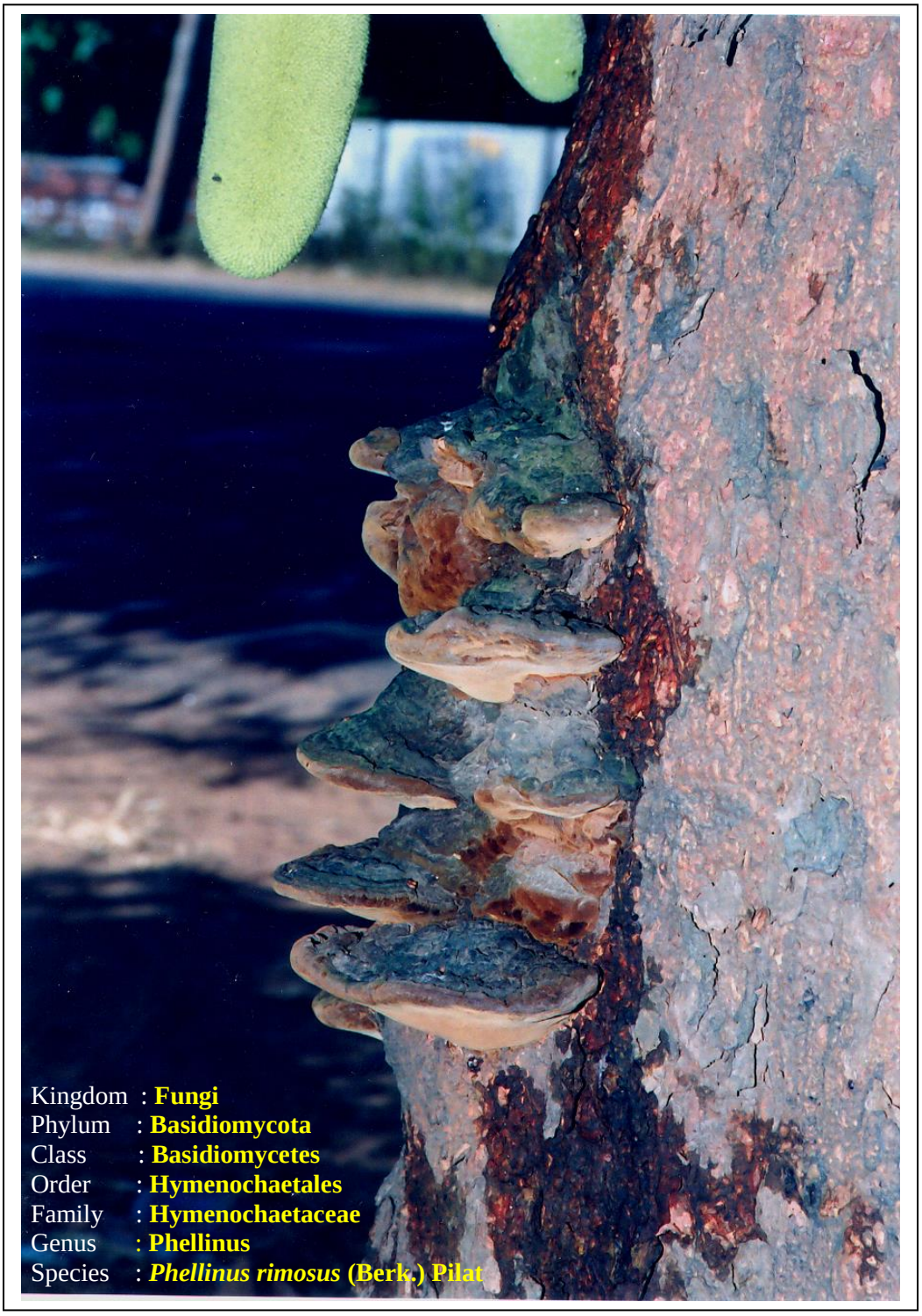

Figure 1.1. Photograph of fruiting body of *Phellinus rimosus* growing on natural habitat

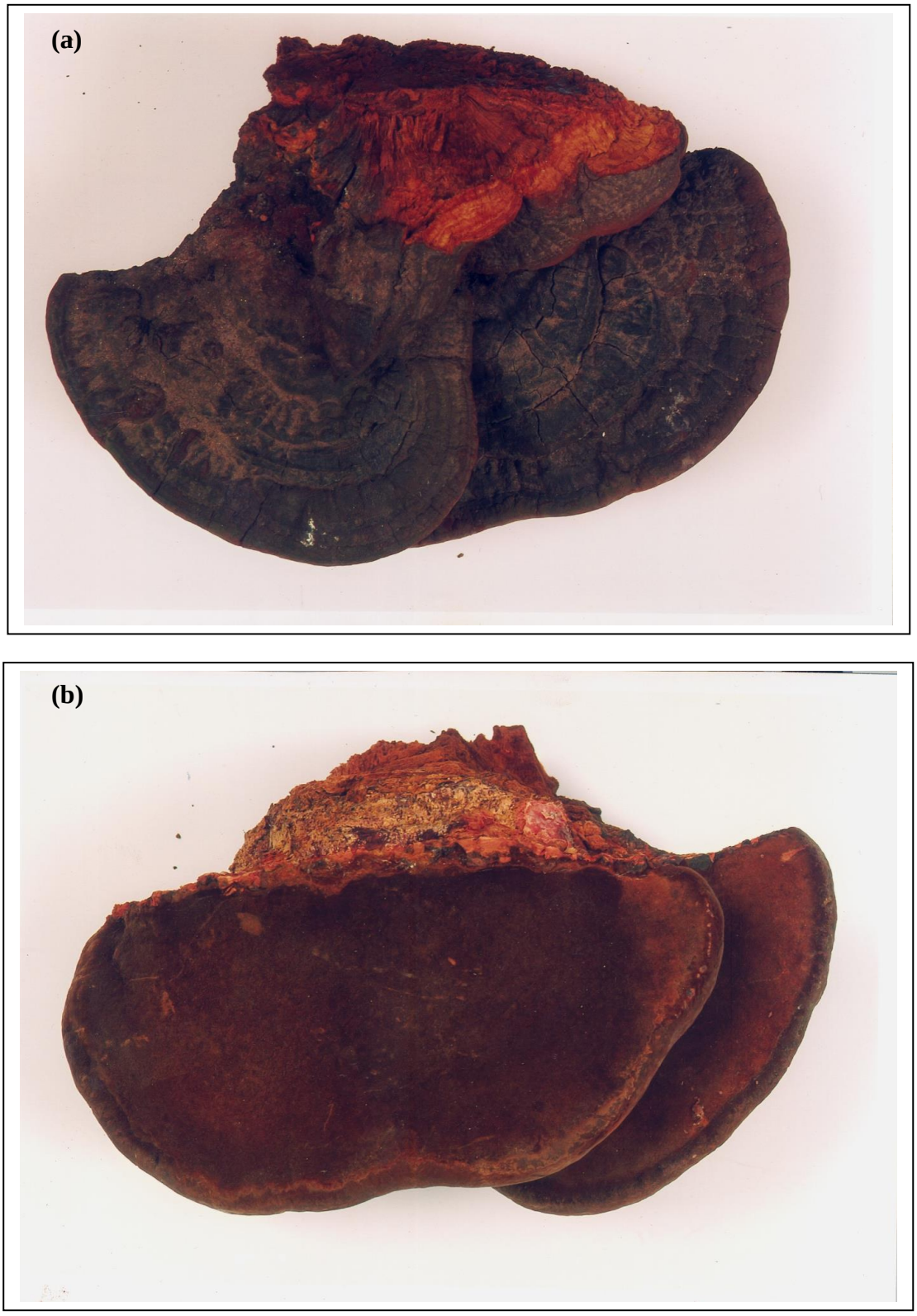

Figure 1.2. Fruiting body of *Phellinus rimosus* (a) dorsal and (b) ventral view

Chapter-2

Review of Literature

Synopsis

This chapter aims to give a basic understanding of the epidemiology and etiology of cancer. The role of free radicals, inflammation and apoptosis in the prevention and treatment of cancer is discussed in detail. This chapter sets out the pharmacological potential of mushrooms as well as the current information on the use of various mushroom polysaccharides in cancer treatment. A brief summary of medicinal properties of P*hellinus* species is outlined. Previous reports pertaining to *P.rimosus* and the objectives of the present study are also addressed.

2.1. Epidemiology of cancer

The overall occurrence of cancer every year is approximately 10.9 million new cases, 6.7 million deaths and 24.6 million persons alive with cancer (within three years of diagnosis) worldwide. The most commonly diagnosed cancers are lung (1.35 million), breast (1.15 million) and colorectal (1 million); the most common causes of cancer death are lung cancer (1.18 million deaths), stomach cancer (700,000 deaths), and liver cancer (598,000 deaths). The most prevalent cancer in the world is breast cancer. There are striking variations in the risk of different cancers by geographic area. Most of the international variation is due to exposure to known or suspected risk factors related to lifestyle or environment, and provides a clear challenge to prevention (American Cancer Society, 2008). Figure 2.1 shows the number of new cases, deaths and persons living with cancer in several larger countries. Every year about 850,000 new cancer cases are diagnosed in India resulting in about 580,000 cancer related death. India has highest number of the oral and throat cancer cases in the world. In males, oral, lungs and stomach cancers are the three most common causes of cancer incidence and death. Cervical, breast and oral cancers are the three main causes of cancer related illnesses and death in females. Overall cervical cancer is the number one cause of cancer death in India (Cancer Support Society, 2008).

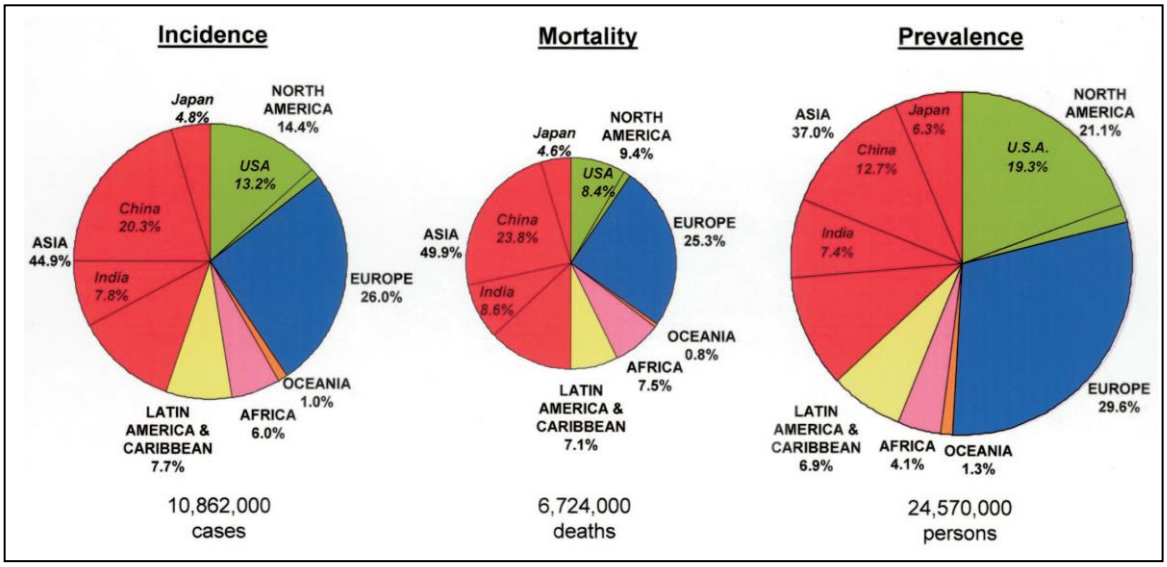

Figure 2.1. Incidence, Mortality and Prevalence by Location
(Downloaded from www.caonline.amcancersoc.org)

2.2. Etiology of cancer

Cancer is a multicausal, multistage group of diseases the mechanisms of which are still only partially known (International Agency for Research on Cancer (IARC), 1992). Many factors, including exposure to viruses, xenobiotic ('foreign') chemicals and radiation contribute to cancer causation (Yuspa, 2000). The conversion of a normal cell to malignant neoplasm is given in Figure 2.2.

2.2.1. Chemical carcinogenesis

A chemical carcinogen is most simply viewed as any chemical agent, process or habit which in humans or animals increases the risk of cancers developing for a particular age group. Neoplasia is a unique and perhaps a most extreme, tissue response to chemical toxicants (Saffiotti, 1977). The first source of our knowledge of chemical carcinogenesis was clinical observations in humans. In 1775, Percival Pott, an eminent English physician and surgeon, observed that the occurrence of cancer of the scrotum was linked to soot exposure in English chimney sweeps. In 1918, Yamagiwa and Ichikawa produced tumors by repeated painting of coal tar on the skin of rabbits. This breakthrough provided the foundation for the isolation, identification, synthesis and biological testing of chemical carcinogens (Okey et al., 2005).

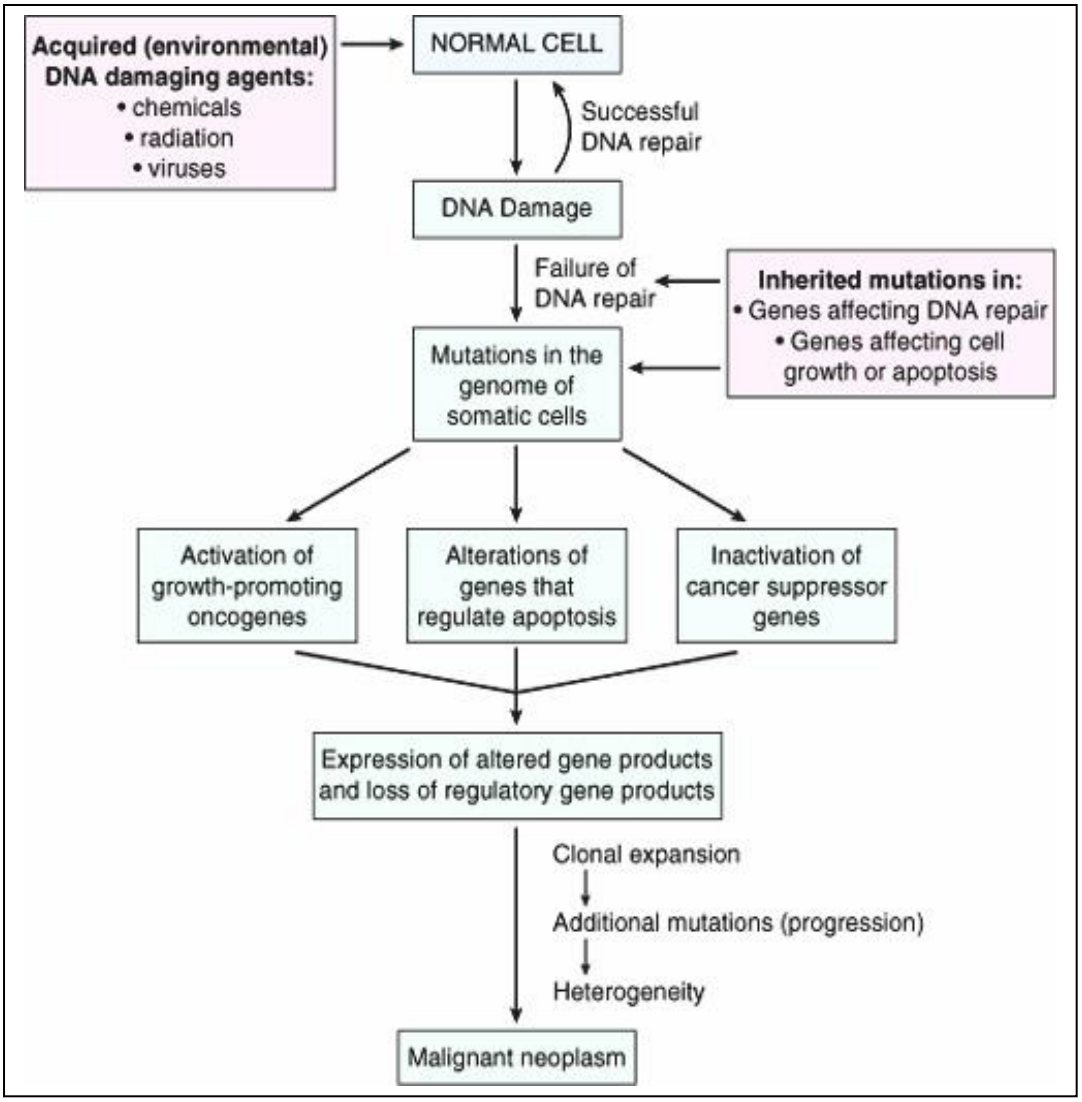

Figure 2.2. Conversion of a normal cell to malignant neoplasm

Most chemicals that were identified as carcinogens in early research are by-products of industrial processes. For example, benzo [*a*] pyrene (BP) and related polycyclic aromatic hydrocarbons (PAHs) arise from partial combustion of petroleum or tobacco; benzidine and other aromatic amines were present in the workplace, as was vinyl chloride. Other chemical carcinogens are present in foodstuffs or in the environment. Examples include aflatoxin B1, a potent liver carcinogen formed by molds that contaminate improperly stored grains, and 2-amino-3-methylimidazo [*4, 5-f*] quinoline (IQ), a heterocyclic amine derived from amino acids during high-temperature cooking (Ames and Gold, 1998). In addition to organic compounds, a number of inorganic elements and their compounds have

been shown to be carcinogenic in both animals and humans (Martell, 1981). At least ten elements or their compounds, including beryllium, iron, cobalt, zinc, lead and platinum, have been shown to be carcinogenic in experimental animals. However, it has been estimated that chemical pollution accounts for less than 1% of human cancer. Some important chemical carcinogens associated with workplace, lifestyle and medical therapy are given in Table 2.1., 2.2.and 2.3.

Table 2.1. Exposures to Chemicals in the Workplace

Agent	**Industries and Trades with Proved Excess Cancers and Exposure**	**Primary Affected Site**
p-Aminodiphenyl	Chemical manufacturing	Urinary bladder
Asbestos	Construction, asbestos mining and milling, production of friction products and cement	Pleura, peritoneum, bronchus
Arsenic	Copper mining and smelting	Skin, bronchus, liver
Alkylating agents (mechloroethamine hydrochloride and bis[chloromethyl]ether)	Chemical manufacturing	Bronchus
Benzene	Chemical and rubber manufacturing, petroleum refining	Bone marrow
Benzidine, β-naphthylamine, and derived dyes	Dye and textile production	Urinary bladder
Chromium and chromates	Tanning, pigment making	Nasal sinus, bronchus
Isopropyl alcohol manufacture	Chemical manufacturing	Cancer of paranasal sinuses
Nickel	Nickel refining	Nasal sinus, bronchus
Polynuclear aromatic hydrocarbons from coke, coal tar, shale, mineral oils, and creosote	Steel making, roofing, chimney cleaning	Skin, scrotum, bronchus
Vinyl chloride monomer	Chemical manufacturing	Liver
Wood dust	Cabinetmaking, carpentry	Nasal sinus

Modified from Cullen et al. (1990)

Table 2.2. Carcinogenic factors associated with lifestyle

Chemical, Physiological Condition or Natural Process	**Associated Neoplasm**
Alcoholic beverages	Esophagus, liver, oropharynx, larynx
Aflatoxins	Liver
Betel chewing	Mouth
Dietary intake (fat, protein, calories)	Breast, colon, endometrium, gallbladder
Reproductive history Late age at first pregnancy Zeo or low parity	 Breast Ovary
Tobacco smoking	Mouth, pharynx, laryns, lung, esophagus, bladder

Modified from Pitot (1986) and Vainio et al. (1991)

Table 2.3. Carcinogenic risks of chemical agents associated with medical therapy and diagnosis

Chemical or Drug	**Associated Neoplasms**
Alkylating agents (cyclophosphamide, melphalan)	Bladder, leukemia
Inorganic arsenicals	Skin, liver
Azathioprine (an immunosuppressive drug)	Lymphoma, reticulum cell sarcoma, skin, Karposi's sarcoma
Chlornaphazine	Bladder
Chloramphenicol	Leukemia
Diethylstibesterol	Vagina (clear cell carcinoma)
Estrogens Premenopausal Postmenopausal	 Liver cell adenoma Endometrium
Methoxypsoralen with ultaviolet light	Skin
Oxymetholone	Liver
Phenacetin	Renal pelvis (carcinoma)
Phenytoin (diphenyhydantoin)	Lymphoma, neuroblastoma
Thorotrast	Liver (angiosarcoma)

Chemical carcinogens act cumulatively. Chemical carcinogens interfere with various stages of the development of cancer and function through modifications of cellular and molecular events. Chemicals, participating in the process of carcinogenesis may function differently. Genotoxic agents are usually chemicals that directly damage DNA, which in turn leads to mutation or clastogenic changes. A second category of carcinogenic compounds (non-genotoxic) function through non-DNA or indirect-DNA reaction mechanisms. These compounds modulate cell growth and cell death; however their mode of action is not yet fully understood (Valko et al., 2006).

2.2.1.1. The multi-step process of chemical carcinogenesis

Epidemiological clues and animal experiments have shown that the process of chemical carcinogenesis consists of multiple and distinct stages, each characterized by different underlying mechanisms, i.e. the multi-stage and –mechanism hypothesis or the "initiation-promotion-progression" model of carcinogenesis (Klaunig and Kamendulis, 2004; Trueba et al., 2004). Initiation involves a non-lethal mutation in DNA that produces an altered cell followed by at least one round of DNA synthesis to fix the damage (e.g. 8-OH-G) produced during the initiation; if dividing cells are damaged for whatever reason, they are able to interrupt temporarily their cell cycle at stage G1, S or G2 ("check points"), repair the damage and resume division. Initiation is irreversible, but not all initiated cells will go on to establish a tumor, because many will die by apoptosis. At least three cellular functions are important in initiation, namely carcinogen metabolism, DNA repair and cell proliferation.

On the other hand, tumor promotion may be modulated by various environmental factors, including diet, age, hormonal balance and sex. This stage dose-dependently requires the continuous presence of the tumor promotion stimulus and therefore it is a reversible process (Loft and Poulsen, 1996). Promotion is that stage in the natural history of neoplastic development which, if existent, is characterized by the reversible expansion of the initiated cell population and the reversible alternation of genetic expression. Progression is the third and final stage of the carcinogenic process (Klaunig and Kamendulis, 2004).

This stage involves cellular and molecular changes that occur from the preneoplastic to the neoplastic stage. Irreversible benign and malignant neoplasms are characteristic of this stage of neoplastic development. Progression has been defined as that stage of carcinogenesis exhibiting measurable and morphological karyotypic changes in the structure of the cell genome (Pitot, 1986). However, it is clear that this process does not always compartmentalize neatly into these three classic stages, and that multiple sequential mutations are required in order to convert a normal cell into a malignant cell (Bertram, 2001; Knudson, 2001).

2.2.1.2. Targets of chemical carcinogens

The relevant targets for mutagenesis by carcinogens include proto-oncogenes and tumor suppressor genes. When proto-oncogenes are activated by a mutational event, signals for cell growth are increased, but for tumor suppressor genes, which regulate cell growth negatively, relevant mutations result in a loss of function (Okey et al., 2005).

a) Oncogenes

Commonly found mutations in chemically induced tumors in rodents are those which activate the *ras* family of oncogenes. For example, rat mammary tumors induced by a single dose of *N*-methyl-*N*-nitrosourea (MNU) contain H-*ras* genes that have been activated by a single point mutation (G-to-A transition) at codon 12 of the gene (Zhang et al., 1991). In papillomas and carcinomas induced in mouse skin by 7,12-dimethylbenz[*a*]anthracene, there is an activating mutation in codon 61 (A-to-T transversion) of the H-*ras* gene (Quintanilla et al., 1986). Genomic analysis of cells derived from neuroblastoma has revealed frequent amplification of the c-myc family member, N-myc. Similar analysis of small cell lung carcinoma showed amplification of all three transforming members of the myc gene family, C-myc, N-myc and L-myc (Oster et al., 2002). Examples of amplification of other oncogenes include the epidermal growth factor receptor (EGFR) in glioblastomas, Her-2/NEU and Cyclin D1 in breast cancer, and C-abl in chronic myeloid leukemia. Bcl-2 oncogene translocation was identified in human follicular lymphomas that juxtaposed the Bcl-2 gene to the immunoglobulin heavy chain gene leading to over expression of Bcl-2. Over expression of Bcl-2 protein

has also been observed in other leukemias, such as B-cell chronic lymphocytic leukemia, acute myeloid leukemia, multiple myeloma, and solid tumors of the breast, bowel, lung and skin (Oster et al., 2005).

b) Anti-oncogenes (Tumor suppressor genes)

The retinoblastoma gene (Rb) was the first human tumor suppressor to be identified as a result of its frequent mutations in familial retinoblastoma, a rare pediatric eye tumor. In addition to retinoblastoma, mutations of Rb can be found in other human malignancies, including osteosarcoma, small cell lung carcinoma, prostate and breast cancer (Weinberg, 1995., Sherr, 1996). In humans, mutations in the tumor suppressor gene p53 are present in more than 50 percent of tumors. The evidence linking particular chemical exposures to cancer has been greatly strengthened by the observation that chemicals leave molecular signatures in the form of characteristic patterns of mutation in p53 and other genes. For example, lung tumors as well as nontumorous lung tissues from smokers with lung cancer carry a high mutational load at codons 157, 248 and 249 in the p53 gene (Hussain et al., 2001). Most p53 mutations in lung cancer are G-to-T transversions, a type of mutation also observed in hepatocellular carcinoma, but rare in other tumors. More than half of the hepatocellular carcinomas (HCC) from aflatoxin-exposed populations in Africa and China have a G-to-T transversion at codon 249 of the p53 gene (Shen and Ong 1996; Smela et al., 2001). Phosphatase and Tensin Homolog Deleted on Chromosome 10 (PTEN) was discovered as a candidate tumor suppressor gene deleted at human chromosome 10q23 in a number of advanced tumors (Li et al., 1997). Mutations of BRCA1, located on human chromosome 17q21, and BRCA-2, located on 13q12-13, account for a majority of familial breast cancer cases. Besides breast cancers, mutations of BRCA1have been found in familial ovarian cancer and prostate cancer, whereas BRCA2 mutations can be found in ovarian, prostate, pancreatic, gall bladder, bile duct and stomach cancer (Welcsh and King, 2001).

2.2.2. Radiation and cancer

Exposure to sufficient doses of ionizing radiation may result in cancer induction. The susceptibility of tissue varies markedly, but all tissues appear to be

at risk (Beebe, 1982), although not necessarily in relation to natural incidence. The cancer that was observed first was skin cancer and an association between radiation and leukemia was suspected some years later. The information about radiation induced leukemogenesis in humans and animals is now extensive, partly because the latent period for leukemia is shorter than that for solid cancers (Upton et al., 1970; Tolle et al., 1982). The association of exposure to greater intensities of sunlight and skin cancer strengthened the evidence that sunlight was a major etiologic factor. In 1928, it was demonstrated that ultra violet radiation (UVR) induced skin cancer in experimental animals; later, the carcinogenic effect was found to be restricted to wavelengths shorter than 320 nm. Recognition of the role of UVR in the induction of this disease and the degradation of the ozone layer, which absorbs UV in sunlight, has led to promotion of reduced levels of sun exposure and more extensive use of sun screen preparations (Okey et al., 2005).

2.2.3. Viruses and cancer

Viruses are implicated in approximately 15 percent of all cancers. Oncogenes and tumor suppressor proteins were first identified through the study of cancer-causing viruses. Research with simian virus 40 led to the discovery of the tumor suppressor gene p53 and retinoblastoma (Rb) (Richardson, 2005). Cervical cancer is the third most common cancer in women worldwide and is a sexually transmitted disease that is associated with high-risk Human papilloma viruses (HPV) types- including HPV16, 18, 31, 33 and 45- that are found in about 90 percent of all cervical cancers (zur Hausen, 2000). Epstein-Barr virus (EBV) infection is associated with three lymphoproliferative diseases of B-cell origin- infectious mononucleosis, Burkitt's lymphoma and lymphoma of the immunocompromised host. There is also a very strong association between EBV infection and undifferentiated nasopharyngeal carcinoma (NPC), and it is implicated in the pathogenesis of Hodgkin's disease (Weinreb et al., 1996). EBV may also be associated with some gastric carcinomas (Takada, 2000). More than 80 percent of individuals with liver cancer have been chronically infected by Hepatitis B virus (HBV). Only two viral genes, HBx and M (perS2/S), are usually retained intact after viral DNA integration. Products of both these genes can

upregulate signal transduction pathways and stimulate transcription of genes characteristic of growth and survival (Hildt et al., 1996; Feitelson, 1999). HBx can interact with and sequester the tumor suppressor, p53, within the cytoplasm, which again would support a role in oncogenesis (Wang et al., 1994; Truant et al., 1995).

HTLV-1 and HTLV-2 (Human T-Cell Leukemia Viruses) are the only retroviruses that are known to lead directly to cancers in humans. HTLV-1 by itself can cause adult T-cell leukemia (ATL), a rare but virulent cancer (Yoshida, 1987). HTLV-2 is much less common than HTLV-1 and can cause Hairy cell leukemia. The related Human Immunodeficiency Viruses (HIV) are associated with tumorigenesis through immune suppression and subsequent reactivation of many of the DNA-containing viruses. Although HIV does not cause cancer directly, it is an immune suppressive virus that can lead to the appearance of several types of malignancies during late-stage infections (Scadden, 2003).

2.3. Role of free radicals in cancer

The generation of Reactive Oxygen Species (ROS) is a consequence of aerobic life and is unavoidable. ROS and reactive nitrogen species (RNS) represent a constant source of assaults upon our genetic material which can be either enhanced or partly reduced by nutritional, hormonal and environmental influences. Overproduction of ROS and RNS through either endogenous or exogenous insults is harmful to living organisms and is termed oxidative and nitrosative stress (Valko et al., 2006).

2.3.1. Sources and reactions of ROS and RNS

ROS and RNS: (i) are generated during irradiation by UV light, by X-rays and gamma rays; (ii) are products of metal-catalyzed reactions; (iii) are present as pollutants in the atmosphere; (iv) are produced by neutrophils and macrophages during inflammation; (v) are by-products of mitochondria-catalyzed electron transport reactions and other mechanisms (Cadenas, 1989). The harmful effects of ROS are balanced by the antioxidant action of non-enzymatic antioxidants in addition to antioxidant enzymes (Halliwell, 1996). Despite the presence of the cell's antioxidant defence system to counteract oxidative damage from ROS, oxidative damage accumulates during the life cycle, and radical-related damage to

DNA, to proteins and to lipids has been proposed to play a key role in the development of age-dependent diseases such as cancer, arteriosclerosis, arthritis, neurodegenerative disorders and other conditions (Halliwell and Gutteridge, 1999).

Superoxide anion ($O_2^{\cdot -}$), arising either through metabolic processes or following oxygen "activation" by physical irradiation, is considered the "primary" ROS, and can further interact with other molecules to generate "secondary" ROS, either directly or prevalently through enzyme or metal-catalyzed processes (Fridovich, 1986). Superoxide radical ion does not react directly with polypeptides, sugars or nucleic acids, and its ability to peroxidise lipids is controversial.

The generation of various free radicals is closely linked with the participation of redox-active metals (Valko et al., 2005). In vivo, under stress conditions, an excess of superoxide releases "free iron" from iron-containing molecules. The released Fe (II) can participate in the Fenton reaction, generating highly reactive hydroxyl radical (Fe (II) + H_2O_2 ⟶ Fe (III) + $^{\cdot}OH$ + OH^-). Thus under stress conditions $O_2^{\cdot -}$ acts as an oxidant of [4Fe-4S] cluster-containing enzymes and facilitates $^{\cdot}OH$ production from H_2O_2 by making Fe(II)available for the Fenton reaction (Stohs and Bagchi, 1995; Leonard et al., 2004). The hydroxyl radical is highly reactive with a half-life in aqueous solution of less than 1ns (Pastor et al., 2000). Thus when produced in vivo it reacts close to its site of formation. Production of $^{\cdot}OH$ close to DNA could lead to this radical reacting with DNA bases or the deoxyribosyl backbone of DNA to produce damaged bases or strand breaks.

Typical of additional radicals derived from oxygen that can be formed in living systems are peroxyl radicals ($ROO^{\cdot}$**).** Peroxyl radicals are high-energy species. The most interesting feature of peroxyl radical is the diversity of those biological reactions in which they participate. The detection and measurement of lipid peroxidation is most frequently cited as evidence to support the iinvolvement of peroxyl radical reactions in human disease and toxicology (Gutteridge, 1995; Cadenas and Sies, 1998). Peroxyl radicals are involved in DNA cleavage and

protein backbone modification. Peroxyl radicals synergistically enhance the induction of DNA damage by superoxide.

Nitric oxide ($NO^{\cdot}$) is an abundant reactive radical that acts as an important oxidative biological signaling molecule in a large variety of diverse physiological processes, including neurotransmission, blood pressure regulation, defence mechanisms, smooth muscle relaxation and immune regulation (Archer, 1993; Forstermann et al., 1998). This small molecule contains one unpaired electron on the antibonding $2\pi_y^*$ orbital and is, therefore, a radical. $NO^{\cdot}$ is generated in biological tissues by specific nitric oxide synthases (NOSs), which metabolize arginine to citrulline with the formation of $NO^{\cdot}$ via a five-electron oxidative reaction (Ghafourifar and Cadenas, 2005). Overproduction of reactive nitrogen species (nitrosative stress) may occur when the generation of RNS in a system exceeds the system's ability to neutralize and eliminate them.

Cells of the immunesystem produce both the superoxide anion and nitric oxide during the oxidative burst triggered during inflammatory processes. Nitric oxide and superoxide anion may react together to produce significant amounts of a much more oxidatively active molecule, peroxynitrite anion ($ONOO^-$), which is an oxidizing free radical that can cause DNA fragmentation and lipid peroxidation(Carr et al., 2000):

$$NO^{\cdot} + O_2^{\cdot -} \longrightarrow ONOO^-$$

Thus $NO^{\cdot}$ toxicity is linked to its ability to combine with superoxide anions.

2.3.2. Oxidative damage to biomolecules

ROS induced DNA damage involves single- or double- stranded DNA breaks, purine, pyramidine or deoxyribose modifications, and DNA cross-links. DNA damage can result either in arrest or induction of transcription, induction of signal transduction pathways, replication errors and genomic instability, all of which are associated with carcinogenesis (Marnett, 2000; Cooke et al., 2003). In addition to ROS, reactive nitrogen species (RNS), such as peroxynitrites and nitrogen oxides, have also been implicated in DNA damage (Brown and Borutaite, 2001). Upon reaction with guanine, peroxynitrite has been shown to form 8-nitroguanine. Due to its structure, this adduct has the potential to induce

G:C ⟶ T: A transversions. ROS are known not only to attack DNA, but additional cellular components such as proteins and lipids, leaving behind reactive species that can, in turn, couple to DNA bases. The most extensively studied DNA lesion is the formation of 8-OH-G (Figure 2.3.). This lesion is important because it is relatively easily formed and is mutagenic and therefore is a potential biomarker of carcinogenesis (Valko et al., 2006).

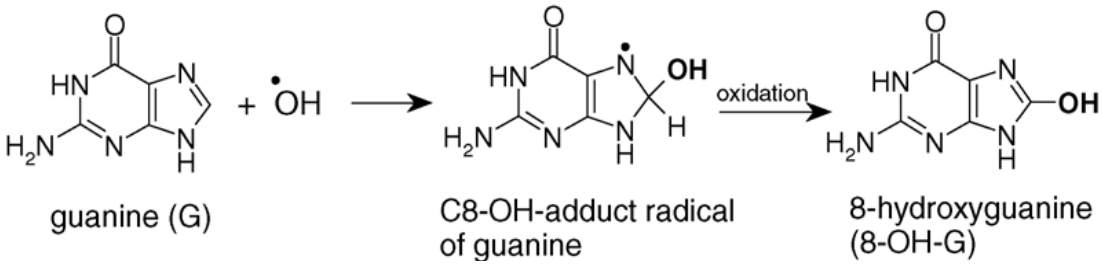

Figure 2.3. Reaction of guanine with hydroxyl radical

Metal-induced generation of oxygen radicals results in the attack of not only DNA in the cell nucleus, but also other cellular components involving polyunsaturated fatty acid residues of phospholipids, which are extremely sensitive to oxidation (Esterbauer et al., 1991). The overall process of lipid peroxidation consists of three stages: initiation, propagation and termination. Once formed, peroxyl radicals ($ROO^{\cdot}$) can be rearranged via a cyclisation reaction to endoperoxides (precursors of malondialdehyde) with the final product of the peroxidation process being malondialdehyde (MDA). MDA is mutagenic in bacterial and mammalian cells and carcinogenic in rats. MDA can react with DNA bases guanine (G), adenine (A) and cytosine (C) to form adducts M_1G, M_1A and M_1C, respectively (Marnett, 1999).

The side chains of all amino acid residues of proteins are susceptible to oxidation by ionizing radiation and by the action of ROS/RNS (Stadtman, 2004). Reactions with hydroxyl radicals lead to abstraction of a hydrogen atom from the protein polypeptide backbone to form a carbon-centered radical, which under aerobic conditions reacts readily with dioxygen to form peroxyl radicals (Stadtman, 1992). The peroxyl radicals are then converted to the alkyl peroxides by reactions with the protonated form of superoxide ($HO_2^{\cdot}$). Highly toxic

peroxynitrite anion $ONOO^-$ is able to nitrosate the cysteine sulphydryl groups of proteins, to nitrate tyrosine and tryptophan residues of proteins and to oxidize methionine residues to methionine sulphoxide. The nitration of tyrosine residues, which is irreversible process, may prevent the phosphorylation or adenylylation of tyrosine residues of regulatory proteins. Cysteine and methionine residues of proteins are particularly susceptible to oxidation by ROS (Levine et al., 1996). Oxidation of proteins is associated with a number of age-related diseases and ageing (Stadtman, 2001).

2.3.3. Antioxidant defence mechanisms in carcinogenesis

Each cell is characterized by a particular concentration of electrons (redox state) stored in many cellular constituents and the redox state of a cell and its oscillation determines cellular differentiation (Filomeni et al., 2002; Moran et al., 2001). Extensive evidence has shown that redox balance is impaired in cancer cells compared with normal cells, which may be related to oncogenic stimulation. The effect of reactive oxygen and nitrogen species is balanced by the antioxidant action of non-enzymatic antioxidants, as well as by antioxidant enzymes. Such antioxidant defences are extremely important as they represent the direct removal of free radicals (pro-oxidants), thus providing maximal protection for biological sites. The most efficient enzymatic antioxidants involve superoxide dismutase, catalase and glutathione peroxidase (Mates et al., 1999). Non-enzymatic antioxidants involve Vitamin C, Vitamin E, carotenoids, thiol antioxidants (glutathione, thioredoxin and lipoic acid), natural flavonoids, melatonin and other compounds (McCall and Frei, 1999). Altered levels of antioxidant enzymes (SOD, catalase, glutathione peroxidase) and non-enzymatic antioxidants (GSH, Vitamin C, thioredoxin) as well as changes in the related signal pathways are evident in many human cancers (McEligot et al., 2005).

2.3.3.1. Enzymatic antioxidants

a) Superoxide dismutase (SOD)

One of the most effective intracellular enzymatic antioxidants is superoxide dismutase (SOD). Superoxide is depleted undergoing a dismutation reaction (Desideri and Falconi, 2003): $2O_2^{\cdot -} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$.

Superoxide dismutase enzymes accelerate this reaction in biological systems by about four orders of magnitude. SOD enzymes work in conjugation with H_2O_2 removing enzymes, such as catalases and glutathione peroxidases (Michiels et al., 1994). While this enzyme was isolated as early as 1939, it was only in 1969 that McCord and Fridovich proved the antioxidant activity of SOD (Mc Cord and Fridovich, 1969).

b) Catalase

Catalase is located in a cell organelle called the peroxisome. The enzyme very efficiently promotes the conversion of hydrogen peroxide to water and molecular oxygen. Catalase has on of the highest turnover rates for all enzymes: one molecule of catalase can convert ~ 6 million molecules of hydrogen peroxide to water and oxygen each minute:

$$2H_2O_2 \xrightarrow{\text{catalase}} 2H_2O + O_2.$$

The significantly decreased capacity of a variety of tumors for detoxifying hydrogen peroxide is linked to a decreased level of catalase (Valko et al., 2006).

c) Glutathione peroxidase

There are two forms of the enzyme glutathione peroxidase, one of which is selenium-independent (glutathione-*S*-transferase, GST) while the other is selenium-dependent (GPx). Glutathione metabolism is one of the most essential of antioxidative defence mechanisms. Humans have four different Se-dependent glutathione peroxidases (Mates et al., 1999). All GPx enzymes are known to add two electrons to reduce peroxides by forming selenoles (Se-OH). The antioxidant properties of these seleno-enzymes allow them to eliminate peroxides as potential substrates for the Fenton reaction. GPx acts in conjuction with the tripeptide glutathione (GSH), which is present in cells in high concentrations. The substrate for catalytic reaction of GPx is H_2O_2, or organic peroxide ROOH. GPx decomposes peroxides to water (or alcohol) while simultaneously oxidizing GSH:

$$2GSH + H_2O_2 \xrightarrow{GPx} GSSG + H_2O$$

$$2GSH + ROOH \xrightarrow{GPx} GSSG + ROH + H_2O$$

Significantly, GPx competes with catalase for H_2O_2 as a substrate and is the major source of protection against low levels of oxidative stress (Valko et al., 2006).

2.3.3.2. Non-enzymatic antioxidants

a) Vitamin C

Vitamin C (ascorbic acid) is a very important and powerful antioxidant that works in aqueous environments of the body, such as is present in lungs and in the lens of the eye. Its primary antioxidant partners are Vitamin E and the caroteniods, as well as working along with the antioxidant enzymes. Vitamin C cooperates with Vitamin E to regenerate α-tocopherol from α-tocopherol radicals in membranes and lipopriteins (Kojo, 2004; Carr and Frei, 1999). The product of ascorbate oxidation by many ROS is the semidehydroascorbate radical ($ASc^{\cdot -}$), a poorly reactive radical that is considered to be a terminal small molecule antioxidant and the level of this radical is a good measure of the degree of oxidative stress in biological systems (Kasparova et al., 2005; Cuzzorcrea et al., 2004). Majority of in vivo studies showed a reduction in markers of oxidative DNA, lipid and protein damage after supplementation with Vitamin C. Recent studies indicate the ability of ascorbic acid to regulate factors that may influence gene expression, apoptosis and other cellular functions (You et al., 2000).

b) Vitamin E

Vitamin E is a fat soluble vitamin that exits in eight different forms. α-Tocopherol is the most active form of vitamin E in humans and is a powerful biological antioxidant which is considered to be the major membrane-bound antioxidant employed by the cell (Burton and Ingold, 1989). Its main antioxidant function is protection against lipid peroxidation (Pryor, 2000). Recent evidence suggests that α-tocopherol and ascorbic acid function together in a cyclic-type of process. During the antioxidant reaction, α-tocopherol is converted to a α-tocopherol radical by the donation of a labile hydrogen to a lipid or lipid peroxyl radical. The α-tocopherol radical can thus be reduced to the original α-tocopherol form by ascorbic acid (Kojo, 2004). Intake of Vitamin E [200 IU (international units)/day] reduced the incidence of colorectal cancer by triggered apoptosis of cancer cells by inducing p21 wafi/cip 1, a powerful cell cycle inhibitor (White et al., 1997). Generally, the protective effect of vitamin E is a result of the inhibition of free radical formation and activation of endonucleases.

c) Thiol antioxidants (glutathione, thioredoxin and lipoic acid)

The major thiol antioxidant is the tripeptide, glutathione. Glutathione (GSH) is a multifunctional intracellular non-enzymatic antioxidant. The antioxidant capacity of thiol compounds is due to the sulphur atom which can easily accommodate the loss of a single electron (Karoui et al., 1996). The main protective roles of glutathione against oxidative stress are: that (i) glutathione is a cofactor of several detoxifying enzymes against oxidative stress, e.g. glutathione peroxidase (GPx), glutathione transferase and others; (ii) GSH participates in amino acid transport through the plasma membrane; (iii) GSH scavenges hydroxyl radical and singlet oxygen directly, detoxifying hydrogen peroxide and lipid peroxides by the catalytic action of glutathione peroxidase; (iv) glutathione is able to regenerate the most important antioxidants, vitamin c and vitamin E back to their active forms; glutathione can reduce the tocopherol radical of Vitamin E directly, or indirectly, via reduction of semidehydroascorbate to ascorbate (Masella et al., 2005).

Thioredoxin provides control over a number of transcription factors affecting cell proliferation and death through a mechanism referred to as redox regulation. Thioredoxin levels are 100 to 1000 fold less than GSH, however, thioredoxin and GSH may have overlapping as well as compartmentalized functions in activation and regulation of transcription factors, including NF-kB and activator protein-1 (AP-1) (Valko et al., 2006). A-Lipoic acid (ALA) is readily absorbed from the diet and is converted rapidly in many tissues to its reduced dithiol form, dihydrolipoic acid (DHLA) (Smith et al., 2004). Both ALA and DHLA are powerful antioxidants (Packer and Suzuki, 1993). Their antioxidant functions involve: (i) quenching of reactive oxygen species; (ii) regeneration of endogenous and exogenous antioxidants involving vitamins C and E and glutathione; (iii) chelation of redox metals including Cu(II) and Fe(II); (iv) repair of oxidized proteins.

d) Carotenoids

Carotenoids (Car) are pigments that are found in plants and microorganisms. Various studies have indicated that carotenoids may prevent or

inhibit certain types of cancer, artherosclerosis, age-related muscular degeneration, and other diseases. The antioxidant activity of carotenoids arises primarily as a consequence of the ability of the conjugated double-bonded structure to delocalize unpaired electrons (Mortensen et al., 2001). This is responsible for the excellent ability of β-carotene to physically quench singlet oxygen without degradation, and for the chemical reactivity of β-carotene with free radicals such as the peroxyl, hydroxyl and superoxide radicals. At sufficiently high concentrations, carotenoids can protect lipids from peroxidative damage (Valko et al., 2006).

e) Flavonoids

One of the most actively studied properties of flavonoids is their protection against oxidative stress (RiceEvans, 2001; Polovka et al., 2003). Flavonoids are ideal scavengers of peroxyl radicals due to their favorable reduction potentials relative to alkyl peroxyl radicals and thus, they are effective inhibitors of lipid peroxidation. Of particular importance is the hydrogen (electron) donating ability of a flavonoid molecule which acts to scavenge a reactive radical species, and is primarily associated with the presence of a B-ring catechol group (dihydroxylated B-ring) (Schroeter et al., 2002). A regular intake of flavonoids, such as polyphenols and quercetin, is linked to lower rates of stomach, pancreatic, lung and possibly breast cancer (Damianaki et al., 2000).

2.4. Association between inflammation and neoplasia

The inflammatory responses could be divided into two categories, acute and chronic. Acute inflammation is a short-lasting (few days) immunological process that in most cases is beneficial to the host due to the clearance of the pathogenic stimulation by the activated innate and adaptive immune cells. Upon clearance, antigenic stimulation disappears, and the immune cells are no longer triggered and return to their quiescent state. In contrast, chronic inflammation is an inflammatory response of prolonged duration (weeks, months, or even indefinite) whose extended time course is provoked by the persistence of the causative inflammatory stimulus. The inflammatory process inevitably causes tissue damage and is accompanied by attempts at ‘healing and repair’. The exact nature, extent and time course of chronic inflammation depends on a balance

between the causative agent and the attempts of the innate and adaptive immune responses to remove it (Baniyash, 2006).

The inter-relationships between different inflammatory settings and cancer are complex, and depend very much on the tumor type, the site in which it arose and the composition of other microenvironmental components at the tumor's vicinity. In many of the malignancies, inflammation is highly associated with tumor initiation and progression. However, it is highly plausible that under certain conditions the inflammatory milieu of tumors actually represents earlier efforts of the host to combat the arising tumor. Eventually, failure to execute surveillance missions and inappropriate control of the inflammatory process may "flip" into an imbalanced condition in which the inflammatory mediators turn into the "enemies within" (Ben-Baruch, 2006).

Malignancy-associated chronic inflammation may be the result of normal protective mechanisms against pathogens. It may also be induced by conditions such as exposure to irritants like asbestos fibers or by alcoholism, and also may result directly from infection of the host cells (Coussens and Werb, 2002; Schwartsburd, 2003; O'Byrne and Dalgleish, 2001; Thun et al., 2004). The tumor microenvironment is composed not only of resident tissue cells, such as fibroblasts and endothelial cells, but also of host leukocytes that have infiltrated the tumor. These include primarily macrophages, which are abundant in many tumor types, as well as lymphocytes, Natural Killer (NK) cells, neutrophils, dentritic cells (DC) and eosinophils (Park et al., 2000; Vicari et al., 2004). Progression into an overt inflammatory process gives rise to increased release of pro-inflammatory mediators of many different types, including cytokines, chemokines, prostaglandins and reactive oxygen/nitrogen species. Although these processes are supposed to be self-limiting, at times they are inappropriately regulated, leading to continuous stimulation and eventually to neoplasia (Ben-Baruch, 2006). Acceleration of neoplastic processes by chronic inflammation was clearly observed in many malignant diseases. For example, *Helicobacter pylori* infection is associated with gastrointestinal carcinogenesis, Schistosomiasis with bladder carcinoma, Hepatitis B virus with hepatocellular carcinoma, alcohol abuse with

pancreatic cancer, asbestos, smoking and silica with lung carcinoma (Kuper et al., 2000; Whitcomb, 2004; Lucia and Torkko, 2004; Ohshima et al., 2003).

Many of the mediators, which are released in dysregulated chronic inflammation, were found to promote cell growth and invasion, to induce mutagenesis and to increase angiogenicity. By virtue of these properties, the inflammatory mediators support transformation and initiation of malignancy, and if sustained, they may also promote progression. In addition, many of the factors that are released by inflammatory cells which are excessively present at the inflammatory milieu induce direct or indirect suppression of immune activities that could have potentially eradicated the evolving tumor (Balkwill and Mantovani, 2001; Brigati et al., 2002; Leek and Harris, 2002; Yu and Rak, 2003). As such, chronic inflammation increases the risk of cancer in inflamed tissues and accordingly is significantly associated with neoplasia.

2.5. Apoptosis and cancer

The control of cell number plays a critical role in organ development and in maintaining the integrity of structural elements. Balance between cell proliferation and cell death represents the central aspect of this regulation, as well as in maintaining homeostasis. Apoptosis or programmed cell death is a mechanism of cell loss that depends on both pre-existing and de novo protein synthesis (Williams and Smith, 1993; Thompson, 1995). Apoptosis plays an important role during development, metamorphosis and in many diseases (Jacobson et al., 1997). In mammalian cells apoptosis can be triggered in response to endogenous stimuli (such as growth factor deprivation) as well as to exogenous stimuli [such as ultraviolet (UV) and γ-irradiation or other DNA damaging agents such as chemotherapeutic drugs]. Apoptosis can also be induced in response to inadequate cell matrix interactions and this specific type of apoptosis is known as *anoikis* (Frisch and Screaton, 2001).

The direct involvement of various human tumor suppressor genes (such as p53, PTEN) and oncogenes (such as AKT, Bcl-2) in the control of apoptosis is a strong demonstration of the requirement for a tight regulation of this cellular process. The regulation of apoptosis in mammalian cells is complex and involves

a variety of molecules, including both anti-apoptotic proteins, such as Bcl-2 and Bcl-$X_{L,}$ and pro-apoptotic proteins, such as Apaf-1 and caspases (Gross et al., 1999; Hengartner, 2000). Mitochondria have also been shown to play a major role in the early steps of apoptosis by releasing factors such as cytochrome *c* and apoptosis-inducing factor (AIF) that activate apoptotic pathways (Hakem and Harrington, 2005).

Dysregulation of cellular pathways resulting in attenuation or enhancement of apoptosis can lead to diseases such as cancer, autoimmunity or neurodegenerative disorders (Johnstone et al., 2002). Tumorigenesis is a multistep process in which acquired mutations result in dysregulation of crucial cellular pathways including apoptosis, cell cycle and repair of DNA damage (Figure 2.4.) (Hanahan and Weinberg, 2000). Loss of expression of the pro-apoptotic genes caspase-8 or Apaf-1, and over expression of the anti-apoptotic Bcl-2 gene are among the defects of apoptosis associated with human cancers. The loss of caspase-8 expression has been found frequently in neuroblastomas and is associated with amplification of the oncogene N-myc (Teitz et al., 2000). Apaf-1 expression is frequently silenced in human melanomas (Soengas et al., 2001). In contrast, Bcl-2 is overexpressed in various types of tumors including human follicular B-cell lymphomas (Mullauer et al., 2001).

The study of apoptosis has gained significant importance in human disease and its clinical management. In situation of tumoral transformation, triggering of apoptosis in malignant cells will be a way of disease control. Apoptotic strategies to kill tumor cells can involve direct induction of pro-apoptotic molecules, modulation of anti-apoptotic proteins, or restoration of tumor suppressor gene functions. Death receptors have been pursued as potential targets for cancer therapy (Fleischer et al., 2006). Mitochondria activation is considered as a key step in the apoptotic process (Thornberry and Lazebnik, 1998), so manipulation of mitochondria activation towards pro-apoptotic initiation has been considered as a potential therapeutic approach.

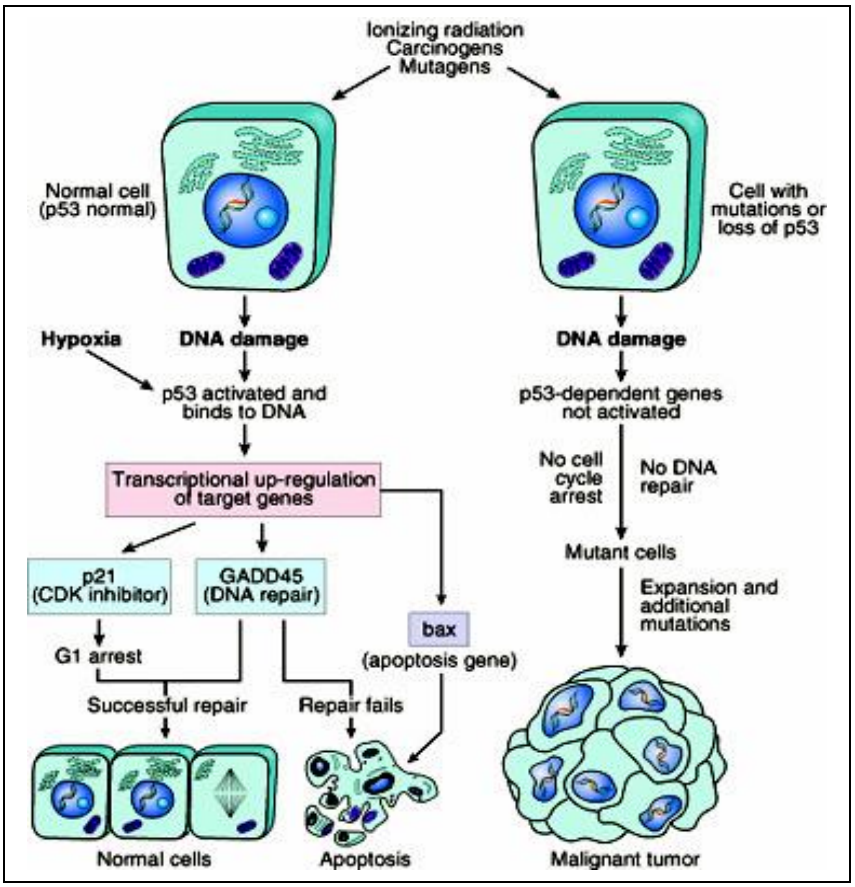

Figure 2.4. Apoptosis and tumorigenesis

2.6. The pharmacological potential of mushrooms

Mushrooms represent a major and yet largely untapped source of powerful new pharmaceutical products (Wasser, 2002). The term 'mushroom' was defined by Chang and Miles as 'a macrofungus with a distinctive fruiting body, which can be either hypogeous or epigeous, large enough to be seen with the naked eye and to be picked by hand' (Chang and Miles, 1992). From a taxonomic point of view, mainly basidiomycetes but also some species of ascomycetes belong to mushrooms (Lindequist et al., 2005). The mushrooms are also called "Macromycetes" (a Latin name) in opposite the term "micromycetes" (a Latin name for the fungi that are invisible with naked eye) (Moradali et al., 2007). The number of mushroom species on earth is estimated to be 140 000, suggesting that only 10% are known. Assuming that the proportion of useful mushrooms among the undiscovered and unexamined mushrooms will be only 5%, which implies 7000 yet undiscovered species will be of possible benefit to mankind (Hawksworth, 2001).

Applying fungi as medicines dates back to3000 BC when macrofungi are used to remedy diseases by mankind especially in the traditional oriental therapies and after discovering of Penicillin (1929), fungi were regarded as rich sources of natural antibiotics and other bioactive compounds. Macrofungi such as *Ganoderma lucidum,Lentinus edodes, Fomes fomentarius, Fomitopsis officinalis* and many others have been used to remedy different diseases for hundreds of years in China, Japan, Korea and Slav regions (Wasser, 2002). Nowadays , constituent molecules of macrofungi organelles and secondary metabolites are known as bioactive compounds that belong to polysaccharides, glycoproteins, proteoglycans, terpenoids, fatty acids, proteins, lectins, etc that possess certain medicinal properties (Table-2.4). The major innunomodulating and anti-tumor effects have been associated with polysaccharides, glycopeptide/protein complexes, proteoglycans, proteins, and triterpenoids (Moradali et al., 2007).

Table 2.4. The different groups of bioactive compounds isolated from mushrooms

Main compound group	**Example**	**Medical potentiality**
Polysaccharides	Grifolan, Lentinan, Schizophyllan	Immunomodulator, Anti-tumor, Antiviral, Antimicrobial
Polysaccharide-peptide	PSP, PSK	Anti-tumor, Antiviral, Antimicrobial, Cytotoxic
Proteins	Fips, Ganoderic acids, Ganoderiol, Ganoderenic acids, Lucidenic acids	Immunomodulator, Anti-HIV activity, Anti-tumor, Cytotoxic
Terpenoids	Ganolucidic acids, Lucidumols, Ganoderols, Applanoxidic acids	Histamine release inhibition, Antihypertension, Anti-inflammatory
Steroids	Polyoxygenated derivatives of ergosterol	Cytotoxic, Anti-tumor, Antibacterial
Fatty acids	Linoleic acid, Palmitic acid, 11-Octadecanoic acid	Antimutagenic, Antibacterial
Organic germanium	Bis-β-carboxyethyl germanium sesquioxide	Anti-tumor, Immunomodulating
Nucleotides	Adenosine	Platelet aggregate inhibition
Polyacetylenic compounds	Biformyne, Agrocybin, Nemotinic acid, Marasmin, Quadrifidins	As antibiotic

Medicinal effects have been demonstrated for many traditionally used mushrooms (Ooi and Liu, 2000), such as extracts of species of *Auricularia*, *Flammulina*, *Ganoderma*, *Grifola*, *Lentinus (Lentinula)*, *Pleurotus*, *Trametes (Coriolus)*, *Schizophyllum*, and *Tremella* (Wasser, 2000) and *Phellinus* (Chung et al., 1993; Kim et al., 2001). Mushroom metabolites are increasingly being utilized to treat a wide variety of diseases without the need to go through phase I/II/III trials as an ordinary medicine, and they are considered as a safe and useful approach for disease treatment. Bioactive metabolites can be isolated from fruiting bodies, pure culture mycelia and culture filtrate (culture broth) (Cristina Lull et al., 2005). At least 651 species and 7 infraspecific taxa representing 182 genera of hetero- and homobasidiomycetes mushrooms contain anti-tumor or immunomodulating metabolites (Wasser, 2002). A lot of scientific investigations have been performed to discover possible functional properties, which could be efficient in possible treatments of diseases like allergic asthma (Li et al., 2000; Liu et al., 2003a; Liu et al., 2003b), food allergy (Hsieh et al., 2003; Li et al., 2001), atopic dermatitis (Kuo et al., 2002), inflammation (Jose et al., 2004; Kim et al., 2004a), autoimmune joint inflammation such as rheumatoid arthritis (Kim et al., 2003a), atherosclerosis (Bobek and Galbavy, 1999, Yamada et al., 2002), hyperglycemia (Gray and Flatt, 1998), thrombosis (Yoon et al., 2003), human immunodefiency virus (HIV) infection (Nanba et al., 2000; Ngai and Ng, 2003), listeriosis (Kodama et al., 2001), tuberculosis (Markova et al., 2003), septic shock (Kim et al., 2003b) and cancer (Kidd, 2000; Fisher and Yang, 2002; Mahajan et al., 2002; Gao and Zhou, 2003; Lee and Nishikawa, 2003; Hattori et al., 2004; Ho et al., 2004; Nakamura, 2004; Shibata et al., 2004).

2.7. Mushroom polysaccharides in the treatment of cancer

Compounds that are capable of interacting with the immune system to upregulate or down regulate specific aspects of the host response can be classified as immunomodulators or biologic response modifiers (BRM). Whether certain compounds enhance or suppress immune responses can depend on a number of factors, including dose, route of administration, and timing of administration of the compound in question. The type of activity can also depend on their mechanism

of action or the site of activity (Wasser, 2002). In this way these compounds' affect on the different cell types involved hematopoietic stem cells, innate (nonspecific) and adaptive (specific) immune systems and also cytokine networks and signaling pathways. Many compounds isolated from macrofungi that are known as immunomodulators that almost related to polysaccharides and their peptide or protein derivatives. These compounds are also worthwhile antiinfective and anti-tumor agents (Figure 2.5.) (Moradali et al., 2007).

Biologically active polysaccharides are widespread among higher basidiomycetes mushrooms, and most of them have unique structures in different species. Moreover, different strains of one Basidiomycetes species can produce polysaccharides with different properties (Wasser, 2002). The mushroom polysaccharides have been effective against esophageal, stomach, prostate and lung cancers. Lucas et al. (1957) demonstrated the anti-tumor effect of higher basidiomycetes (specifically extracts of fruiting bodies of *Boletus edulis*). Yohida et al. in 1962 isolated from *Lampteromyces japonicus* (kowamura) Sing, an agent active against Ehrlich carcinoma of the mouse. Gregory (1966) experimented on more than 7000 cultures of higher basidiomyces for anti-tumor activity against rodent tumor systems. Positive inhibitory effects were obtained using fermentation media materials against sarcoma 180, mammary adenocarcinoma 755, and leukemia L-1210. Ikekawa et al. (1968) reported that an essence obtained from the fruit body of edible mushrooms exhibited remarkable host-mediatory anti-tumor activity against grafted cancer in animals such as sarcoma 180. Daba (1998) and Daba et al. (2002) reported that *Pleurotus Ostreatus* mushrooms cutivated on date waste possess a potent anti-tumor activity against Ehrlich ascites carcinoma. Further biochemical studies were carried out by the authors on the effect of mushrooms and isolated polysaccharides on the tumors transplanted in mice. The anti-tumor essence was later discovered to be a type of β-D-glucan, a polysaccharide yielding D-glucose only by acid hydrolysis (Mizuno, 1999). The polysaccharides of mushrooms occur mostly as glucans, some of which are linked by β-(1-3), (1-6) glycosidic bonds and α-(1-3) glycosidic bonds but many are true heteroglycans. More than 50 mushroom species have yielded potential

immunoceuticals that exhibit anticancer activity in vitro or in animal models. Six of these polysaccharides that have been investigated in human cancers include Lentinan, Schizophyllan, Active hexose correlated compounds (AHCC), Maitake D-fraction, polysaccharide-K (PSK) and polysaccharide-P (PSP) (Table 2.5.)[Daba and Ezeronye, 2003].

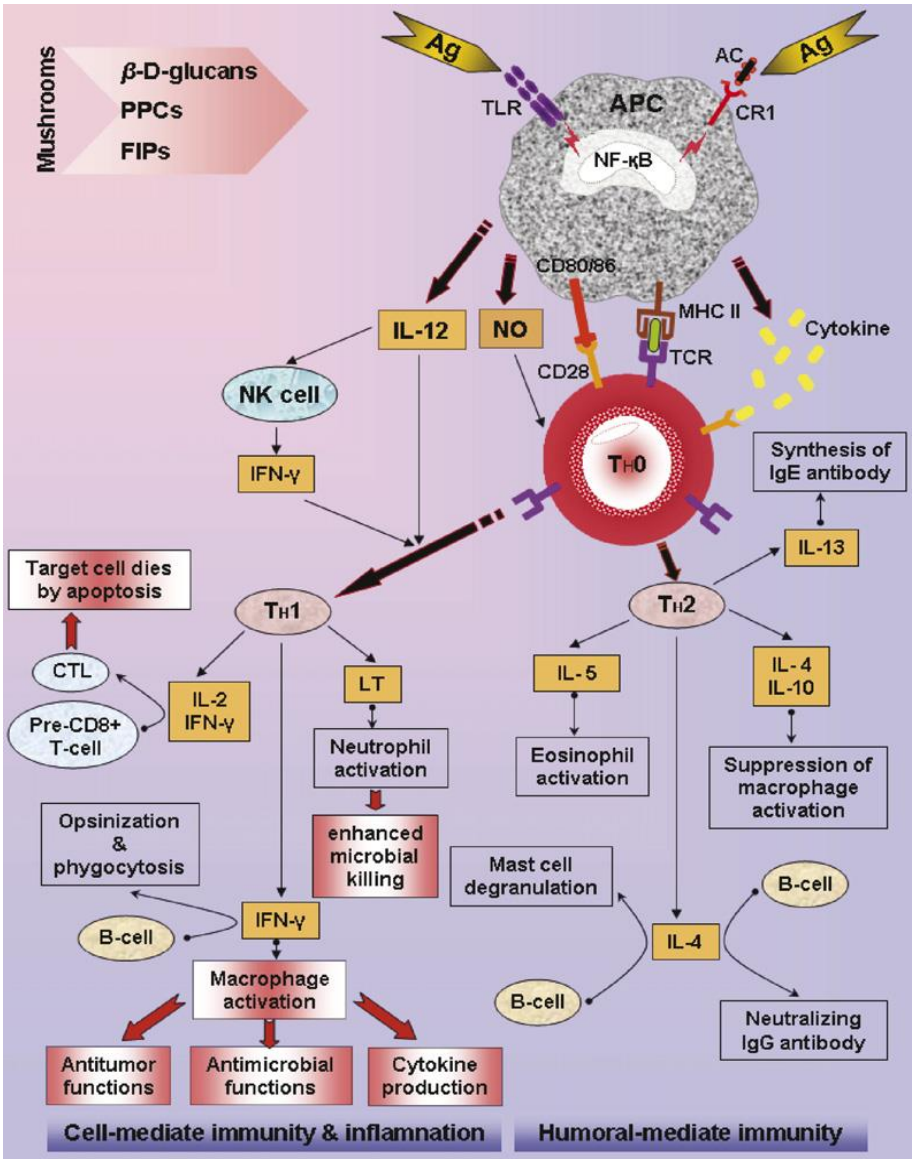

Figure 2.5. Schematic representation of the affecting of immunomodulatory macrofungi metabolites on the adaptive immune system leading to activation of antimicrobial and antitumor pathways. Ag, antigen; PPCs, polysaccharide-peptide/protein complexes; FIPs, fungal immunomodulatory proteins; APC, antigen-presenting cell; TLR, toll-like receptor; CR1, complement receptor 1; AC, activated complement; NF-kB, nuclear factor kappa B; CD, cluster designation; MHC II, major histocompatibility complex; TCR, T-cell receptor; NO, nitric oxide; IL, interleukin; IFN-γ, interferon γ; LT, lymphotoxin; TH, helper T cell; NK cell, natural killer cell; CTL, cytotoxic T lymphocyte.

Table 2.5. Some anti-tumor polysaccharides isolated from macrofungi

Compound	Origin	Molecular structure
Lentinan	*Lentinus edodes*	1,6-Monoglucosylbranched 1,3-β-D-glucan
Schizophyllan	*Schizophyllum commune*	1,6-Monoglucosylbranched 1,3-β-D-glucan
AHCC	A proprietary extract from several species of basidiomycete mushrooms, including *Lentinus edodes*	1,3-α-D-glucan
Maitake D-fraction	*Grifola frondosa*	1,6-Monoglucosylbranched 1,3-β-Dglucan
PSK	*Coriolus versicolor*	1,3 and 1,6-monoglucosylbranched 1,4-β-Dglucan with binding to aspartic, glutamic and other acidic amino acids
PSP	*Coriolus versicolor*	Resemble to PSK structure but is riched in glutamic and aspartic acids

β-D-glucans have a different mode of action from the conventional chemotherapeutic agents in that it is immunotherapeutic, inhibiting the growth of cancer cells by activating and reinforcing the host immune system. For example, Lentinan is understood as a pure β-D-glucan that affects and stimulates the key immune mechanisms to mediate destruction of tumor cells (Figure 2.6.). Such mechanisms are also looked upon for peptide or protein derivates of β-D-glucans (Chihara, 1992a). Of course, there are other pathways and mechanisms that macrofungi can prevent and suppress tumor cell growth. It has proved that different compounds of mushrooms act via different pathways recognized as enhancing detoxification of carcinogens, increased expression and activity of Phase II enzymes, inhibition of organ exposure of carcinogens due to reduced absorption or increased excretion, decreased expression and activity of Phase I enzymes, decreased formation of toxic metabolites and adduct formation with macromolecules, antioxidative and radical-scavenging effect, antipromotion effect, anti-inflammation, induction of differentiation, direct cytotoxicity, induction of

cell cycle arrest, antiproliferation and modulation of signaling transduction molecules, antiprogression and tumor growth inhibition, antimetastasis and anti-angiogenesis (Wasser, 2005).

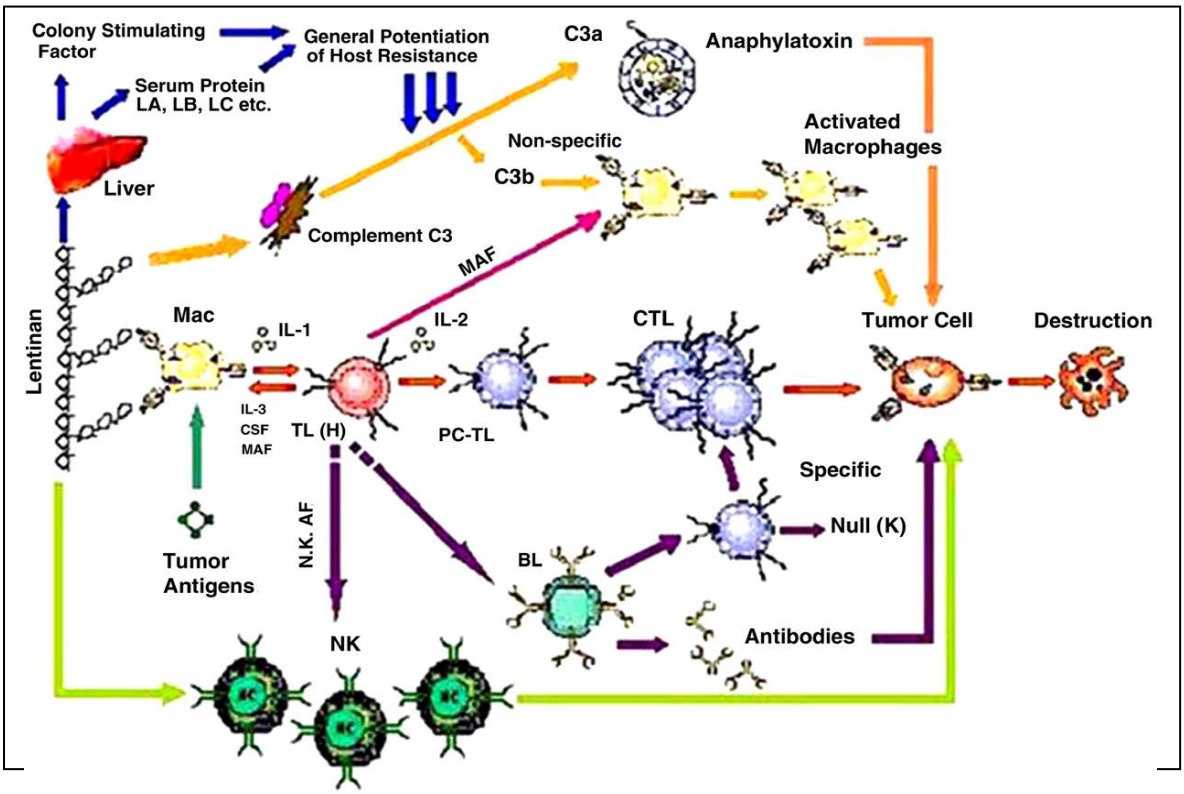

Figure 2.6. Mechanisms of antitumor activity of lentinan as a β-D-glucan (according to Chihara [1992a]). Mac, macrophages; TL(H), T-lymphocyte (helper); NK, natural killer cells; IL-1, -2 and -13, interleukin-1, -2, -13; CSF, colony-stimulating factor; MAF, macrophage-activating factor; PC-TL, precytolytic T lymphocyte; CTL, cytolytic (cytotoxic) T lymphocyte; BL, B lymphocyte.

2.8. Medicinal properties of *Phellinus* species

There are approximately 220 known species of *Phellinus* mushrooms in the world. Various *Phellinus* mushrooms like *P. igniarius, P. hartigii, P. gilvus, P.pini, etc.* are known to have different medicinal effects such as anti-tumor and immuno-stimulating activities ((Ayer et al., 1996; Jung et al., 1997; Rew et al., 2000; Shibata et al., 1968; Shon and Nam, 2001). *Phellinus baummi* extracts are reported to possess high antioxidant and free radical scavenging activity (Shon et al., 2003). Polysaccharides isolated from *Phellinus gilvus* significantly inhibited benzo(a)pyrene-induced forestomach carcinogenesis in mice by down-regulating

mutant p53 expression (Bae et al., 2005). *Phellinus linteus* is a well-known species of the genus *Phellinus,* which attracts great attention due to its potent anti-tumor effect and other medicinal values. The anti-tumor activity of the polysaccharides from the fruiting body of this mushroom was first reported in 1968 (Ikekawa et al., 1968), thereafter a wide variety of further reports have been documented by many investigators (Kang et al., 1997; Chi et al., 1996; Chung et al.,1993; Han et al., 1999; Lee et al., 1996; Song et al., 1995). Polysaccharide of *P. linteus* (PL) showed the hypoglycemic effect, which was investigated in streptozotocin-induced diabetic rats, and also decreased total cholesterol, triacylglycerol and aspartate aminotransferase activity (Kim et al., 2001). *P. linteus* extract acts as an activator of catalase by regulating its expression at the translational or transcriptional level (Park et al., 2001).

In a comparative study of polysaccharides from Basidiomycetes, the polysaccharide of *P. linteus* was the most potent one and produced growth inhibition of 96.7% in Sarcoma 180 transplanted immunocompetent ICR mice (Song et al., 1995). The active polysaccharide purified from mycelial culture of *P. linteus*, also stimulates humoral and cell-mediated immunity, and exhibits a wider range of immunostimulation and anti-tumor activity than other polysaccharides isolated from Basidiomycetes (Kim et al., 1996). PL was also suggested to be used in immunochemotherapy of cancer because of its effective activities on tumor growth and metastasis through the immunopotentiation of the patients without toxicity (Han et al., 1999). Many investigators have been demonstrated that the mechanism of *Phellinus* mushroom anti-tumor action involves multiple processes. Results of the study carried out by Kim et al. (2003c) suggest that PL is a biological response modifier that stimulates proliferation and expression of co-stimulatory molecules in B cells, probably by regulating protein tyrosine kinase (PTK) and protein kinase C (PKC) signaling pathways. Another study says that the tumoricidal activity of peritoneal macrophages (PM) cultured with PL against B16 melanoma cells was enhanced in a dose-dependent manner. This study concluded that PL act as an effective immunomodulator and enhances the anti-tumoral activity of PM through the up regulation of nitric oxide (NO) and

Tumor necrosis factor-α (TNF- α) (Kim et al., 2004b). Extract of *P. linteus* was reported to possess antimutagenic activities and play a role in the prevention of cancer by inducing NAD(P)H:quinine oxidoreductase and glutathione S-transferase activities and increasing glutathione level (Shon and Nam, 2001). Li et al. (2004) reported the direct cytotoxicity of PL againt cancer cells. This study says that PL inhibited cellular proliferation of SW480 human colon cancer cells by induction of apoptosis and G_2 /M phase arrest.

2.9. Previous work

Phellinus rimosus is a less extensively studied species of the genus *Phellinus.* Basidiocarps of this mushroom have been reported to be used by some tribes in Kerala for curing mumps (Ganesh, 1988). The significant antioxidant and anti-inflammatory activities of methanolic extract of *P. rimosus* was first reported by Ajith and Janardhanan, (2001). The ethyl acetate extract of *P. rimosus* exhibited significant hepatoprotective effect against CCl_4 induced acute hepato toxicity in rats. The extract also possessed significant free radical scavenging activity in a concentration dependent manner that could exert a beneficial effect against hepatotoxicity in experimental animals (Ajith and Janardhanan, 2002a). The ethyl acetate extract of *P. rimosus* was also found to be effective in the amelioration of cisplatin induced nephrotoxicity in mice (Ajith and Janardhanan, 2002b). Ajith and Janardhanan (2003) have also reported the cytotoxic and anti-tumor activities of ethyl acetate, methanol and aqueous extracts of *P. rimosus.* This study showed that ethyl acetate and methanol extracts possessed in vitro cytotoxic activity against Dalton's lymphoma ascites (DLA) and Ehrlich's ascites carcinoma (EAC) cell lines. All the three extracts were highly effective in inhibiting growth of solid tumor induced by DLA cell line in mice. The ethyl acetate extract was also effective in preventing the EAC induced ascites tumor development in mice.

2.10. Present study

The approach to treat advanced cancer using natural medicines has drawn much attention recently (Vickers, 2000). The search for novel polysaccharides with anti-tumor properties stems from the basic shortcomings of existing cancer

chemotherapy and radiotherapy. In particular, the great majority of chemical compounds, which have been identified as cytotoxic to cancer cells, are also toxic to normal cells (Borchers et al., 1999). Hence, the discovery and identification of safer new drugs, without severe side effects, has become an important goal of research in the biomedical sciences. Although there are reports on the therapeutic effects of various extracts of *P. rimosus*, no attempt has been made to study the anti-neoplastic properties of the isolated active polysaccharides from this mushroom and their mechanism of action is not known. The present study aims at the isolation and characterization of polysaccharides from *P. rimosus* and to study its anti-neoplastic properties using in vitro and animal experimental models. The main objectives are as given below:

- Isolation and characterization of crude polysaccharide from the fruiting bodies of *P. rimosus.*
- Evaluation of the anti-neoplastic properties and the mechanism of action of the crude polysaccharide isolated from *P. rimosus.*
- Isolation and characterization of neutral polysaccharide from the crude polysaccharide of *P. rimosus* and study of its anti-neoplastic properties.

Chapter-3

Materials and methods

Synopsis

A description of test animals, cell lines, chemicals and instruments used in the present study is given this chapter. Preparation of the hot water extract of saprocarps of *P.rimosus*, quantitative estimation of carbohydrate by Phenol sulphuric acid and Anthrone methods, protein estimation by Bradford method are fully described. Statistical method used for the analysis of experimental data is also explained.

3.1. Materials

3.1.1. Test animals

Albino mice and rats were purchased from Small Animal Breeding Centre, Kerela Agricultural University, Mannuthy, Thrissur, India and housed under controlled conditions with free access to standard rodent chow and water *ad libitum*. Animal experiments were carried out according to the guidelines of Committee for the Prevention of Control and Supervision of Experiments on Animals (CPCSEA) and approval of Institutional Animal Ethic Committee.

3.1.2. Cell lines

Ehrlich's ascites carcinoma (EAC) and Dalton's lymphoma ascites (DLA) cell lines were obtained from Cancer Institute, Adayar, Chennai. The cells were maintained by intraperitoneal (i.p.) passages in Swiss albino mice. HCT 116 cells (human colon cancer cell line) were obtained from the National Centre for Cell Science, Pune, India. The cells were grown in monolayer culture in Dulbecco's Modified Eagle's Medium (Life Technologies, USA) containing 10% fetal bovine serum (Sigma, USA) and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin) in a humidified atmosphere of 5% CO_2 at 37 ^oC.

3.1.3. Chemicals

Phenol, chloroform ($CHCl_3$), formaldehyde, hydrogen peroxide (H_2O_2), sodium nitroprusside and sodium nitrite were purchased from Merck India Ltd., Mumbai. Ascorbic acid , 1,1-diphenyl 2-picryl hydrazyl (DPPH), ferric

chloride, methanol, myoglobin, sodium acetate, Trolox (6-hydroxi-2,5,7,8-tetra methyl chroman-2-carboxylic acid), 2,4,6-tripyridyl-s-triazine (TPTZ), 2-thiobarbituric acid, triphenyl phospene (TPP), trifluro acetic acid (TFA), carrageenan and 3-(4-5 dimethylthiozol-2-yl) 2-5 diphenyl tetrazolium bromide (MTT) were obtained from Sigma Chemical Co., USA. Sodium azide (NaN_3), reduced glutathione (GSH), 5,5-diothiobis-(2-nitrobenzoic acid) (DTNB), nitroblue tetrazolium (NBT), riboflavin, glucose-6-phosphate, sulphanilamide, nicotinamide adenine dinucleotide phosphate (disodium) (NADP), reduced NADP (NADPH) and Diethyl aminoethyl (DEAE) cellulose were purchased from Sisco Research Laboratories, Mumbai. Blue dextran, dextran T500, dextran (Mw:60,000), Sephadex G100 and Dialysis membrane -150 were from Hi-Media. Cisplatin was purchased from Dabur India Ltd, New Delhi. Dulbecco's Modified Eagle Medium (DMEM) was from Gibco™, and 10 % Fetal Bovine Serum (FBS) obtained from Life Technologies, Grand Island, USA. All other chemicals and reagents used were analytical reagent grade.

3.1.4. Instruments used

The following instruments were used for the study

Cold Lab	: LKB, Bromma
Cooling centrifuge	: Remi, Chennai
Deep freezer (-70 and -20^{o}C)	: Remi and Tropicana
Electronic balance	: Contech instrument company, Mumbai
Lyophilyser	: Labconco, Missouri
pH meter	: Elico IL 120
Research Microscope	: Meiji, Japan and Labex, India
Spectrophotometer	: Elico SL 159 and Elico SL 177
Tissue homogenizer	: ***
Fluorescence microscope	: TE-Eclipse 300
FT - IR Spectroscope	: Magna- IR 550
NMR Spectroscope	: Varian, USA
MALDI –TOF mass spectroscope	: Micromass TofSpec 2E
Aminoacid analyser	: Shimadzu HPLC-LC10As

3.2. Methods

3.2.1. Preparation of the hot water extract

Saprocarps of *P.rimosus* growing on the jackfruit tree trunks were collected from the outskirts of Thrissur, Kerala, India. The voucher specimen was deposited in the Herbarium of Centre for Advanced Studies in Botany, University of Madras, Chennai, India (HERB. MUBL 3171). The saprocarps were cut into small pieces, dried at 40-50ºC for 48 hrs and powdered. Powdered material was defatted with petroleum ether using a Soxhlet apparatus for 8-10 hrs. The defatted material was extracted with hot water at 95ºC for 8-9 hrs, repeatedly thrice. After each extraction, the soluble polymers were separated from residues by filtration and extracts were combined, concentrated under reduced pressure.

3.2.2. Carbohydrate estimation by Phenol sulphuric acid method

Quantitative examination of carbohydrate content was carried out according to Dubois's et al. method (1956) using glucose as the standard.

Principle

In hot acidic medium carbohydrate is dehydrated to hydroxymethyl furfural. This forms a colored complex with phenol and has absorption maximum at 490 nm.

Procedure

The stock standard solution was prepared by dissolving 100mg of glucose in 100 ml of distilled water. 10ml of this stock solution was made up to 100ml with distilled water to prepare the working standard. 0.1, 0.2, 0.4, 0.6, 0.8 and 1ml of standard solution was taken in six test tubes. 0.1 and 0.2ml of the test solutions having unknown concentrations were added to the test tubes, marked as T1 and T2. All the solutions were made up to 1ml with distilled water. The blank containing 1ml of distilled water was also taken. To all tubes, 1ml of 5% phenolic solution was added. Then 5ml of concentrated sulphuric acid was added to all tubes without disturbing the solution and shaken well. After 10min, the contents of the tube were mixed well and placed in a water bath at 25-30^0 C. Optical density was measured at 490nm. A calibration curve was constructed by plotting optical density reading on Y-axis against standard glucose mg/tube. The amount

of total carbohydrate present in the test solutions were calculated using the standard graph.

3.2.3. Carbohydrate estimation by Anthrone method

Anthrone method (Yemm and Wills, 1954) was utilized to monitor the presence of carbohydrate in the test solutions.

Principle

Carbohydrate reacts with concentrated sulphuric acid to give derivative of furfural which on reaction with anthrone forms a bluish green colored complex and has absorption maximum at 630nm.

Procedure

Anthrone reagent is prepared by dissolving 0.2g anthrone in 100ml of chilled concentrated sulphuric acid. To all the test tubes 4ml of anthrone reagent was added. Then without disturbing the reagent in the test tubes, 0.5ml of the test solution having unknown concentration of carbohydrate was added. All the solutions were made up to 1ml using distilled water. The blank containing 1ml of distilled water was also taken. Then the contents of the tubes were mixed well and heated in a boiling water bath for 8min. It was then immediately cooled and optical density was measured at 630nm.

3.2.4. Protein estimation by Bradford method

Quantitative examination of protein content was carried out using Protein Estimation Kit (GENEI Cat # KT33) by Bradford Macro Method (Bradford, 1976). Bradford's assay is a rapid and accurate method for estimation of protein concentration. Moreover, when compared with the Lowry's method it is subject to less interference by common reagents and nonprotein components of biological sample.

Principle

The assay relies on the binding of the dye Coomassie blue G250 to protein. The quantity of protein can be estimated by determining the amount of dye in theblue ionic form. This is usually achieved by measuring the absorbance of the solution at 595nm.

Procedure

Standard protein is prepared by reconstituting one vial containing 5mg Bovine serum albumin (BSA) with 1ml distilled water to get 5mg/ml. Just before use 0.2ml of 5mg/ml BSA solution was diluted with 0.8ml of distilled water to get 1mg/ml standard protein solution. 10, 20, 40, 60 and 80µl of standard BSA solution were taken in five test tubes. 40 and 60µl of the test solutions having unknown concentrations were added to the test tubes, marked as T1 and T2. All the solutions were made up to 200µl with distilled water. The blank containing 200µl of distilled water was also taken. To all the tubes 2ml of Bradford's reagent was added, mixed well and kept for 10min. Optical density was measured at 595nm. A calibration curve was constructed by plotting optical density reading on Y-axis against standard protein µg/tube. The amount of total protein present in the test solutions were calculated from the standard graph.

3.2.5. Statistical analysis

The data were analyzed by one-way analysis of variance (ANOVA) using MSTAT-C, Software package, UK. If found significant pair wise comparison of polysaccharide treated groups with the control group was done by Dunnett's-*t* test (Cochran and Cox, 1957).

Critical difference (c.d) $= t \times \sqrt{(2 \times EMS / r)}$

where t is the Dunnett's *t* value for $P < 0.01$ or $P < 0.05$, EMS is the error mean square value and r is the number of observations / group. Value is found to be significant if the difference between average value of the control group and treated group was greater than the c.d.

For comparison of normal with control group critical difference (lsd) was used. $P < 0.05$ was found to be significant.

Chapter- 4

Isolation and characterization of crude polysaccharide-protein complex from the fruiting bodies of *Phellinus rimosus*

Synopsis

A protein bound polysaccharide was isolated from the medicinal mushroom, *Phellinus rimosus* (Berk) Pilat. The isolation of a water soluble polysaccharide-protein complex from *P. rimosus* (PPC-*Pr*) was achieved by hot water extraction, filtration, solvent precipitation, deproteinization, dialysis and freeze-drying. The proximate analysis showed that PPC-*Pr* was composed of 54.8 % polysaccharide and 28.6 % protein. The molecular weight determination of the compound was accomplished by gel filtration chromatography using Sephadex G 100. The molecular weight of PPC-*Pr* was approximately 1,200,000 Da. The paper chromatography and thin layer chromatography (TLC) analysis of PPC-*Pr* after acid hydrolysis with trifluroacetic acid showed that it was composed of D-glucose as the only monosaccharide unit. The amino acid profile analysis of PPC-*Pr* revealed that it contained large amounts of aspartic acid, glutamic acid, alanine, glycine and serine. Results thus, suggested that PPC-*Pr* is a D-glucan- protein complex.

4.1. Introduction

In recent decades, polysaccharides isolated from botanical sources like mushrooms, algae, lichens and higher plants have attracted a great deal of attention in the biomedical arena because of their broad spectrum of therapeutic properties and relatively low toxicity (Tzianabos, 2000; Paulsen, 2001; Wasser, 2002). Hot water extracts of many mushrooms (which mainly contain polysaccharides) used in traditional Chinese medicine and other folk medicines have long been said to be efficacious in the treatment of various diseases including many forms of cancer. The use of medicinal mushroom extracts in the fight against cancer is well known and documented in China, Japan, Korea, Russia and now increasingly in the USA (Mizuno et al., 1995a). However, it is only within the last three decades that chemical technology has been able to isolate the relevant compounds and use them

in controlled experiments. They have been extensively screened for medical properties especially for anticancer application (Mizuno, 1999). Important pharmacological activities like anti-cancer and immunomodulatory are due to the high molecular weight polysaccharides from the cell walls, either free or complexed with proteins that can be extracted with hot water (Politi et al ., 2006). There is a broad similarity in the various methods that have been developed to extract the anti-cancer polysaccharides from mushroom fruit-bodies, mycelium and liquid media (Mizuno, 1999).

Polysaccharides extracted from mushrooms have significant therapeutic potential and represent a rich source for future discovery and development of novel compounds of medical value. Several groups of polysaccharides from medicinal mushrooms are attractive because they have potent biological and pharmacological activities including anti-tumor, immunomodulating, anti-inflammatory, cardiovascular, hypocholesteric, antiviral, antibacterial, antiparasitic, antifungal, antidiabetic and hepatoprotective activities (Mizuno, 1999; Wasser and Weis, 1999; Wasser, 2002; Rowan et al., 2003; Smith et al., 2003; Hobbs, 2005; Ohno, 2005; Yap and Ng, 2005; Wasser and Didukh, 2005). In a recent review Reshetnikov et al., (2001) have listed 650 species and 7 intraspecific taxa from 182 genera of higher Hetero- and Homo- basidiomycetes that contain pharmacologically active polysaccharides that can be derived from fruit-bodies, culture mycelium and culture broths. In general, there is normally a higher level and number of different polysaccharides extracted from fruit-bodies than from the other cultural sources. While our understanding of the mechanism of action of these substances is still developing, it appears that one of the primary mechanisms involves nonspecific induction of the immune system (Tzianabos, 2000). Indeed, the basic mechanism of the immunostimulatory, anti-tumor, bactericidal and other therapeutic effects of polysaccharides is thought to occur via macrophage stimulation and modulation of the complement system (Chihara, 1992b; Wang et al., 1997). Consequently, modulation of innate immunity has a significant impact on the host's ability to respond rapidly and potently to a diverse array of pathogens (Beutler, 2004).

A protein bound polysaccharide was isolated from the medicinal mushroom, *Phellinus rimosus* (Berk) Pilat. The final lyophilized material was analysed for solubility, total carbohydrate content, total protein content, molecular weight, constituent monosaccharides and amino acids. The findings are presented in this chapter.

4.2. Materials and methods

4.2.1. Isolation of polysaccharide-protein complex (PPC-*Pr*) from *P.rimosus*

Hot water extract of *P.rimosus* was prepared as described in the section 3.2.1. Isolation of polysaccharide-protein complex from *P.rimosus* (PPC-*Pr*) was carried out by the method of Mizuno (2000) with slight modifications (Figure 4.1.). Briefly, hot water extract was precipitated by the addition of 5x volume of chilled ethanol and kept at 4°C for 48 hrs; followed by centrifugation at 10,000 rpm for 20 min, repeated twice, washed successively with ethanol. Precipitate

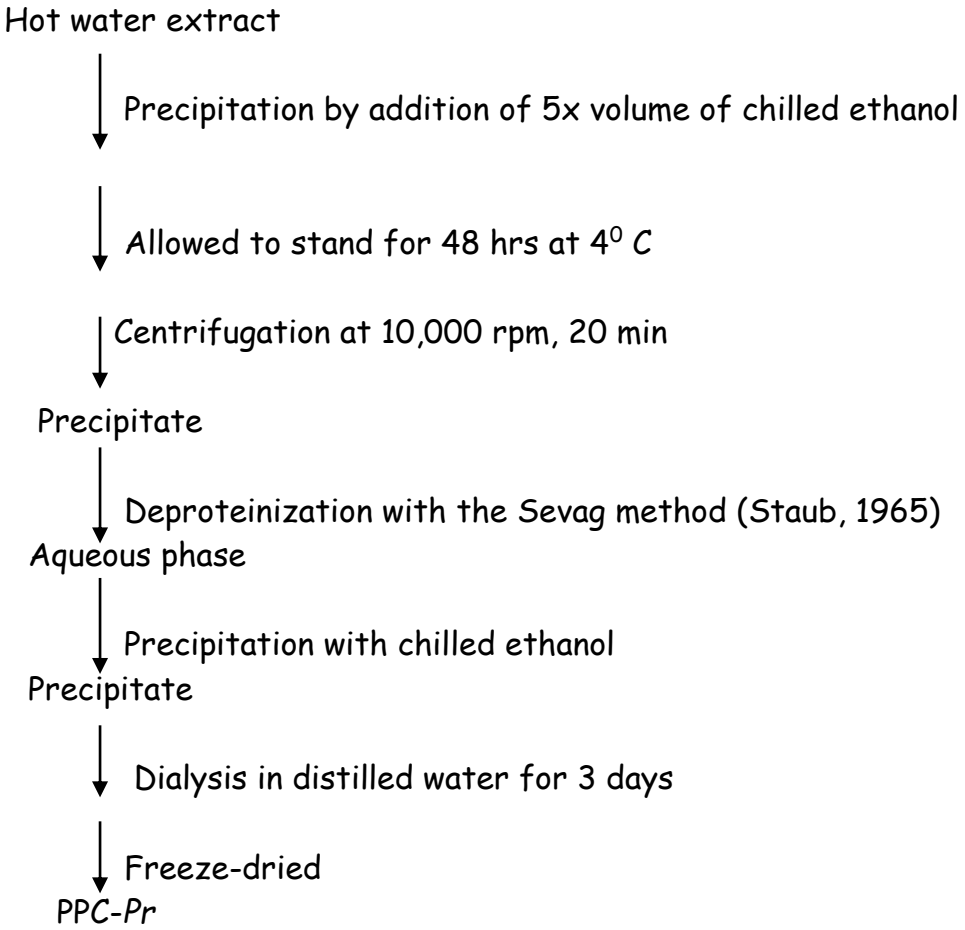

Figure 4.1. Procedure for isolation of polysaccharide-protein complex (PPC-*Pr*) from the fruiting-body of *P. rimosus*.

obtained was deproteinized with the Sevag method (Staub 1965). In this method the aqueous sugar solution was treated with Sevag reagent containing 1-butanol: chloroform in 1:4 ratio; after full oscillation and centrifugation, the supernatant solution was retreated in this manner until there was no free protein. The aqueous layer was removed and again precipitated with chilled ethanol and centrifuged after 48 hrs. Thus obtained precipitate was dialyzed in distilled water for 3 days using dialysis tubing with a M_r cut-off 12,000, followed by lyophilisation to obtain the PPC-*Pr*.

4.2.2. Estimation of total carbohydrate content

Quantitative examination of carbohydrate content was done by Phenol sulphuric acid method using glucose as the standard, as described in section 3.2.2.

4.2.3. Determination of monosaccharide components by Partition (Paper and Thin layer) chromatography

Principle

Partition chromatography is the distribution of a solute between two liquid phases. The distribution of solutes between the two phases is based primarily on solubility differences. The ratio of the concentrations of the solute in the two phases is called partition coefficient. The position of each component of a mixture is quantified by calculating the distance traveled by the component relative to the distance traveled by the solvent, which is called relative mobility (R_f value). The R_f for a substance is a constant for a certain set of experimental conditions.

Procedure

In order to detect the monosaccharide profile, 1 mg of PPC-*Pr* was hydrolyzed with 2M trifluoroacetic acid (TFA). The residue obtained after hydrolysis was dissolved in 10% isopropanol and analyzed by paper chromatography (PC) using Whatman No:1 filter paper and also by thin layer chromatography (TLC) using precoated silica gel plates. The chromatograms were developed with butanol: acetic acid: water (2: 1: 1) solvent system. After developing, the chromatograms were dried in air and sprayed with the aniline diphenylamine phosphoric acid reagent containing 2ml aniline, 2gm diphenylamine and 10ml of 85% phosphoric acid in 100ml of acetone. The plates

were then heated at 85^0c for 10 min and spot development was observed. D-Glucose, D-Fructose, D-galactose, D-mannose and D-xylose were used as standards.

4.2.4. Estimation of total protein content

Total protein content was determined by the Bradford method using bovine serum albumin as the standard, as described in section 3.2.4.

4.2.5. Determination of total amino acid profile

Principle

The amount of each amino acid present with in a given protein does not vary from molecule to molecule and can provide useful information about the nature of the protein molecule. In a typical analysis of the amino acid content of a protein, peptide bonds are first be broken by acid hydrolysis 6N HCl at 110ºC for 24 hour so that the released amino acid can be assayed.

Procedure

About 10 mg of sample was weighed accurately into a heat sealable test tube. 10ml of 6 N HCl was added and the tubes were heat sealed after filling pure nitrogen gas. Hydrolysis was carried out in a hot air oven at 110ºC for 24hours. After the hydrolysis, the contents were removed quantitatively and filtered into a round bottom flask through Whatmann filter paper No.42. The contents of the flask were flash evaporated to remove trace of HCl and the process repeated for 2-3 times with added distilled water. The residue was made up to 1.0ml with 'A' buffer containing Tri Sodium citrate 32.7g, Methanol of 140ml, Perchloric acid 16.6ml, made up to 2L (pH 3.2).

The sample thus prepared was filtered again through a membrane filter and 0.45µl of this was injected to Shimadzu HPLC-LC10As Amino acid analyzer consisting of column packed with a strongly acidic cation exchange resin i.e. strene divinyl copolymer with sulphonic group. The column used is Na type [i.e. ISC-07/S1504 Na] having a length of 19cm and diameter 5mm. The mobile phase consists of two buffers, Buffer B (Tri Sodium citrate 117.6g, Boric acid 24.8g, 4N NaOH 45ml, pH 10) and Buffer C (0.05N HCl). The oven temperature was maintained at 60^OC. The amino acids were eluted from the column by stepwise

elution i.e. acidic amino acid first followed by neutral and basic amino acids. The amino acid analysis was done with non-switching flow method and fluorescence detection after post column derivatization with O-phthalaldehyde. In the case of proline and hydroxy proline, imino group was converted to amino group with sodium hypochlorite. Amino acid standard was also run to calculate the concentration of the amino acid depending on the standard chromatogram. The result were quantified and represented in percentage.

4.2.6. Determination of molecular weight by Gel exclusion chromatography using Sephadex G 100

Principle

Gel exclusion chromatography (also called gel filtration, molecular sieve or gel permeation chromatography) exploits the physical property of molecular size to achieve separation. The stationary phase consists of inert particles that contain small pores of a controlled size. Solute molecules larger than the pores cannot enter the interior of the gel beads, so they are not slowed in their progress through the column and elute rapidly in a single zone. Small molecules capable of diffusing in and out of the beads are delayed in their journey through the column bed. Molecules of intermediate size migrate through the column at a rate somewhere between those for large and small molecules. Therefore, the order of elution of the various solute molecules is directly related to their molecular dimensions.

Procedure

The molecular weight of the PPC-*Pr* was determined by Gel filtration chromatography using Sephadex G-100 column. Sephadex G-100 was allowed to swell by autoclaving for 1-2 hrs. The gel slurry was poured into a graduated cylinder and water equivalent to two times the gel volume was added. The cylinder was inverted several times and the gel was allowed to settle. After 90 to 95% of the gel has settled, the supernatant was decanted, again water was added, and the settling process was repeated to remove air bubbles and fine particles. Then the gel was packed in to a column of size 2.2 x 50 cm. PPC-*Pr* dissolved in deionized water was applied to the column and elution was carried out with the

same at a flow rate of 1ml/min. Fractions of 20ml were collected and Anthrone method (Yemm and Wills, 1954) described in Section 3.2.3. was used to monitor the presence of polysaccharide in the eluted fractions. Blue Dextran (M.W. 2,000,000 Da), Dextran T-500 (M.W. 500,000 Da) and Dextran (M.W. 60,000 Da) were used as molecular weight markers. A calibration curve was plotted by taking the elution volumes of standard Dextrans along X-axis and their molecular weights along Y-axis. The elution volume is the volume of the eluting buffer necessary to remove the particular solute from a packed column. The elution volume of PPC-*Pr* was also measured and its molecular weight was then read directly from the standard graph.

4.3. Results

The polysaccharide-protein complex (PPC-*Pr*) was isolated by repeated solvent precipitation, deproteinization, dialysis and freeze-drying of aqueous extract of *P.rimosus.* From 100g of powdered fruiting body of *P.rimosus*, 8g of PPC-*Pr* was obtained as brown colored powder form. On reaction with Phenol sulphuric acid reagent, PPC-*Pr* showed characteristic color reactions indicating the presence of polysaccharides. The total polysaccharide present in PPC-*Pr* was 54.8 % (Table 4.1.). For the determination of monosaccharide constituents of PPC-*Pr*, the R_f value of hydrolysate was compared with the R_f values of standard monosaccharides obtained by Paper and Thin layer chromatography. The R_f values of standards on Paper chromatography were: D-Glucose 0.479, D-Fructose 0.508, D-galactose 0.404, D-mannose 0.517 and D-xylose 0.528. On TLC analysis, the R_f values produced by standards were: D-Glucose 0.457, D-Fructose 0.436, D-galactose 0.447, D-mannose 0.500 and D-xylose 0.553. Paper chromatography analysis showed that the hydrolysis product of PPC-*Pr* had only single spot with R_f value 0.479, which coincided with the R_f value of glucose (Figure 4.2.). This result was confirmed by TLC analysis in which also PPC-*Pr* produced only single spot with R_f value 0.457, which coincided with the R_f value of glucose (Figure 4.3.). Thus glucose was found to be the only monosaccharide component of PPC-*Pr*.

Table 4.1. Total polysaccharide and protein contents of the PPC-*Pr*

Contents of *P.rimosus*	Conc. in mg/ml	Percent (%)
Polysaccharide	0.548	54.8
Protein	0.286	28.6

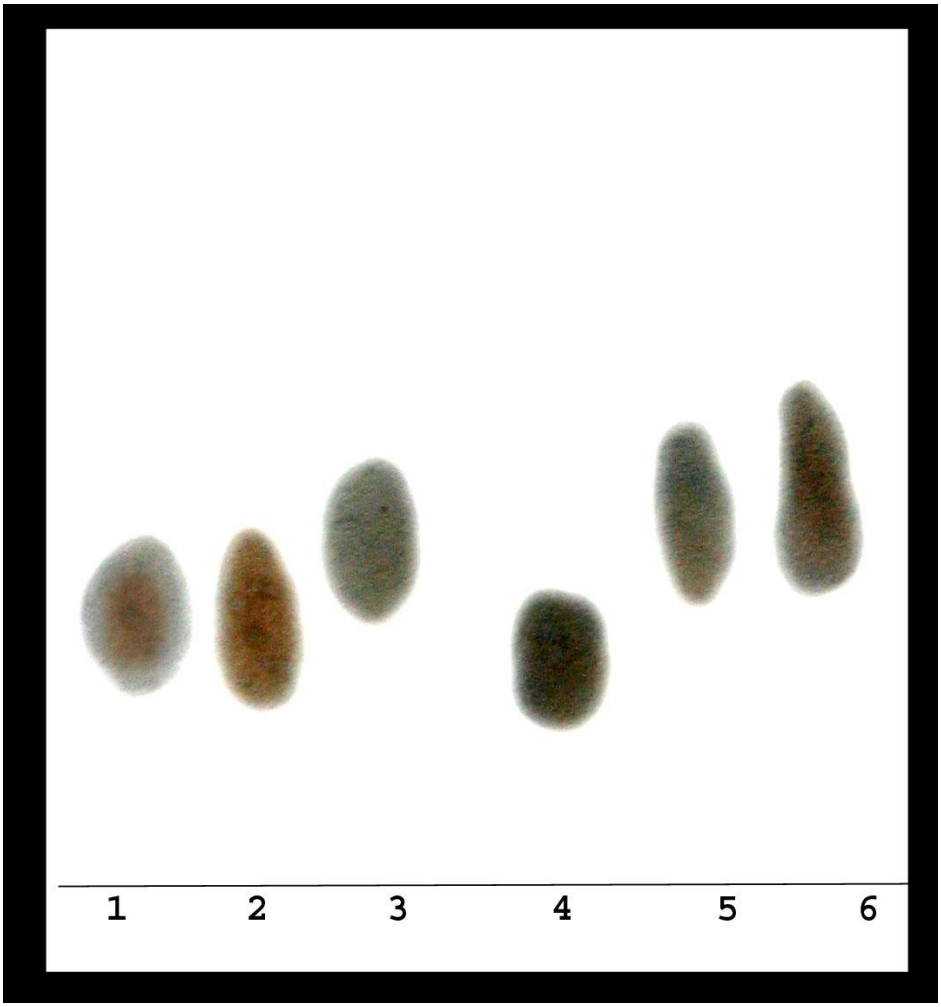

Figure 4.2. Paper chromatographic pattern of monomer forms of PPC-*Pr* from *P.rimosus* using Whatman No:1 filter paper. Solvent system-butanol: acetic acid: water (2: 1: 1), Spray reagent-aniline diphenylamine phosphoric acid reagent. Spot (1) PPC-*Pr;* spot (2), (3), (4), (5) and (6) Glucose, Fructose, Galactose, Mannose and Xylose respectively, used as standards.

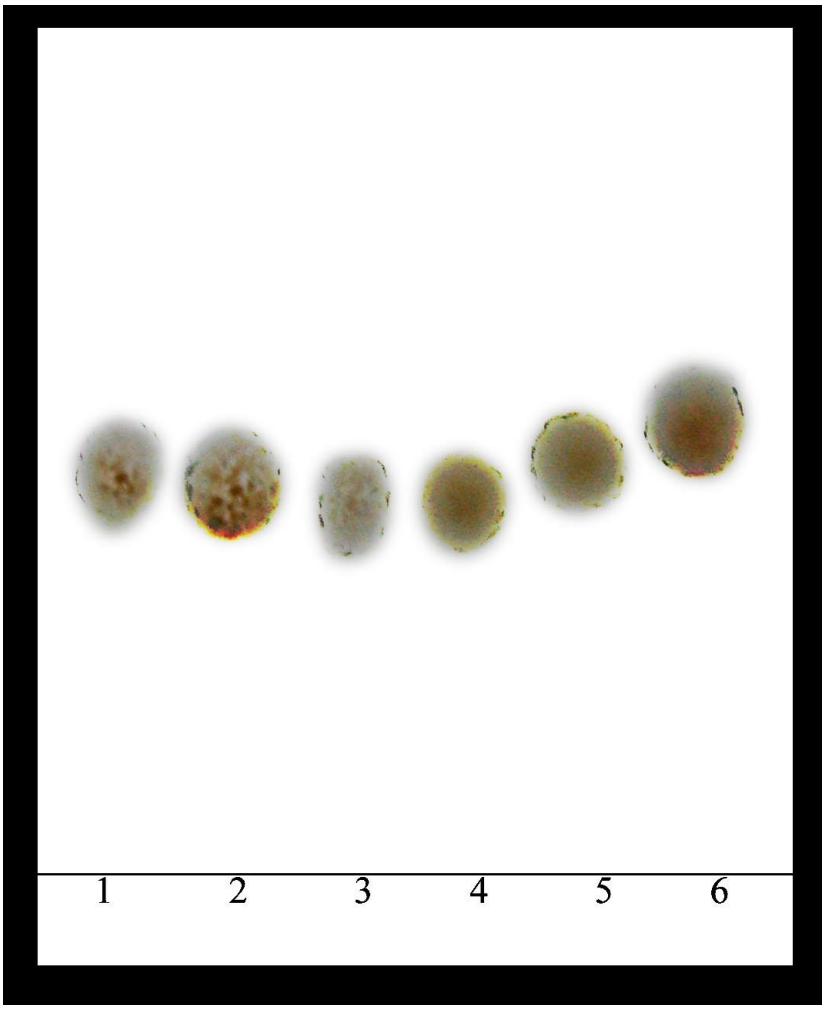

Figure 4.3. TLC pattern of monomer forms of PPC-*Pr* from *P.rimosus* using silica gel G. Solvent system-butanol: acetic acid: water (2: 1: 1), Spray reagent-aniline diphenylamine phosphoric acid reagent. Spot (1) PPC-*Pr;* spot (2), (3), (4), (5) and (6) Glucose, Fructose, Galactose, Mannose and Xylose respectively, used as standards.

PPC-*Pr* also produced characteristic color on reaction with Bradford's reagent, indicating the presence of protein and the protein content was found to be 28.6 % (Table 4.1.). The composition of hydrolyzed amino acids in PPC-*Pr* is shown in Table 4.2. Its protein portion predominantly consisted of aspartic acid, glutamic acid, glycine, alanine and serine. The molar ratios were 14.1%, 12.4%, 11.6%, 11.6% and 11.2% respectively. The bulky side chains of alanine and gycine were considered important in promoting hydrophobic interactions with biomolecules. The elution profile of gel filtration chromatography is shown in Figure 4.4. The elution volume of standard size markers was in 60 ml (Blue

Table 4.2. Aminoacid composition of hydrolysed PPC-*Pr* from *P.rimosus*

Aminoacid	**M%**
Aspartic Acid	14.1
Threonine	8.3
Serine	11.2
Glutamic acid	12.4
Proline	0.2
Glycine	11.6
Alanine	11.6
Cystine	0.4
Valine	7.6
Methionine	1.3
Isoleucine	3.6
Leucine	5.4
Tyrosine	1.6
Phenylalanine	3.1
Histidine	2.9
Lysine	0.5
Arginine	2.9

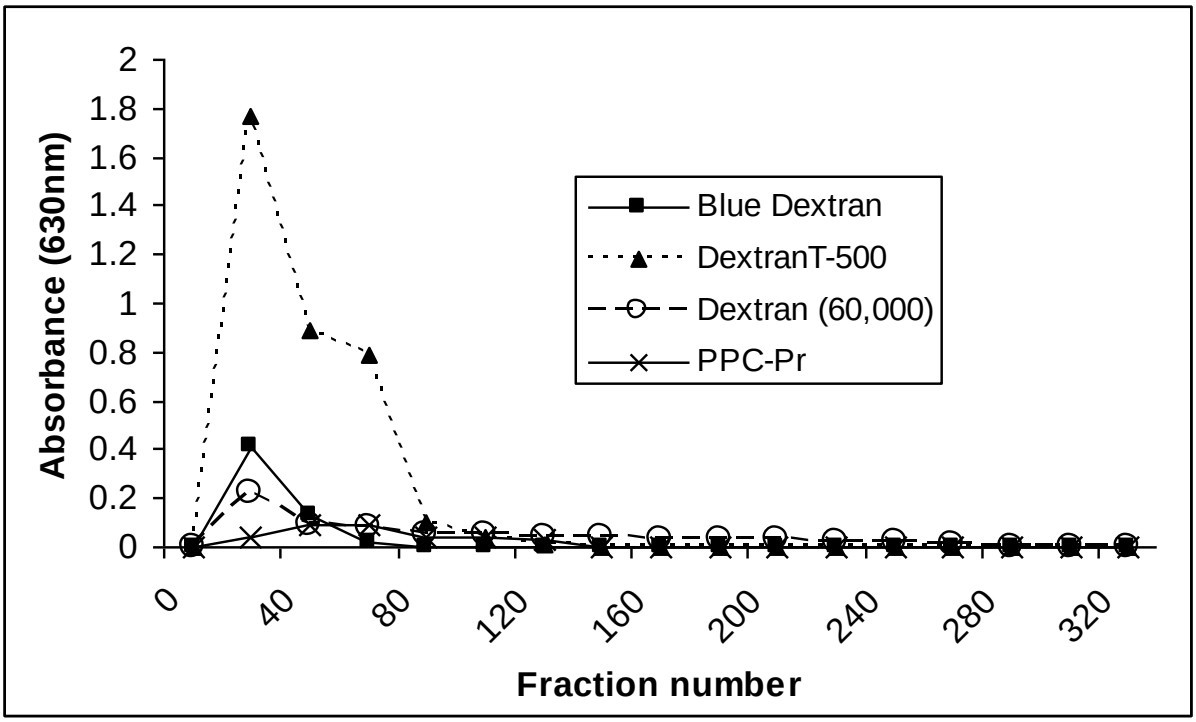

Figure 4.4. Gel filtration of PPC-*Pr* isolated from fruiting body of *P.rimosus* on Sephadex G-100 column (2.2 x 50 cm). The column was eluted with deionized water and O.D. of collected fractions was measured at 630 nm by the Anthrone method.

Dextran), 140 ml (Dextran T-500), and 300 ml (Dextran-M.W. 60,000). The PPC-*Pr* was 120 ml of elution volume. Sephadex G 100 gel chromatography gave a value of 1,200,000 Da for the molecular mass of PPC-*Pr*, when compared to dextrans of known molecular weight.

4.4. Discussion

Higher Basidiomycetes mushrooms represent an unlimited source of antitumor or immunostimulating polysaccharides. For almost 40 years, medicinal mushrooms have been intensively investigated for their medicinal effects in *in vivo* and *in vitro* model systems, and many new antitumor and immunomodulating polysaccharides have been identified and put into practical use (Zhang et al. 2007). The main antitumor compounds presently isolated from mushroom fruit bodies, mycelial biomass or liquid culture broth are either water soluble β-D-glucans, β-D-glucans with heterosaccharide chains of xylose, mannose, galactose or uronic acid or β-D-glucan-protein complexes, proteoglycans.

Polysaccharides are a structurally diverse group of biological macromolecules of widespread occurrence in nature. They are composed of repetitive structural features that are polymers of monosaccharide residues joined to each other by glycosidic linkages. Concentration of polysaccharides in certain medicinal mushroom species can be related to the stage of development of the mushroom fruit-body and also to the time after harvest and subsequent storage conditions (Minato et al., 1999; 2001). The basic β-D-glucan is a repeating structure with the D-glucose units jointed together in linear chains by beta-bonds. Molecular weight, degree of branching, number of substituents, as well as ultra structure, including the presence of single and triple helices, significantly affect the biological activities of β-glucans (Adachi et al. 2002). Polysaccharide-peptides or glycoproteins are polypeptide chains or small proteins to which polysaccharide β-D-glucan chains are stably attached (Boldizsar et al., 1998). As a general rule the protein-linked glucans have a greater immunopotential activity than the corresponding glucans.

The PPC-*Pr* isolated from *P.rimosus* showed the presence of both polysaccharide and protein. Though the protein content of PPC-*Pr* was relatively

high, it could be deemed to be a protein-bound polysaccharide because the Sevag method has been repeated many times to remove free proteins. Amino acid analysis also revealed the abundance of proteins. Paper chromatography as well as the TLC analysis of acid hydrolyzed product showed that the PPC-*Pr* contained only glucose as monosaccharide unit. The results thus indicate that PPC-*Pr* belong to D-glucan-protein complexes or proteoglycans. An analysis of structural features of the various anti-tumor mushroom extracts showed that all the compounds responsible for anti-tumor activity in mushrooms were high molecular weight glucans linked in 1-3 and 1-6 linkages that would provide solubility and anti-tumor activity (Mascarenhas, 1994). The PPC-*Pr* was found to be completely soluble in water and the compound had high molecular weight as indicated by gel filtration chromatography. Greater solubility in water, high molecular weight and protein bound nature of PPC-*Pr* can contribute towards its anti-tumor activity.

Chapter - 5

Anti-tumor activity of PPC-*Pr*

Synopsis

Anti-tumor activity of PPC-*Pr* was studied using the Ehrlich's ascites carcinoma (EAC) and Dalton's lymphoma ascites (DLA) murine cell line. The PPC-*Pr* was found to be effective in increasing the life span of EAC cell line induced ascitic mice. PPC-*Pr* administration at a dose of 25 and 50 mg/kg increased the life span of animals by 38.86% and 66.07% respectively. PPC-*Pr* was also found to have significant preventive and curative effects on solid tumor induced by DLA cell line. It was found that PPC-*Pr* at a dose of 50 mg/kg was able to prevent the tumor proliferation as effectively as the standard reference drug cisplatin. The experimental results thus, indicated that protein bound polysaccharide (PPC-*Pr*) isolated from *P.rimosus* possessed profound antitumor activity. The findings suggest the potential therapeutic use of this compound as an antitumor agent.

5.1. Introduction

Cancer is the second largest cause of death worldwide. The major modules for treating cancer till now include surgery, radiation and chemotherapy. Conventional cancer chemotherapy is used to kill or disable tumor cells while preserving the normal cells in the body by the application of synthetic compounds (Filder and Ellis 2000). These agents have a narrow margin of safety, and the therapy may fail due to drug resistance and dose-limiting toxicities. Hence attempts are being continued to develop new therapeutically useful anticancer agents.

The medicinal use of mushrooms has a very long tradition in the Asian countries; they have been utilized in China, Japan and other Asian countries for two thousand years for edible and medicinal uses. Recently, a number of bioactive molecules, including anti-tumor substances, have been identified in numerous mushroom species (Mizuno et al., 1995b). Polysaccharides, proteo-polysaccharides and their derivatives from the mushroom have been recognized to be the potent immuno-stimulatory and anti-tumor active compounds (Kim et al.

2003d). Antitumor polysaccharides from mushrooms are considered as biological response modifiers (BRM) based on their action mechanism. They are known to have minimal adverse effects and drug-induced sufferings.

Ikekawa et al. (1969) published one of the first scientific reports on antitumor activities of essences obtained from fruiting bodies of mushrooms belonging to the family Polyporaceae (Aphyllophoromycetideae) and a few other families, against Sarcoma 180 transplanted tumors in animals (Ikekawa et al. 1982; Ikekawa et al. 1992; Ikekawa 2001). Soon thereafter the first three major drugs were developed from medicinal mushrooms. All three were polysaccharides, specifically β-glucans; Krestin from cultured mycelial biomass of *Tremetes versicolor* (Turkwey Tail), Lentinan from fruiting bodies of *Lentinus edodes* (Shiitake), and Schizophyllan from the liquid cultured broth of *Schizophyllum commune* (Split Gill).

Here we studied the effect of PPC-*Pr* on increasing the life span of Ehrlich's ascites carcinoma (EAC) cell line induced mice and also its effect on preventing and curing the solid tumors induced by Dalton's lymphoma ascites (DLA) cell line. PPC-*Pr* was found to have significant activity against both the murine tumor cell lines. The results are presented in this chapter.

5.2. Materials and methods

5.2.1. Test animals

Female Swiss albino mice (6-8 weeks old) of weight 20 ± 5g were used for the anti-tumor studies.

5.2.2. Cell lines

Ehrlich's ascites carcinoma (EAC) and Dalton's lymphoma ascites (DLA) cells were maintained by intraperitoneal (i.p.) passages in female Swiss albino mice. Ascetic fluid was aspirated on the 15th day after i.p. inoculation. The cells were washed thrice and the cell suspension was diluted to 1x10^6 viable cells/0.1 ml and used for the studies.

5.2.3. Isolation of PPC-*Pr* from *P.rimosus*

PPC-*Pr* was isolated from the hot water extract of *P.rimosus* as described in section 4.2.1.

5.2.4. Ascites tumor model

Animals were divided into four groups of six animals in each group. All animals were injected i.p. with $1x10^6$ viable EAC cells in PBS. Group1, maintained as control, was administered with the vehicle (sterile normal saline, i.p.) alone. Group2, maintained as standard, received the reference drug Cisplatin at a dose of 2 mg/kg body weight (i.p.). Group3 and 4 received the test compound PPC-*Pr* at a dose of 25 and 50 mg/kg body weight (i.p.). The drug administration was started 24h after the tumor implantation, continued for ten days with one dose in each alternate day and the total number of dose given was five. The mortality rate was noted in each group and the percent increase in life span (ILS) was calculated using the formula:

Percentage (%) ILS = (T-C / C) x100

where, T is mean survival time of treated group and C that of control group (Ajith and Janardhanan 2003).

5.2.5. Solid tumor model (Preventive effect)

Animals were divided into four groups of six animals in each group. Viable DLA cells ($4x10^6$) in PBS were transplanted into the right groin of mice. Drug administration at 25 and 50 mg/kg body weight was started (i.p.) 24 h after tumor implantation and continued with one dose in each day for 10 consecutive days. Standard group received Cisplatin at a dose of 4 mg/kg body weight (i.p). The tumor development on animals in each group was determined by measuring the diameter of tumor growth in two perpendicular planes using Vernier calipers twice a week for 5 weeks. The tumor volume was calculated using the formula $V = 4/3\ \pi\ a^2b\ /\ 2$ where, a is minor diameter and b is major diameter. At the end of the fifth week, animals were sacrificed under anesthesia using diethyl ether, tumor expirated and weighed. The percent inhibition was calculated by the formula: (1-*B/A*) x100 where, *A* is average tumor weight of the control group and *B* that of the treated group (Ajith and Janardhanan 2003).

5.2.6. Solid tumor model (Curative effect)

Curative effect of PPC-*Pr* was tested after tumor development in mice (Ajith and Janardhanan 2003). Solid tumor in mice was induced as in above

experiment. After 15 days, animals with tumor size around 1.1 ± 0.1 cm^3 were divided into four groups as mentioned earlier. Drug was administered at 25 and 50 mg/kg body weight as i.p. once daily for 10 consecutive days. Tumor diameter was measured using Vernier calipers twice a week for a period of 3 weeks and volume was calculated. At the end of the experiment, animals were sacrificed, tumor expirated and weighed. The percent inhibition was calculated as mentioned in the earlier experiment. The growth delay was determined as the difference in the number of days required for the control versus treated tumors to increase in volume twice. The tumor curative effect of PPC-*Pr* was determined by the reduction in tumor volume or weight.

5.3. Results

In the ascites tumor model, PPC-*Pr* significantly increased the life span of EAC induced ascetic mice. PPC-*Pr* administration at a dose of 25 and 50 mg/kg increased the life span of animals by 38.86% and 66.07% ($P<0.001$) respectively (Table 5.1). The PPC-*Pr* showed significant preventive activity against DLA induced solid tumor model in a dose-dependent manner (Table 5.2). The PPC-*Pr* at a dose of 25 mg/kg and 50 mg/kg could prevent 59.3% and 82.2% of solid tumor growth respectively in tumor induced mice. The weight and volume of tumor in PPC-*Pr* treated groups at the end of fifth week was significantly lower ($P<0.001$) than the control group. It was found that PPC-*Pr* at a dose of 50 mg/kg was able to prevent the tumor proliferation as effectively as the standard reference drug cisplatin (Figure 5.1).

The curative effect of PPC-*Pr* treatment on the growth of DLA induced solid tumor is shown in Table 5.3. The results showed that administration of PPC-*Pr* significantly reduced the tumor weight in a dose-dependent manner, with inhibition rates of 54.21 and 60.30 % at doses of 25 and 50 mg/kg respectively, when the tumor weight was recorded on day 21. The inhibition rate of standard drug Cisplatin was 59.26%. PPC-*Pr* at a dose of 50 mg/kg was found to be as effective as the reference drug Cisplatin in curing the developed tumor in mice. PPC-*Pr* also showed a growth development delay of 8.85 and 13.12 days at doses of 25 and 50 mg/kg respectively (Figure 5.2).

Table 5.1. Effect of PPC-*Pr* on EAC induced Ascites tumor model

Group	Treatments (mg/kg)	Survival time (days)	% increase in life span	Mortalilty at 30th day
Control	Normal Saline	19.66±1.21		6/6
Cisplatin	2	34.33±0.82*	78.03	0/6
PPC-*Pr*	25	27.30±1.37*	38.86	6/6
PPC-*Pr*	50	32.65±2.58*	66.07	2/6

Values are mean ± SD, n = 6; *P<0.001 significant with respect to control.

Table 5.2. Effect of PPC-*Pr* on DLA induced preventive solid tumor model

Group	Treatments (mg/kg)	Vol. on 5th week (cm^3)	Weight of tumor (g)	% of inhibition
Control	Normal Saline	4.75±0.38	4.67±0.21	
Cisplatin	4	0.10±0.10	0.58±0.03*	87.6
PPC-*Pr*	25	1.88±0.40	1.90±0.14*	59.3
PPC-*Pr*	50	0.12±0.12	0.83±0.02*	82.2

Values are mean ± SD, n = 6; * P<0.001 significant with respect to control.

Table 5.3. Effect of PPC-*Pr* on DLA induced curative solid tumor model

Group	Treatments (mg/kg)	Growth delay (days)	Weight of tumor (g)	% of inhibition
Control	Normal Saline		4.98±0.67	
Cisplatin	4	12.75	2.03±0.19*	59.26
PPC-*Pr*	25	8.85	2.28±0.11*	54.21
PPC-*Pr*	50	13.12	1.98±0.16*	60.30

Values are mean ± SD, n = 6; * P<0.001 significant with respect to control.

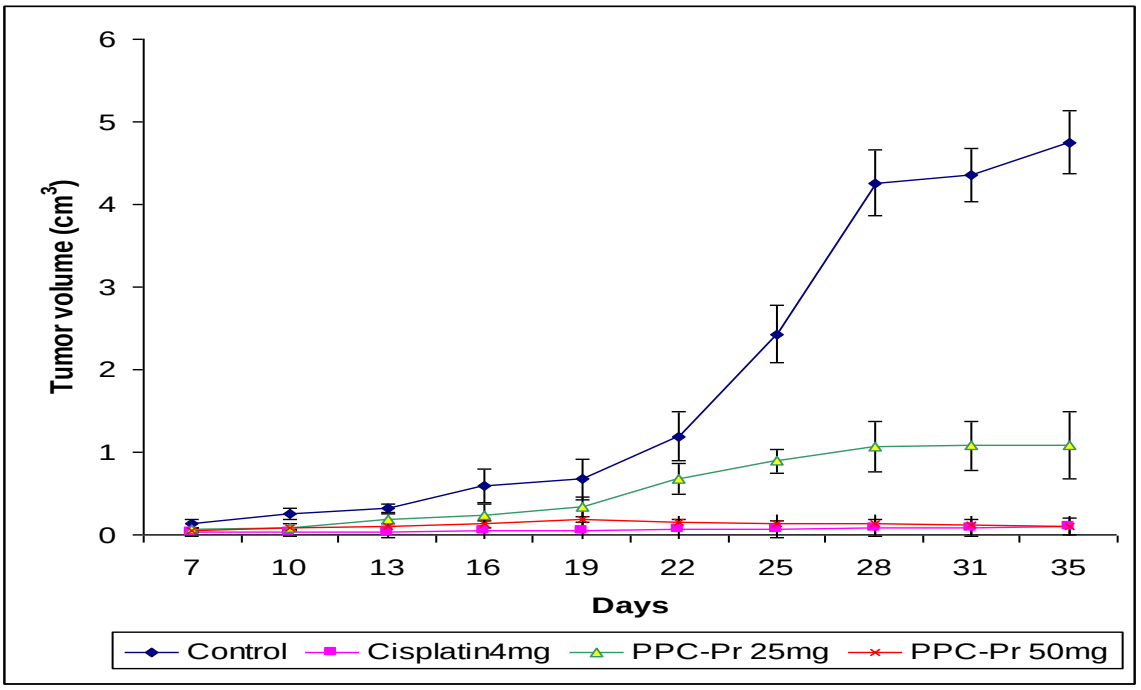

Figure 5.1. Inhibition of tumor development by PPC-*Pr* at dose of 25 and 50 mg/kg in DLA induced preventive solid tumor model. Control group received vehicle (normal saline) only. Cisplatin at a dose of 4 mg/kg was used as standard. Drug was administered as i.p. for 10 consecutive days starting 24 h after tumor implantation. Values are mean ± SD, n=6.

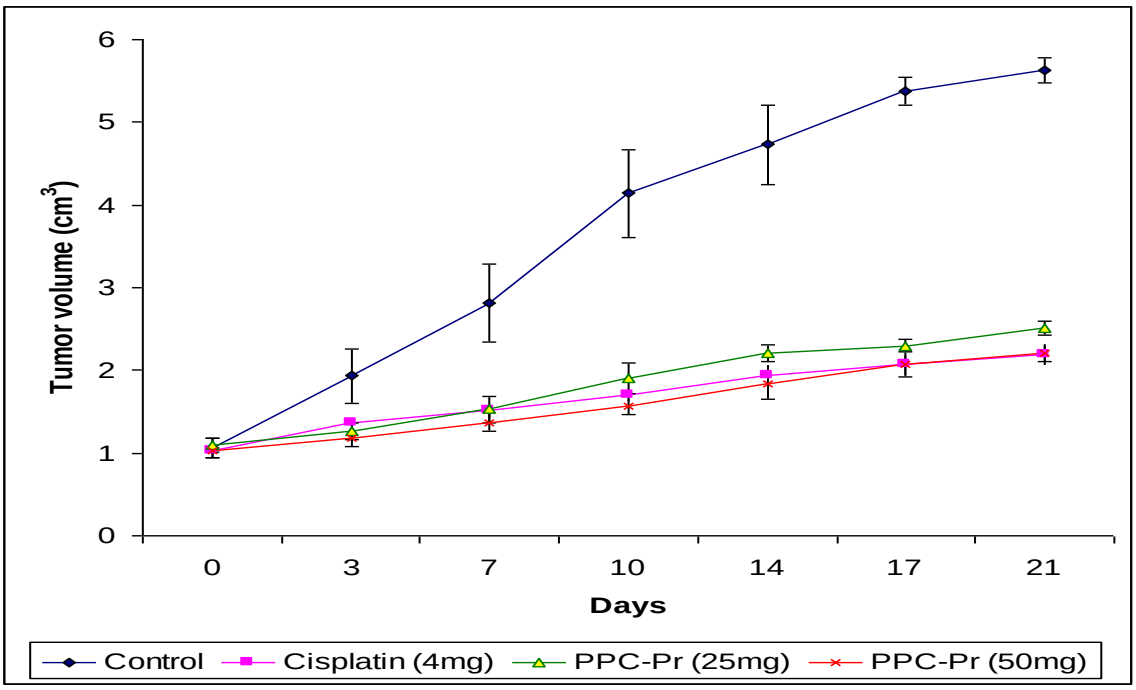

Figure 5.2. Tumor growth development delay by PPC-*Pr* at dose of 25 and 50 mg/kg in DLA induced curative solid tumor model. Control group received vehicle (normal saline) only. Cisplatin at a dose of 4 mg/kg was used as standard. Drug was administered as i.p. for 10 consecutive days, when tumor size was around 1.1 ± 0.1 cm^3. Values are mean ± SD, n=6.

5.4. Discussion

During the evolution of neoplastic diseases, the body's natural defenses are normally lowered, while the activity of suppressor cells and the blocking antibodies are increased (Gazit and Kedar 1994). This phenomenon facilitates tumor establishment and growth. Along with this imbalance in the tumor-host relationship, there is secondary immunodeficiency due to conventional cancer treatment, such as chemotherapy and radio therapy. Another mode of tumor therapy, immunotherapy, seeks to correct this immunological deterioration in the host's favor, providing it with an efficient tumor immuno-surveillance capacity. Some polysaccharides or polysaccharide-protein complexes from mushrooms are able to stimulate the non-specific immune system and to exert antitumor activity through the stimulation of the host's defence mechanism. The drugs activate effector cells like macrophages, T lymphocytes and NK cells to secrete cytokines like TNF-α, IFN-γ, IL-β, etc., which are antiproliferative and induce apoptosis and differentiation in tumor cells (Wasser and Weis 1999b; Reshetnikov et al. 2001). β-D glucans are known to induce a biological response by binding to membrane complement receptor type 3 (CR3, alphaMβ2 integrin or CD 11b/CD 18) on immune effector cells. The ligand-receptor complex can be internalized. The intercellular events that occur after glucan-receptor binding have not been fully determined till now (Zhou and Gao 2002).

Several species of *Phellinus* including *P. igniarius, P. hartigii, P. gilvus, P. pini*, etc. are reported to have different medicinal effects such as anti-tumor and immuno-stimulating activities (Ayer et al. 1996; Jung et al. 1997). The active polysaccharide purified from *Phellinus linteus* stimulates humoral and cell mediated immunity, and exhibits a wider range of immunostimulation and anti-tumor activity than other polysaccharides isolated from Basidiomycetes (Kim et al. 1996). Polysaccharide isolated from P. linteus was also suggested to be used in immunochemotherapy of cancer because of its effective activities on tumor growth and metastasis through the immunopotentiation of patients without toxicity (Han et al. 1999).

There is no available report on the anti-tumor activity of polysaccharides isolated from *P.rimosus.* This particular study showed that D-glucan-protein complex isolated from *P.rimosus* significantly inhibited the tumor development and growth. The protein-linked glucans are found to be the better immunopotentiators than the corresponding glucans. Since PPC-*Pr* is a protein bound glucan, it can cause immunostimulation, which is supposed to be the main mechanism of action behind the anti-tumor activity of polysaccharides. Higher anti-tumor activity seems to be correlated with higher molecular weight, lower level of branching and greater water solubility of β-glucans (Zjawiony 2004). PPC-*Pr* which is completely soluble in water has a higher molecular weight of ~ 1,200,000. Although the mechanism of anti-tumor effect remains to be elucidated, it might be related to the greater solubility, high molecular weight and protein bound nature of PPC-*Pr*.

Chapter - 6

Anti-oxidant activity of PPC-*Pr*

Synopsis

There is increasing evidence indicating that free radicals have a wide variety of pathological effects. These species may be either oxygen derived reactive oxygen species (ROS) or nitrogen derived reactive nitrogen species (RNS). The free radical scavenging activity of PPC- P*r* was determined by evaluating superoxide radical, hydroxyl radical, nitric oxide, and 1,1-diphenyl 2-picryl hydrazyl (DPPH) radical scavenging activities, inhibition of lipid peroxidation and ferric reducing antioxidant power (FRAP). PPC- P*r* at a concentration of 2 mg/ml showed 93.18% of superoxide radical scavenging activity. PPC-*Pr* also showed marked ability to scavenge hydroxyl radicals and nitric oxide with increasing concentrations. The DPPH scavenging activity of PPC-P*r* was tested with increasing concentrations and it was observed that PPC-P*r* possessed significant activity with IC_{50} value of 0.058 mg/ml. PPC-*Pr* when assayed for FRAP showed significant ferric reducing antioxidant ability. PPC-*Pr* was also found to inhibit the lipid peroxidation considerably and the IC_{50} value was 0.253 mg/ml. The inhibitory effect of PPC-P*r* was increased with increasing concentrations.

6.1. Introduction

Chemical compounds and reactions capable of generating potential toxic oxygen species/free radicals are referred to as pro-oxidants. On the other hand, compounds and reactions disposing off these species, scavenging them, suppressing their formation or opposing their actions are called anti-oxidants. In a normal cell, there is an appropriate pro-oxidant: anti-oxidant balance. However, this balance can be shifted towards the pro-oxidant when production of oxygen species is increased or when levels of anti-oxidants are diminished. This state is called oxidative stress and can result in serious cell damage if the stress is massive or prolonged. Oxidative stress is implicated in the etiopathogenesis of a variety of human diseases (Frei, 1994; Peterhans, 1997; Beck and Levander, 1998; Domenico et al., 1998).

During the last two decades, there has been a growing interest in studies that concern with the prevention of uncontrolled oxidative process leading to various diseases in living system. Several studies have shown the role of oxidative stress in the causation and progression of different diseases including atherosclerosis, carcinogenesis, neurodegenerative diseases, chronic inflammatory diseases, radiation damage, aging and various other pathobiological effects (Frei, 1994; Peterhans, 1997; Beck and Levander, 1998; Domenico et al., 1998; Treitinger et al., 2000; Beck, 2000). A major development of carcinogenesis research has been the discovery of significant levels of DNA damage by oxyradicals of endogenous cellular source. Reactive oxygen and nitrogen species play an important role in carcinogenesis. ROS induce DNA damage, as the reaction of free radicals with DNA includes strand breaks, base modification and DNA-protein cross-links. Commonly observed base modification is the formation of 8-oxoguanine (Kasai and Nishimura, 1984) which induces Guanine: Cytosine to Thymine: Adenine transversions at the DNA replication stage (Floyd, 1990), an important process in carcinogenesis and tumor development.

Many synthetic antioxidant components exhibited toxic or mutagenic effects that have directed the attention to the naturally occurring antioxidants in recent years. Polysaccharides, which are widely distributed in nature, have been demonstrated to play an important role as free-radical scavenger for the prevention of oxidative damage. The free radical scavenging activity of PPC-*Pr* from *P.rimosus* was studied using different in vitro techniques and found to be an effective anti-oxidant. The findings are presented in this chapter.

6.2. Materials and methods

6.2.1. Isolation of PPC-*Pr* from *P.rimosus*

PPC-*Pr* was isolated from the hot water extract of *P.rimosus* as described in section 4.2.1.

6.2.2. Superoxide radical scavenging activity

Superoxide radical ($O_2^{\cdot}$) generated from the photo reduction of riboflavin and was detected by NBT reduction method (McCord and Fridovich, 1969). The reaction mixture contained, EDTA (6mM) containing 3µg NaCN; riboflavin (2µg);

NBT (50µg); KH_2PO_4-Na_2HPO_4 buffer (67mM, pH 7.8) and various concentrations of PPC-*Pr* in a final volume of 3 ml. The tubes were illuminated under incandescent lamp for 15 min. The optical density (OD) at 530 nm was measured before and after illumination. The inhibition of superoxide radical generation was determined by comparing the absorbance values of the control with that of treatments. Vitamin-C was used as standard.

6.2.3. Inhibition of lipid peroxidation

Lipid peroxidation was induced by Fe^{2+} -ascorbate system (Bishayee and Balasubramanian, 1979) in the rat liver homogenate in the presence and absence of PPC-*Pr* to form thiobarbituric acid reacting substance (TBARS). TBARS is measured according to the method of Ohkawa et al (1979). The reaction mixture contained rat liver homogenate 0.1 ml (25% w/v) in Tris-HCl buffer (20mM, pH 7.0); KCl (30 mM); $FeSO_4$ $(NH_4)_2$ $SO_4.6H_2O$ (0.16 mM); ascorbate (0.06mM); and various concentrations of PPC-*Pr* in a final volume of 0.5ml. The reaction mixture was incubated for 1 hour at 37 ^{0}C. After the incubation period, 0.4 ml was removed and treated with 0.2 ml SDS (8.1%); 1.5 ml TBA (0.8%); and 1.5 ml acetic acid (20%, pH 3.5). The total volume was made up to 4ml by distilled water and then kept in a water bath at 95-100 ^{0}C for 1 hour. After cooling, 1.0 ml of distilled water and 5.0 ml of *n*-butanol and pyridine mixture (15:1 v/v) were added to the reaction mixture, shaken vigorously and centrifuged at 4000 rpm for 10 min. The organic layer was removed and its absorbance at 532 nm was measured. Inhibition of lipid peroxidation was determined by comparing the O.D. of the treatments with that of control. Vitamin-E was used as standard.

6.2.4. Hydroxyl radical scavenging activity

Hydroxyl radicals generated from Fe^{3+}- ascorbate-EDTA-H_2O_2 system (Fenton's reaction) was estimated by its degradation of deoxyribose that resulted in thiobarbituric acid reactive substance (TBARS) (Elizabeth and Rao, 1990). The reaction mixture contained deoxyribose (28 mM); $FeCl_3$ (1 mM); KH_2PO_4-KOH buffer (20 mM, pH 7.4); EDTA (1 mM); H_2O_2 (1 mM); ascorbic acid (0.1mM) and various concentrations of PPC-*Pr* in a final volume of 1.0 ml. The reaction mixture was incubated for 1 hour at 37 ^{0}C. The TBARS formed was estimated by

TBA method (Ohkawa et al., 1979). The hydroxyl radical scavenging activity was determined by comparing absorbance of control with that of treatments. Vitamin-E was used as standard.

6.2.5. Nitric oxide scavenging activity

The nitric oxide scavenging activity was measured according to the method of Marcocci et al (1994). Immediately before the experiment, 10 mM stock solution of sodium nitroprusside was prepared in PBS (pH 7.4). Various concentrations of PPC-*Pr* and sodium nitroprusside in PBS (10mM) in a final volume of 3ml were incubated at 25 ^{o}C for 150min. After incubation, 0.5ml of the reaction mixture was removed and mixed with 0.5ml of Griess reagent (1% sulphanilamide, 2% o-phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride). The absorbance of the chromophore was read immediately at 546 nm against reagent blank. The nitric oxide scavenging activity was determined by comparing the absorbance of control with that of treatments. Vitamin-C was used as standard.

6.2.6. DPPH free radical scavenging activity

In this method a commercially available and stable free radical 2,2-diphenyl-1-picrylhydrazil ($DPPH^{+}$) soluble in methanol, was used (Aquino et al., 2001). DPPH in its radical form has an absorption peak at 515 nm, which disappeared on reduction by an antioxidant compound. An aliquot (25µl) of the PPC-P*r* was added to 1 ml of freshly prepared DPPH solution (0.25 g/l in methanol). The samples were kept for 20 min in dark and the decrease in the absorbance was measured at 515 nm. Vitamin-C was used as standard.

6.2.7. Ferric reducing antioxidant power (FRAP) assay

The ferric reducing ability was measured at low pH. The stock solution of 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) in 40 mM HCl, 20 mM $FeCl_3.6H_2O$ and 0.3 M acetate buffer (pH 3.6) were prepared. The FRAP reagent contained 2.5 ml TPTZ solution, 2.5 ml ferric chloride solution and 25 ml acetate buffer. It was prepared freshly and warmed to 37^{0}C. Then 900 µl of distilled water and 30 µl of test sample/methanol/distilled water/ standard solutions were added. The reaction mixture was then incubated at 37^{0}C for 30 min. An intense blue colour complex

was formed when ferric tripyridyl triazine (Fe $^{3+}$ TPTZ) complex was reduced to the ferrous (Fe^{2+}) form and the absorption at 595 nm was recorded (Benzie and Strain, 1996).

6.3. Results

The free radical scavenging activity or antioxidant activity of PPC-*Pr* expressed as IC_{50} values are presented in Table 6.1. Superoxide radical can be generated by photochemically-reduced flavins and it can reduce nitroblue tetrazolium to blue formazan. The formation of formazan resulted in a rise in absorbance at 530 nm, which indicated the generation of superoxide radicals. By the addition of PPC-P*r*, the formation of formazan was inhibited in a dose dependent manner (Figure 6.1.). This showed the superoxide radical scavenging activity of PPC-*Pr*. The concentration of PPC-*Pr* required to scavenge 50% superoxide anion generated (IC_{50}) was found to be 0.606 ± 5.80 mg/ml.

Table 6.1. In vitro antioxidant activity of PPC-P*r*, IC $_{50}$ (mg/ml)

Activities	IC $_{50}$	Standard	
		Vitamin C	Vitamin E
Super oxide radical scavenging	0.606 ± 5.8	0.027± 4.90	--
Lipid peroxidation inhibition	0.253 ± 7.6	--	0.120 ±9.80
Hydroxyl radical scavenging	0.430 ± 5.0	--	0.032 ± 0.38
Nitric oxide scavenging	0.998 ± 2.3	0.067± 0.98	--
DPPH free radical scavenging	0.058 ± 2.9	0.003± 2.90	--

Values are mean ± SD, n=3

PPC-*Pr* was effective in inhibiting the lipid peroxidation induced by Fe^{2+}-ascorbate system in rat liver homogenate. The generation of malondialdehyde (MDA) and related substances that reacted with thiobarbituric acid was found to be inhibited by PPC-*Pr*. This indicated the significant lipid peroxidation inhibiting activity of PPC-*Pr*. The inhibitory effect of PPC-P*r* was increased with increasing concentrations (Figure 6.2.). The IC_{50} of PPC-P*r* required to inhibit the lipid peroxidation was found be 0.253 ± 7.6 mg/ml. The IC_{50} value of standard Vitamin E was 0.120 ± 9.8.

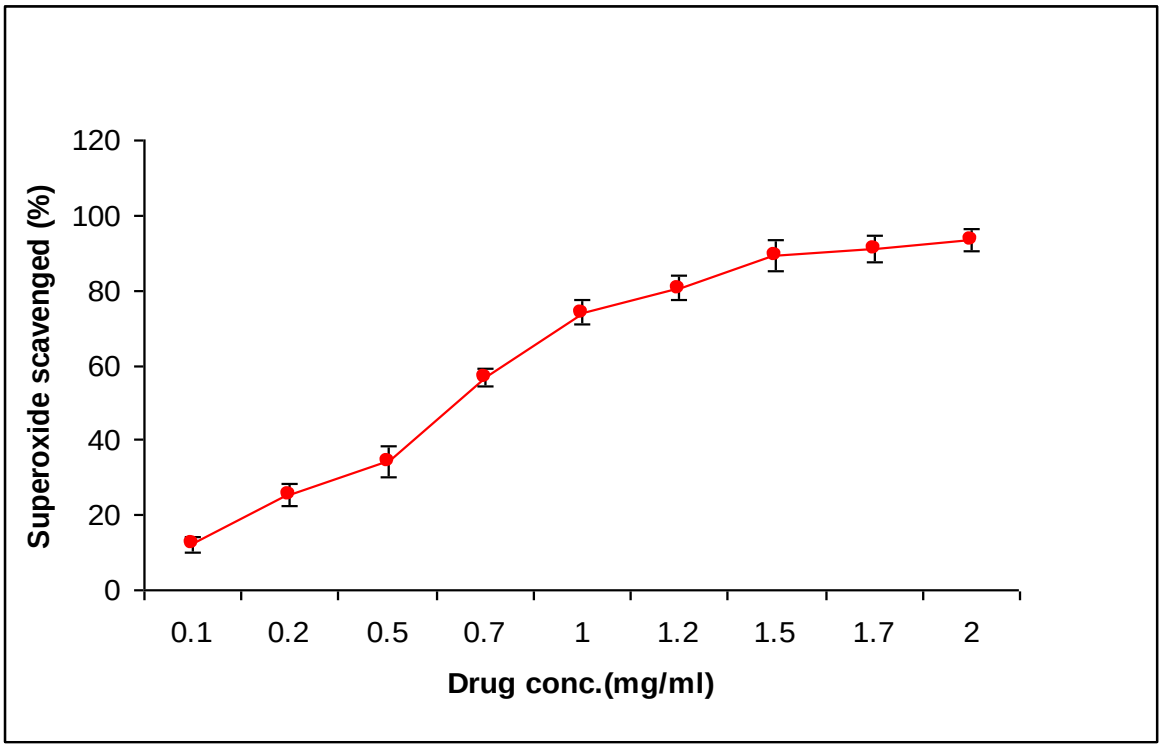

Figure 6.1. Superoxide radical scavenging activity of PPC-*Pr.* Values are mean ± SD, n=3.

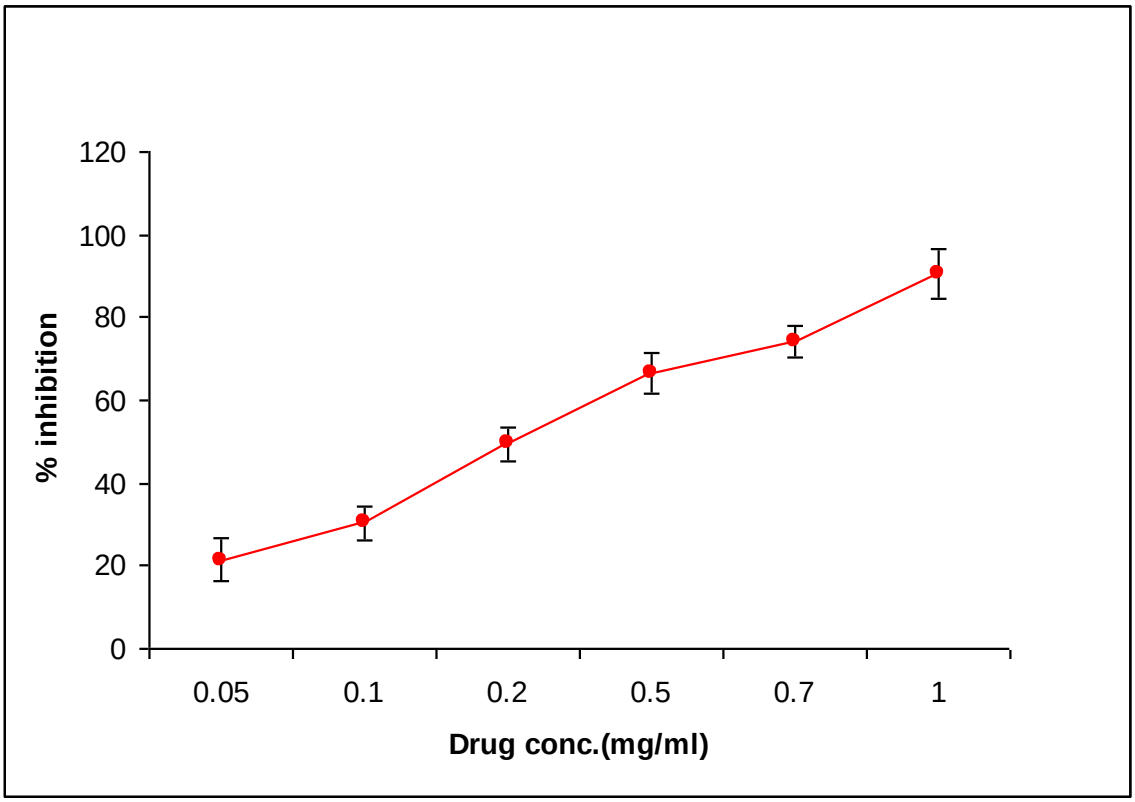

Figure 6.2. Inhibition of lipid peroxidation by PPC-P*r.* Values are mean ± SD, n=3.

The degradation of deoxyribose to TBARS by hydroxyl radical generated from Fe^{3+}-ascorbate-EDTA-H_2O_2 system was significantly decreased by the addition of PPC-P*r* (Figure 6.3.). The IC_{50} of PPC-P*r* required to scavenge the generated hydroxyl radical was found to be 0.430 ± 5.0 mg/ml. PPC-P*r* also inhibited the nitric oxide released form the sodium nitroprusside in a dose dependent manner (Figure 6.4.). The IC_{50} of PPC-P*r* required to scavenge the generated nitric oxide was found to be 0.998 ± 2.3 and that of standard Vitamin C was 0.067 ± 0.98.

The DPPH free radical scavenging activity of PPC-P*r* was tested with increasing concentrations and it was observed that PPC-P*r* possessed significant activity. PPC-P*r* at a concentration of 0.5 and 1 mg/ml showed 72.56% and 90.29% scavenging activity respectively (Figure 6.5.). The IC_{50} of PPC-P*r* in DPPH scavenging activity was found to be 0.058 ± 2.9. PPC-*Pr* when assayed for FRAP showed significant ferric reducing antioxidant ability. The ferric reducing anti-oxidant power of PPC-P*r* was found to increase in a concentration dependent manner (Figure 6.6.).

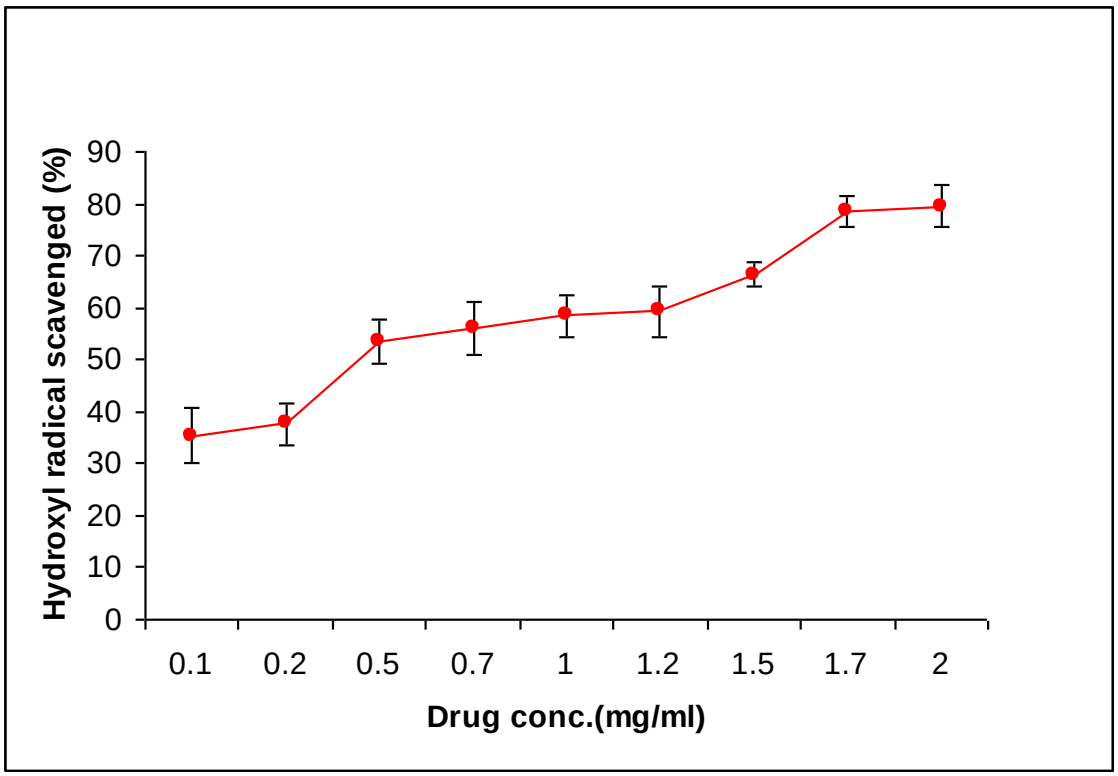

Figure 6.3. Hydroxyl radical scavenging activity of PPC-P*r*. Values are mean ± SD, n=3.

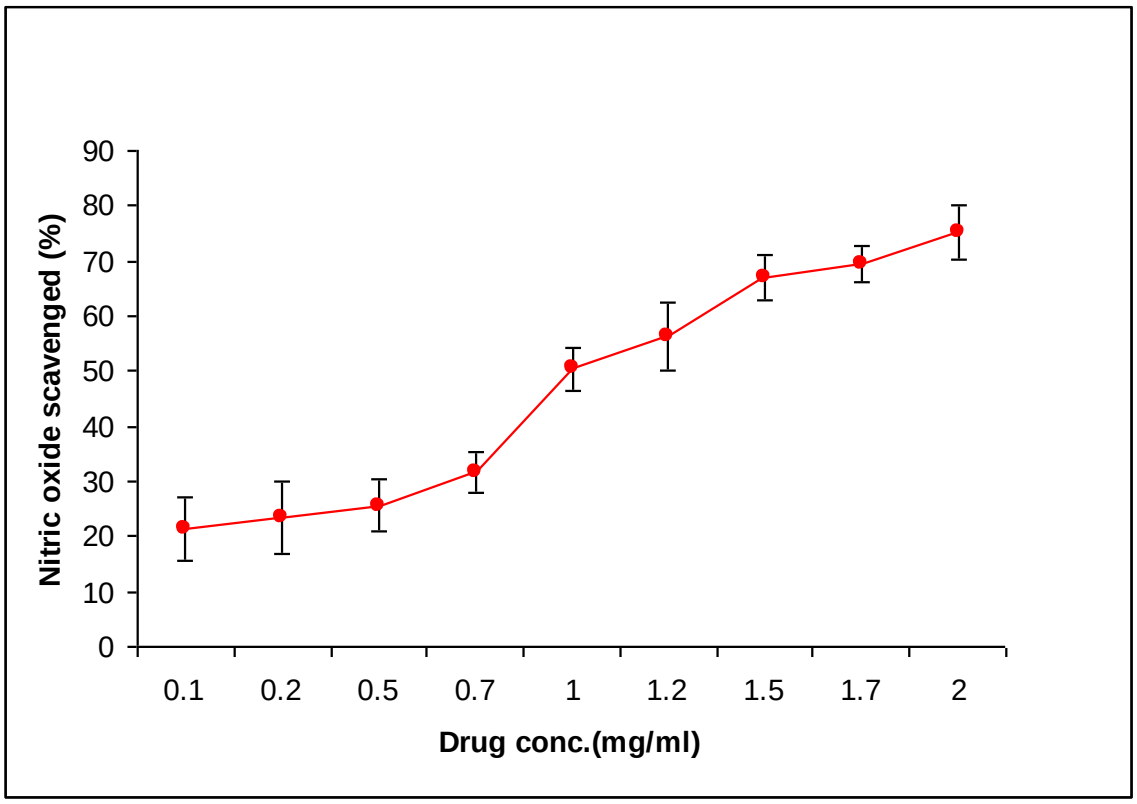

Figure 6.4. Nitric oxide radical scavenging activity of PPC-P*r*. Values are mean ± SD, n=3.

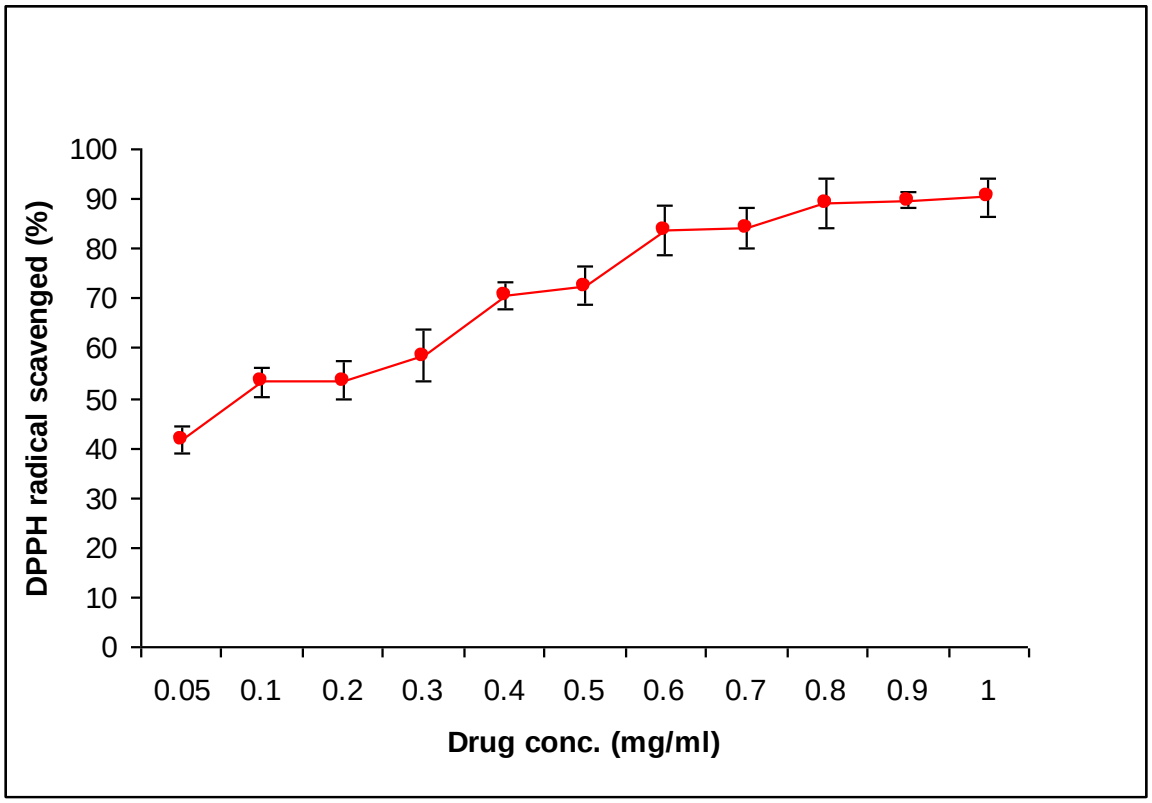

Figure 6.5. DPPH free radical scavenging activity of PPC-P*r*. Values are mean ± SD, n=3

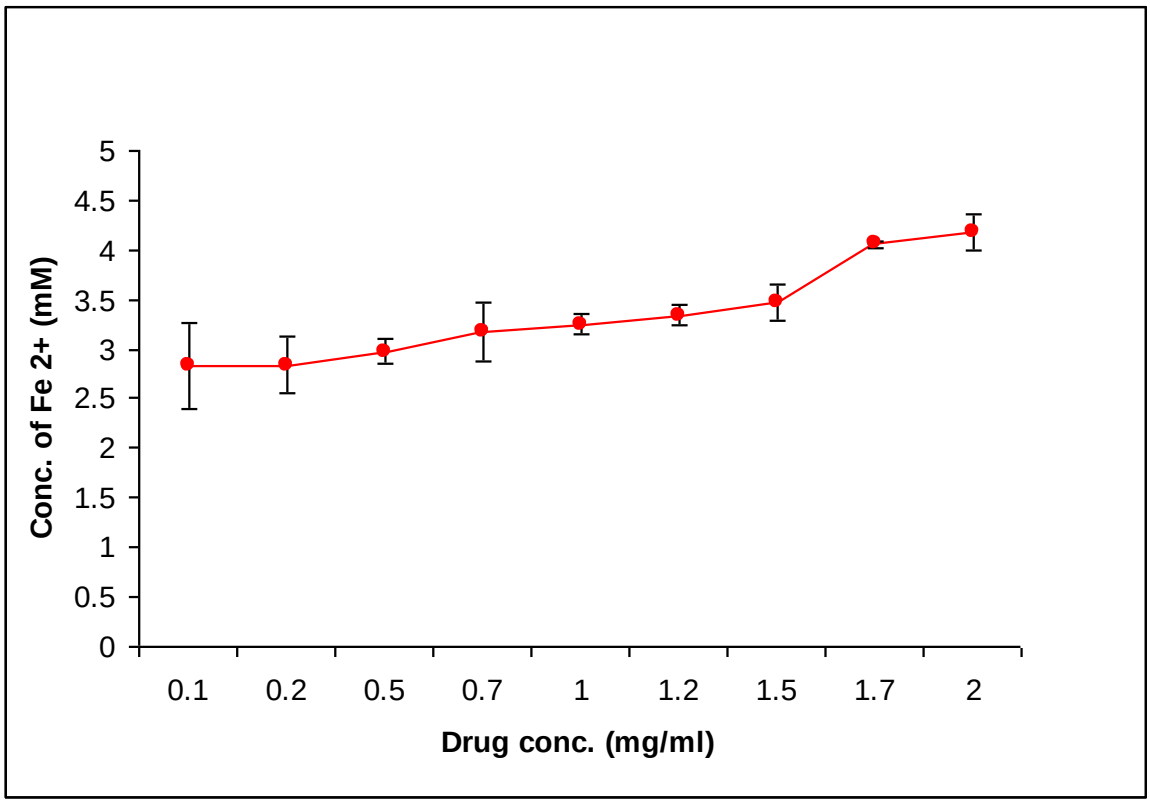

Figure 6.6. Ferric reducing antioxidant power assay of PPC-P*r.* Values are mean ± SD,n=3

6.4. Discussion

There is increasing evidence indicating that free radicals have a wide variety of pathological effects. The free radicals are species with very short half life, high reactivity and damaging activity towards macromolecules like proteins, DNA and lipids. These species may be either oxygen derived reactive oxygen species (ROS) or nitrogen derived reactive nitrogen species (RNS). Superoxide and hydroxyl radicals are the two most representative free radicals. Superoxide radical is normally formed first, and its effects can be magnified because it produces other kinds of free radicals and oxidizing agents. However, the damaging action of hydroxyl radical is the strongest among free radicals. Many synthetic chemicals such as phenolic compounds are found to be strong radical scavengers, but they usually have side effects (Liu et al., 1997). Lipid peroxidation which involves a series of free radical-mediated chain reaction processes is also associated with several types of pathological manifestations (Perry et al., 2000).

Non- enzymatic antioxidants react with pro-oxidants and inactivate them. In this redox reaction, antioxidant acts as a reductant. In this context, the antioxidant power can be referred to as reducing ability. In FRAP assay, an easily reducible oxidant FeIII is used in excess. On reduction of Fe III- TPTZ complex by antioxidant, Fe II- TPTZ is formed which can be measured spectrophotometrically at 595 nm (Benzie and Strain, 1996). Another mode of action of antioxidants is to scavenge free radicals to suppress chain initiation and /or break the chain propagation reactions. In the DPPH assay, the ability of antioxidant to scavenge stable purple colored primary DPPH radical is assayed (Aquino et al., 2001).

Present study revealed the profound antioxidant activity of PPC-*Pr* isolated from *P.rimosus*. PPC-*Pr* was found to be effective in scavenging superoxide, hydroxyl,nitric oxide radicals and also the DPPH free radical. It significantly inhibited the lipid peroxidation and FRAP assay showed the reducing ability of PPC-*Pr*. The association of protein with the polysaccharide molecules is known to potentiate their free radical scavenging activity. For example, lentinan and schizophyllan, which contain only trace amounts of peptides in the polysaccharide samples, have almost no scavenging effect, whereas krestin and polysaccharopeptide, which are obtained from mushrooms, such as *Ganoderma lucidum* and *Grifola umbellate* which have lower polysaccharide/ peptide ratios, exhibited the strongest scavenging effects (Liu et al., 1997). This indicates that protein bound polysaccharides have higher free radical scavenging activity than polysaccharides alone. Hence, the protein bound nature of PPC-P*r* might be contributing significantly for its profound free radical scavenging activity.

Mushrooms usually contain a wide variety of free radical scavenging molecules, such as polysaccharides and polyphenols (Liu et al., 1997; Mau et al., 2002). The mechanism underlying the free radical scavenging activity exerted by polysaccharides is still not fully understood. Tsiapali et al., (2001) speculated that the abstraction of anomeric hydrogen from monosccharides was the reason for the free radical scavenging ability. Polysaccharides enhanced antioxidant activity over monosaccharides because polysaccharides exhibited the greater ease of abstraction

of the anomeric hydrogen from one of the internal monosaccharide units. Hence, the antioxidant mechanism may be due to the supply of hydrogen by PPC-P*r*, which combines with radicals and itself forms a stable radical to terminate the radical chain reaction. The other possibility is that PPC-P*r* can combine with the radical ions that are necessary for radical chain reaction; then the reaction is terminated. However, this needs further confirmation by adequate experimental results.

Chapter - 7

Anti-inflammatory and in vivo anti-oxidant activities of PPC-*Pr*

Synopsis

The anti-inflammatory activity of the polysaccharide-protein complex (PPC-*Pr*) from *Phellinus rimosus* (Berk) Pilat was determined by carrageenan, dextran induced acute and formalin, Freund's complete adjuvant (FCA) induced chronic inflammatory models. The PPC- Pr showed strong anti-inflammatory activity in both the acute and chronic inflammatory models, comparable to the activity of clinically used standard drug, diclofenac. Free radicals have long been implicated in change of connective tissues in inflammation. Improperly regulated and prolonged chronic states of inflammation can lead abnormalities in cell growth and developing tumors. In the present study, in vivo anti-oxidant status in FCA induced chronic inflammation in rats was evaluated. Lipid peroxides formation as well as altered levels of endogenous scavengers like superoxide dismutase, glutathione peroxidase and reduced glutathione were taken as an indirect *in vivo* evidence for the participation of free radicals in the progression of chronic inflammation. There was a significant increase in lipid peroxide level in plasma of FCA induced control rats. The antioxidant enzymes superoxide dismutase and glutathione peroxidase were also elevated in adjuvant induced rats. Blood glutathione was decreased in control group. The treatment with various concentrations of PPC-*Pr* reduced the above alterations produced in FCA induced animals in a dose dependent manner. In this respect, PPC-*Pr* with least side-effects can be a possible alternative for non-steroidal anti-inflammatory drugs (NSAID's) which are widely used clinically for inflammatory diseases.

7.1. Introduction

Acute inflammation is an immediate response of living tissue towards local injury. The response is initiated by a variety of chemical mediators, which are released in the injured tissue (Ryan and Majno, 1977; Sin and Ang, 1990; Sin et al., 1985). The action of these mediators would result in plasma exudation and emigration of peripheral blood leucocytes into the injured area. Active

phagocytosis occurs in the inflamed tissue (Sin et al., 1984) and thereby generates toxic-free radicals to destroy phagocytosed materials (Biemond et al., 1986; Janoff and Carp, 1982). However, excessive production of the free radicals if released can be deleterious, causing problems such as rheumatoid arthritis where a high level of oxidants is present in the synovial fluid (Biemond et al., 1986).

Chronic inflammation may develop either as a progression from acute inflammation if the original stimulus persists or after repeated episodes of acute inflammation. Etiological agents that can produce chronic inflammation include infectious organisms that can avoid or resist host defense mechanisms and so persist in the tissue for a prolonged period such as *Mycobacterium tuberculosis* that is able to survive within phagocytic cells (Kusner, 2005). When improperly regulated, chronic states of inflammation lead to various interconnected abnormalities such as local and systemic immunosuppression, persistent tissue damage, opportunistic infections, abnormalities in cell growth and developing tumors. Free radicals have also been implicated in damages of connective tissues in inflammation.

Production of free radicals as superoxide, oxygen radicals, nitric oxide and hydrogen peroxide (commonly designated as reactive oxygen species, ROS) was shown to play an important role in tissue damage and loss of function in a number of tissues and organs. Although the organisms possess efficient free radical scavenging mechanisms and anti-ROS defense systems, in a number of circumstances the balance between ROS production and elimination is displaced in favour of the damaging actions of ROS. This is the case in a number of inflammatory processes as well as in age-associated diseases as pulmonary emphysema or atherosclerosis. Excessive production of free radicals if released can be deleterious, causing problems such as rheumatoid arthritis, a chronic inflammatory state, where a high level of oxidants is present in the synovial fluid (Biemond et al., 1986). At present, pharmacological treatments for inflammatory disorders are generally palliative and antisymptomatic and none is effective at treating the underlying pathology. Hence such treatments are incapable of affecting the progression of the disease. Therefore, there is need for alternative

therapies help to treat the painful symptoms of inflammation, and possibly slow disease progression.

No information is available on the anti-inflammatory activity of polysaccharides or polysaccharide-protein complex isolated from *P.rimosus.* The polysaccharide-protein complex (PPC-*Pr*) from *Phellinus rimosus* showed strong anti-inflammatory activity in both the acute and chronic inflammatory models. PPC-*Pr* was also found to possess significant in vivo anti-oxidant activity in FCA induced chronic mice. Results of the study are given in this chapter.

7.2. Materials and methods

7.2.1. Isolation of PPC-*Pr* from *P.rimosus*

PPC-*Pr* was isolated from the hot water extract of *P.rimosus* as described in section 4.2.1.

7.2.2. Test animals

Female Swiss albino mice (6-8 weeks old) of weight 20 ± 5g were used for the dextran, carrageenan and formalin induced acute and chronic inflammatory models. For Freund's complete adjuvant (FCA) induced chronic inflammatory model, female Wistar albino rats weighing 130-150g were used.

7.2.3. Determination of anti-inflammatory activity

7.2.3.1. Carrageenan induced acute paw oedema model

Animals were divided into 4 groups of six animals each group. Pedal inflammation in Swiss albino mice was induced by subcutaneous injection of 20µl of freshly prepared 1% suspension of carrageenan in normal saline into the right hind paw of the mice (Winter et al., 1962). The paw thickness was measured using vernier calipers before and 3 hr after carrageenan challenge in each group. Animals were pre-medicated intraperitonially 1hr before the induction of oedema. Group1, which served as control received only the vehicle (normal saline). Group 2 served as positive control and received the reference drug Diclofenac at a dose of 10 mg/kg. Group 3 & 4 received PPC-*Pr* at a dose of 25mg/kg and 50 mg/kg respectively.

7.2.3.2. Dextran induced acute paw oedema model

Animals were treated as in the case of Carrageenan induced paw oedema

model, except that instead of carrageenan, dextran was used to induce the oedema (Ajith and Janardhanan, 2001).

7.2.3.3. Formalin induced chronic paw oedema model

Animals were treated as in the above models, except that 20 µl of freshly prepared 2% formalin was used as oedematogenic agent and the drug treatment was continued for 6 consecutive days (Ajith and Janardhanan, 2001).

7.2.3.4. Freund's complete adjuvant (FCA) induced chronic inflammatory model

All groups were administered with the mentioned doses of drugs intraperitonially. First dose was given one hour prior to the induction of chronic inflammation. Chronic inflammation was induced in rats by injection of 0.1 ml of Freund's complete adjuvant (FCA) into the sub planar region in the right hind paw (Gokhale et al., 2002). The administration of the drug was continued once daily for 6 days. The thickness of the injected paws was measured using vernier calipers before and every 3 days after the induction of arthritis till day 21. On day 21, the severity of the secondary lesions is evaluated visually and scored.

In all the above models the degree of oedema formation was assayed as increase in paw thickness. The increase in paw thickness and percent inhibition were calculated as follows:

Increase in paw thickness in control group/ treated group (P_C/P_T) = Pt-Po

$$\text{Percent inhibition} = \frac{PC - P_T \times 100}{P_C}$$

where Pt is paw thickness at time t, Po is initial paw thickness, P_C is the increase in paw thickness of the control group and P_T is the increase in paw thickness of the treated group.

7.2.4. Determination of in vivo anti-oxidant activity

In vivo anti-oxidant status was studied in FCA induced chronic rats. At the end of the experimental period, the animals were killed under anaesthesia and blood samples were collected by heart puncture. The chosen biochemical markers were plasma levels of lipid peroxides as well as the activities of the enzymes

Superoxide dismutase (SOD), Glutathione peroxidase (GPx) and reduced Glutathione (GSH) in blood.

7.2.4.1. Determination of lipid peroxidation in plasma

Lipid peroxidation in plasma was determined as thiobarbituric acid-reacting substances (TBARS) according to the spectrophotometric method of Ohkawa et al., (1979), after precipitating the protein according to the method of Satoh (1987). Lipids were isolated by precipitating them with plasma protein using 0.02 % trichloroacetic acid (TCA). To 0.5 ml plasma, 2.5 ml of 0.02 % TCA was added and the tube was left to stand for 10 min at room temperature. After centrifugation at 3500 rev/min for 10 min, the precipitate was washed once with 0.05 M H_2SO_4. The precipitate was suspended in 1 ml distilled water. The level of lipid peroxidation was measured as malondialdehyde (MDA) by reacting with TBA in acetic acid solution as described in section 6.2.3. The result was expressed as nmol/ ml of plasma.

7.2.4.2. Determination of Superoxide dismutase (SOD) activity in blood

SOD activity in blood was determined according to the method of McCord and Fridovich (1969), after removing the haemoglobin by the method of Miami and Yoshikawa (1979). 0.1 ml of the heparinised blood was haemolysed by 0.9 ml of cold water at 4° C. The haemolysate was treated with 0.25 ml of chloroform ($CHCl_3$) and 0.5 ml of ethanol with vigorous mixing to remove the haemoglobin. The mixture was centrifuged at 15,000 rpm for 60 min. The 0.025 ml of the clear supernatant was used for SOD assay. The reaction mixture contained, 0.025 ml of the clear supernatant, EDTA (6mM) containing 3µg NaCN; riboflavin (2µg); NBT (50µg); KH_2PO_4-Na_2HPO_4 buffer (67mM, pH 7.8) and various concentrations of PPC-*Pr* in a final volume of 3 ml. The tubes were illuminated under incandescent lamp for 15 min. The optical density (OD) at 530 nm was measured before and after illumination. The inhibition of superoxide radical generation was determined by comparing the absorbance values of the control with that of treatments. The volume of the sample required to scavenge 50% of the generated superoxide anion was considered as 1 unit of enzyme activity and was expressed as U/g Hb.

7.2.4.3. Determination of reduced glutathione (GSH) content in blood

GSH was determined after preparing a haemolysate of blood in distilled water. A 20 % haemolysate of heparinised blood was prepared in distilled water. 0.3 ml of the sample was mixed with 0.7 ml of 0.2 M sodium phosphate buffer (pH 8) and 0.6 mM dithionitrobenzene (DTNB) (prepared in 0.2 M buffer, pH 8). The acid soluble sulfhydryl groups (non-protein thiols of which more than 93 % is reduced glutathione) form a yellow colored complex with DTNB. After 10 min, absorbance of the yellow colored complex was measured at 412 nm against a blank which contained 0.3 ml of 5% TCA in place of the sample (Moron et al., 1979). A standard graph was prepared using different concentrations (10-50 nmols) of GSH in 0.3 ml of 5 % TCA. The GSH content was calculated with the help of this standard graph and expressed as nmol/mg protein.

7.2.4.4. Determination of glutathione peroxidase (GPx) activity in blood

The activity of GPx was determined according to the method of Hafemann et al., (1974). 0.02 ml of heparinised blood was treated with 0.1 ml of 5 mM GSH, 0.1 ml of 1.25 mM H_2O_2, 0.1 ml of 25 mM NaN_3 and phosphate buffer (0.05 mM, pH 7) in a total volume of 2.5 ml at 37° C for 10 min. The reaction was stopped by adding 2 ml of 1.65 % HPO_3^{2-} and the reaction mixture was centrifuged at 1500 rpm for 10 min. 2 ml of the supernatant was mixed with 2 ml of 0.4 M Na_2HPO_4 and 1 ml of 1mM DTNB. The absorbance of the yellow colored complex was measured at 412 nm after incubation for 10 min at 37° C, against distilled water. One unit of enzyme activity is defined as decrease in log GSH by 0.001/min after subtraction of the decrease in log GSH/min for the non-enzymatic reaction and is expressed as U/g Hb.

7.3. Results

The PPC-*Pr* showed significant anti-inflammatory activity against acute and chronic models of inflammation in a dose dependent manner (Table 7.1.). In both the carrageenan and dextran acute inflammatory models, the activity of PPC-*Pr* at a dose of 25 & 50 mg/kg was found to be higher than that of the reference drug, diclofenac at a dose of 10 mg/kg (Figure 7.1. and 7.2.). The PPC-*Pr* was also effective in ameliorating formalin induced chronic inflammation ($P<0.001$).

The inhibitory activity of PPC-*Pr* at a dose of 50 mg/kg was found to be higher than the activity of reference drug, diclofenac (Figure 7.3.).

Table 7.1. Effect of PPC-*Pr* on carrageenan, dextran, formalin and Freund's complete adjuvant induced paw oedema models

Groups	**Treatment** (mg/kg) i.p.	**Initial paw thickness** (cm)	**Final paw thickness** (cm)	**Increase in paw thickness** (cm)	**Percent inhibition (%)**
Carrageenan model [a]					
Control	Normal saline	0.255±0.013	0.427±0.034	0.175± 0.039	--
Diclofenac	10	0.235± 0.008	0.340± 0.017	0.105±0.021*	40
PPC-*Pr*	25	0.235± 0.010	0.313 ±0.025	0.078±0.023*	55.24
PPC-*Pr*	50	0.232 ±0.008	0.275 ±0.012	0.043 ±0.008*	75.24
Dextran model [b]					
Control	Normal saline	0.215±0.006	0.343± 0.009	0.128± 0.013	--
Diclofenac	10	0.225±0.006	0.288± 0.005	0.063± 0.005*	50.78
PPC-*Pr*	25	0.213±0.005	0.256± 0.005	0.045± 0.006*	64.84
PPC-*Pr*	50	0.223±0.015	0.238± 0.009	0.015± 0.006*	88.28
Formalin model [c]					
Control	Normal Saline	0.243±0.008	0.423±0.030	0.180± 0.032	--
Diclofenac	10	0.235± 0.010	0.332± 0.016	0.097± 0.024*	46.29
PPC-*Pr*	25	0.227± 0.005	0.327 ±0.015	0.100±0.020*	44.44
PPC-*Pr*	50	0.218 ±0.008	0.280 ±0.028	0.062 ±0.034*	65.74
Frund's Complete Adjuvant model [d]					
Control	Normal Saline	0.350±0.008	0.735± 0.013	0.385± 0.019	--
Diclofenac	10	0.350±0.008	0.583± 0.009	0.233± 0.017*	39.61
PPC-*Pr*	25	0.332±0.011	0.524± 0.011	0.208± 0.016*	45.97
PPC-*Pr*	50	0.334±0.013	0.490± 0.016	0.156± 0.022*	59.48

Values are mean ± SD, n = 6; [a, b] - Paw thickness after 3 hrs; [c] - Paw thickness on day 6;
[d] - Paw thickness on day15; * p values <0.001 compared to control

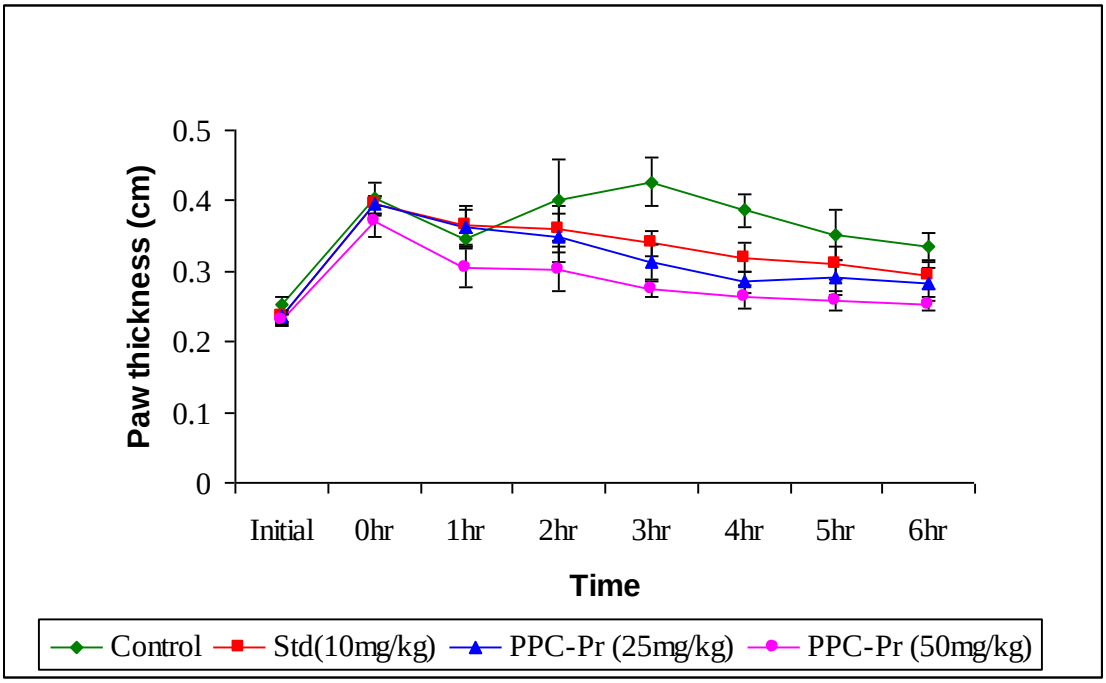

Figure 7.1. Inhibition of carrageenan induced acute inflammation by PPC-*Pr*. Control group received vehicle (normal saline) only. Diclofenac at a dose of 10 mg/kg was used as standard. PPC-*Pr* at dose of 25 and 50 mg/kg was administered as i.p. 1 hr before the induction of oedema. Values are mean ± SD, n=6.

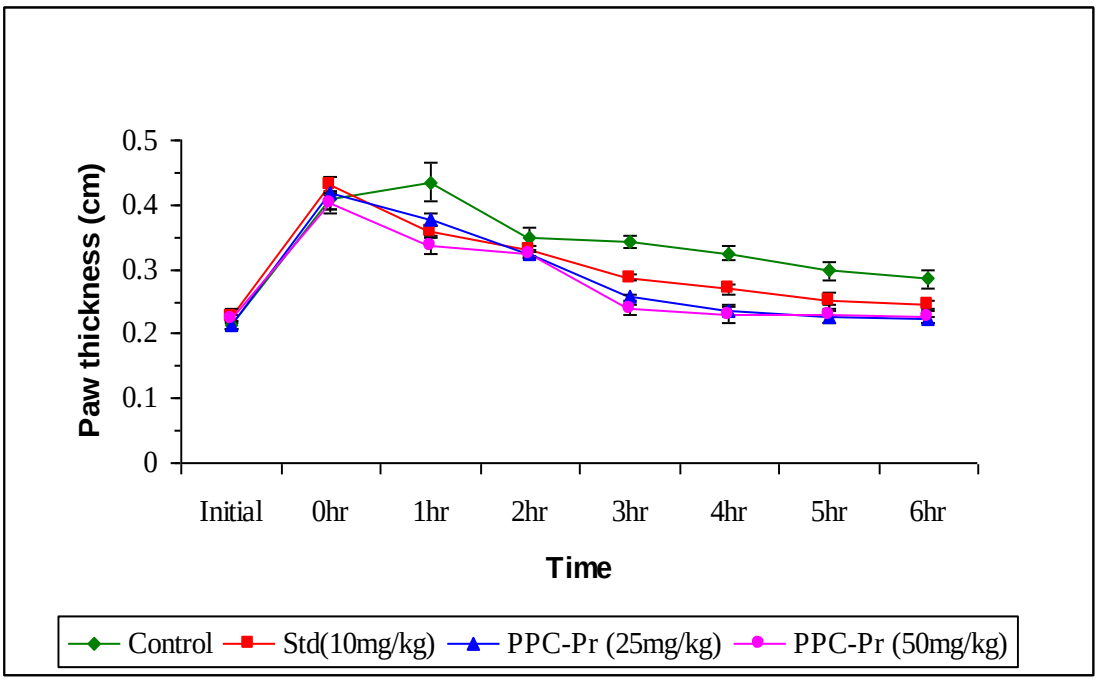

Figure 7.2. Inhibition of dextran induced acute inflammation by PPC-*Pr*. Control group received vehicle (normal saline) only. Diclofenac at a dose of 10 mg/kg was used as standard. PPC-*Pr* at dose of 25 and 50 mg/kg was administered as i.p. 1 hr before the induction of oedema. Values are mean ± SD, n=6.

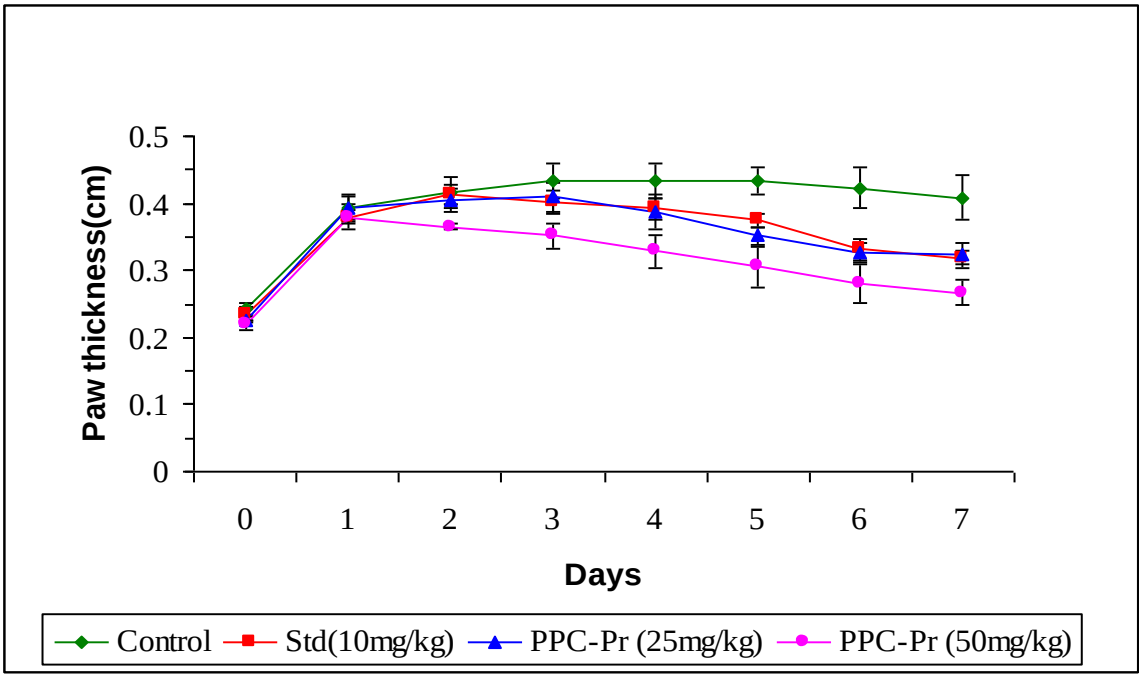

Figure 7.3. Inhibition of formalin induced chronic inflammation by PPC-*Pr*. Control group received vehicle (normal saline) only. Diclofenac at a dose of 10 mg/kg was used as standard. PPC-*Pr* at dose of 25 and 50 mg/kg was administered as i.p. 1 hr before the induction of oedema and continued for 6 consecutive days. Values are mean ± SD, n=6.

The present investigation also showed that PPC-*Pr* possessed significant activity against FCA indued chronic inflammation, in a dose dependent manner (Table 7.1.). Sub planar injection of Freund's complete adjuvant in the rat hind paw led to the development of inflammation which reached a peak oedema on day 15 of the injection. The reference drug, diclofenac, was found to inhibit this oedema to an extent of 39.61%. The test compound, PPC-*Pr* at the doses of 25 & 50 mg/kg, i.p. showed 45.97% and 59.48% inhibition respectively (Figure 7.4.). PPC-*Pr* was also found to be effective in reducing the development of the secondary lesions in arthritic rats (Table 7.2.). Figure 7.5. shows the effect of PPC-*Pr* on lipid peroxidation in plasma of the control and experimental groups. FCA reagent administration to rats was accompanied by a four fold increase in MDA level in control group as compared to the normal non-arthritic group. The above changes were altered with the administration of different doses of PPC-*Pr* and the plasma lipid peroxide level was almost normalized. Figure 7.6 and Figure. 7.7 represent the effect of antioxidant enzymes in blood. The enzymes SOD and

GPx were significantly increased (P<0.01) in control. Treatment with PPC-*Pr* significantly reduced the level of enzymes. The effect produced by PPC-*Pr* on GPx at a dose of 50 mg/kg was even better, since the level of enzyme was brought almost to nearly that of the normal rats. Changes in the reduced glutathione in blood are presented in Figure 7.8. Reduced glutathione was reduced significantly in the blood of control group (P<0.01). Administration of PPC-*Pr* at a dose of 50 mg/kg elevated the glutathione level to that of the normal group.

Table 7.2. Number of Freund's complete adjuvant- induced arthritic rats with developed secondary lesions

Groups	Treatment (mg/kg) i.p.	Redness on ears	Swelling on nose	Inflammation on forepaws	Inflammation on hind paw other than induced	Marked inflammation on induced hind paw
Control	Normal Saline	2/6	3/6	3/6	3/6	6/6
Diclofenac	10	1/6	0/6	2/6	2/6	2/6
PPC-*Pr*	25	0/6	1/6	2/6	2/6	2/6
PPC-*Pr*	50	0/6	0/6	2/6	1/6	1/6

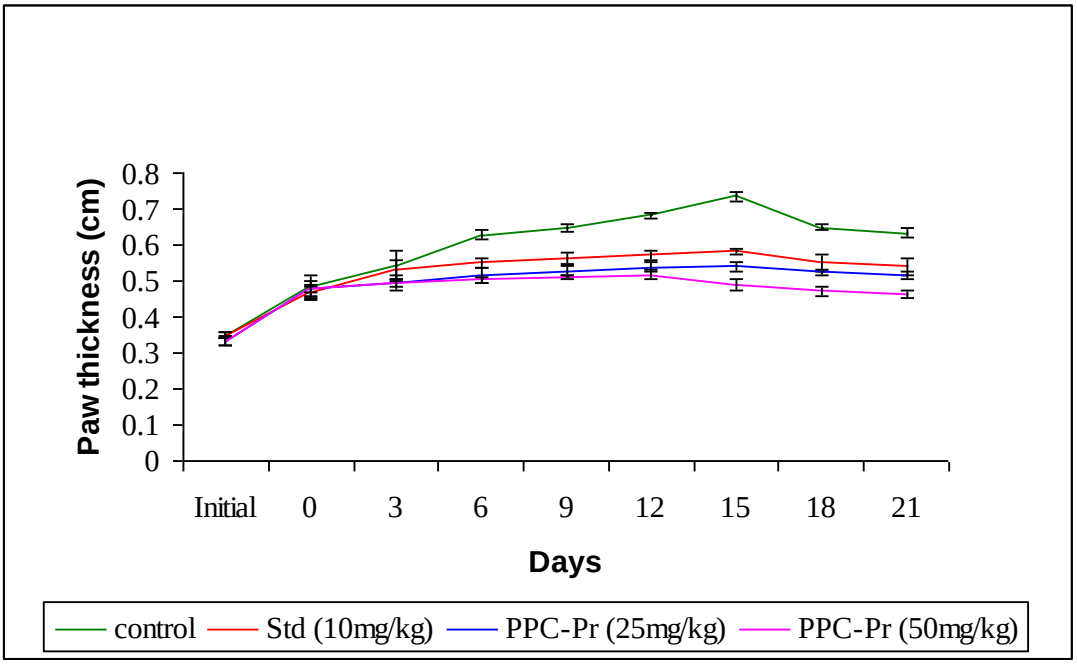

Figure 7.4. Inhibition of Freund's complete adjuvant induced chronic inflammation by PPC-*Pr*. Control group received vehicle (normal saline) only. Diclofenac at a dose of 10 mg/kg was used as standard. PPC-*Pr* at dose of 25 and 50 mg/kg was administered as i.p. 1 hr prior to the

induction of oedema and continued once daily for 6 days. Values are mean ± SD, n = 6. * p values < 0.01 in comparison to control.

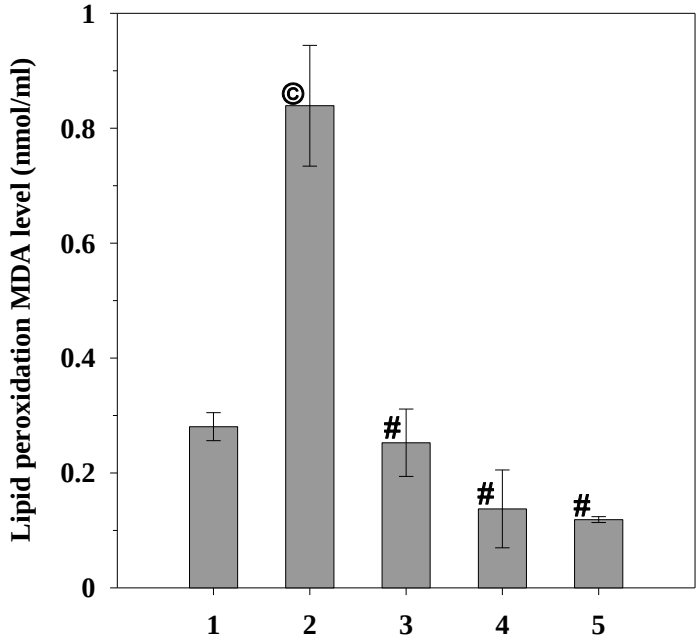

Figure 7.5. Effect of PPC-*Pr* on lipid peroxidation in plasma of normal and experimental rats. Labels on the X-axis as follows: Normal group (1); control group (2); FCA treated group with diclofenac 10 mg/kg (3); PPC-*Pr* 25 mg/kg (4) and PPC-*Pr* 50 mg/kg (5). Values are mean ± SD for six animals in each group. © represents significant difference from the normal group at P <0.01; # significant difference from the control group at P <0.01.

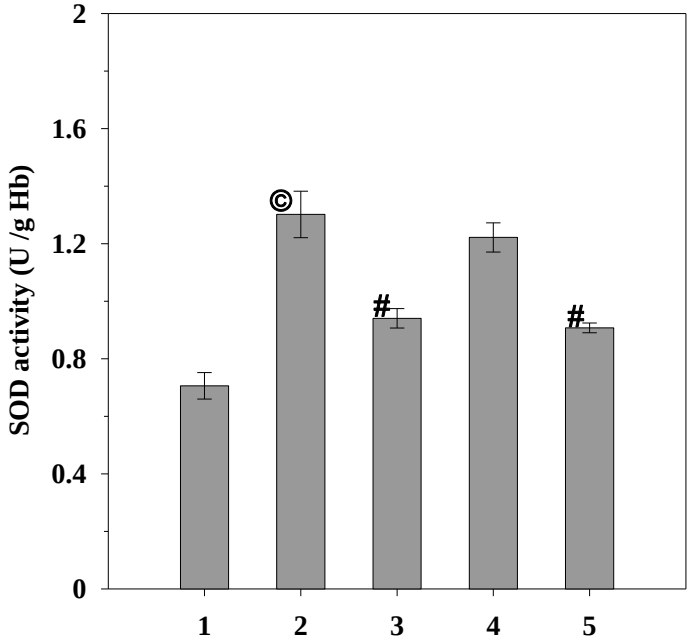

Figure 7.6. Effect of PPC-*Pr* on Superoxide dismutase activity in blood of normal and experimental rats. Labels on the X-axis as follows: Normal group (1); control group (2); FCA treated group with diclofenac 10 mg/kg (3); PPC-*Pr* 25 mg/kg (4) and PPC-*Pr* 50 mg/kg (5). Values are mean ± SD for six animals in each group. © represents significant difference from the

normal group at P <0.01; # significant difference from the control group at P <0.01.

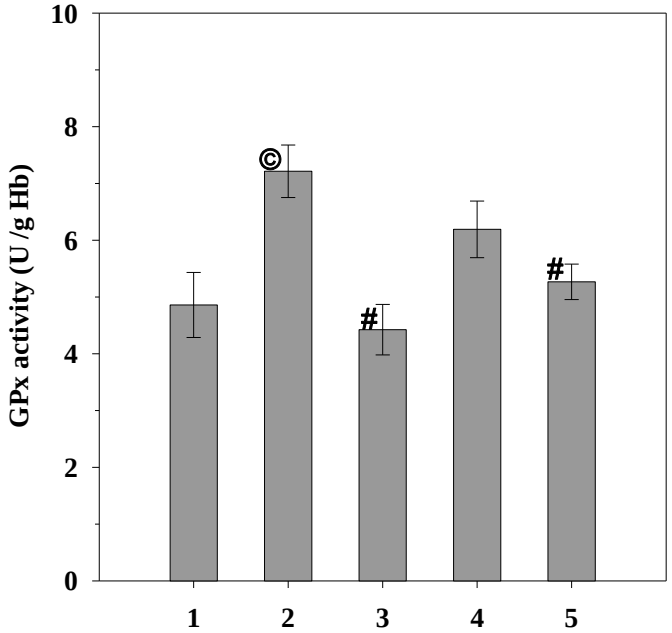

Figure. 7.7. Effect of PPC-*Pr* on Glutathione peroxidase activity in blood of normal and experimental rats. Labels on the X-axis as follows: Normal group (1); control group (2); FCA treated group with diclofenac 10 mg/kg (3); PPC-*Pr* 25 mg/kg (4) and PPC-*Pr* 50 mg/kg (5). Values are mean ± SD for six animals in each group. © represents significant difference from the normal group at P <0.01; # significant difference from the control group at P <0.01.

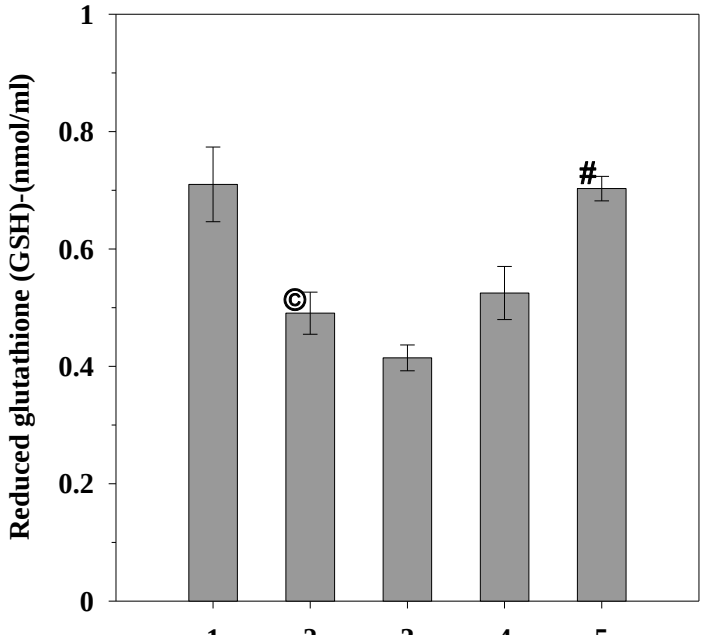

Figure. 7.8. Effect of PPC-*Pr* on reduced glutathione in blood of normal and experimental rats. Labels on the X-axis as follows: Normal group (1); control group (2); FCA treated group with diclofenac 10 mg/kg (3); PPC-*Pr* 25 mg/kg (4) and PPC-*Pr* 50 mg/kg (5). Values are mean ± SD for six animals in each group. ©

represents significant difference from the normal group at P <0.01; # significant difference from the control group at P <0.01.

7.4. Discussion

The development of carrageenan induced oedema is biphasic; the first phase is attributed to the release of histamine, 5-HT and kinins, while second phase is related to the release of prostaglandins (Brooks and Day, 1991). Dextran induced paw oedema is known to be mediated both by histamine and serotonin. Formalin induced paw oedema is one of the most suitable methods to screen chronic anti-inflammatory agents as it closely resembles human arthritis (Gutteridge et al., 1983). The nociceptive effect of formalin is also biphasic, an early neurogenic component followed by a later tissue mediated response.

Freund's adjuvant induced chronic inflammation shares many features of human rheumatoid arthritis. It involves most of the joints and associated tissues. Free radicals have long been implicated in change of connective tissues in inflammation and arthritis. Oxygen free radicals and H_2O_2 are closely involved in the pathogenesis of rheumatoid arthritis. Granulocytes are strongly increased in number in this disease; they produce large amounts of $\cdot O_2^-$ and H_2O_2 during phagocytosis of immune complexes and other materials. Oxygen free radicals are responsible for at least a part of the joint destruction (Biemond et al., 1984). FCA induced inflammation also produces secondary lesions after a delay of approximately 11 to 12 days which are characterized by inflammation of non-injected sites, a decrease of weight and immune response.

Free radicals are continuously generated in almost all aerobic cells. If they are not scavenged by the comprehensive array of endogenous antioxidant mechanisms, tissue damage occurs. ROS are the mediators of inflammation and through their interaction with platelets, neutrophils, macrophages and other cells can involve the synthesis of eicosanoids and the activation and release of various cytokines, propagating the inflammatory process from one organ system to another. This results in tissue oxidative stress and multiple-system organ failure (Parke and Parke, 1995). Recent studies have shown that inflammation of many of the tissues is accompanied by up regulation of an inducible isoform of nitric oxide (NO) synthase (iNOS)[1], that is capable of producing excess NO for a prolonged period (Lirk et al., 2002). Although host defense and cytoprotective functions of

NO have been demonstrated (Granger et al., 1988; James, 1995; Alam et al., 2002; Nathan and Shiloh, 2000; Mannick et al., 1994), accumulating evidence indicates that NO-derived reactive nitrogen species (RNS) such as peroxynitrite ($ONOO^-$), nitroxyl (HNO), N_2O_3 and nitrogen dioxide ($^{\cdot}NO_2$) also have pathogenic potential in various diseases (Beckman and Koppenol, 1996; Rubbo et al., 1996; Stamler et al., 1992), possibly through induction of oxidative, nitrative and nitrosative damage to nucleic acids, proteins and lipids. It has been proposed that DNA and tissue damage induced by RNS may contribute to increased mutation rates, genome instability, apoptosis and associated tissue regeneration and cell proliferation, all of which can drive carcinogenesis.

It has been reported that inflammatory stimuli trigger an oxidative burst, resulting in the formation of several reactive oxygen species, namely, superoxide anion, hydrogen peroxide, hydroxyl radical and singlet oxygen. Once generated, free radicals provoke deleterious effects on various cellular targets, foremost among which are membrane lipids, leading to an extensive process of lipid peroxidation (Symons et al ., 1988). In the present study, lipid peroxides formation as well as altered levels of endogenous scavengers were taken as an indirect in vivo evidence for the participation of free radicals in the progression of inflammation. Increment in level of lipid peroxides in synovial fluid, serum and tissue of arthritic animals as well as patients suffering from arthritis has been demonstrated. Furthermore, aggravation of arthritis was shown to be associated with enhancement of lipid peroxidation (Symons et al ., 1988). SOD can change superoxide anion radical ($\cdot O_2^-$) to hydrogen peroxide. GPx is also a detoxifying enzyme, changing the peroxides to water. An increase in SOD has been observed in control FCA rats (Figure 7.6.). Increased production of NADPH from HMP shunt during chronic inflammation may cause an increase in SOD activity. This increase in enzyme activity appears to be protective against the extracellular oxygen free radicals. GPx protects the cell against damage resulting from the increased disease-associated peroxides by enhancing the GPx catalysed peroxide destruction (Kasama et al., 1988). The enhanced GPx activity observed is presumably due to the increased level of lipid peroxides that has been detected in

the present study. Administration of PPC-*Pr* to FCA induced rats caused a significant decrease in elevated SOD and GPx activities. In this study, blood GSH content of control was reduced considerably compared to the normal. The large difference points to the great consumption of GSH to counteract free radicals produced during chronic inflammation. Decrease in blood GSH level observed in control FCA rats may be due to increase in GPx activity which removes peroxides. GSH is not being replaced adequately, since glutathione reductase activity is affected. Hence the redox balance of the cell is upset and cell damage results (Braven et al., 1989). Administration of PPC-*Pr* to FCA induced rats caused a significant elevation in GSH level. Thus, our results indicate that the in vivo antioxidant activity of PPC-*Pr* might be, at least in part, an important component of its anti-inflammatory properties.

A number of non-steroidal anti-inflammatory drugs (NSAID's) are widely used clinically to treat inflammatory disease conditions. Despite their great number, their therapeutic efficacy seems to be hampered by the association of a number of undesired, and often serious, side effects. Therefore, natural products with very little side-effects are desirable to substitute these synthetic compounds for therapeutic use. Protein bound polysaccharides appear to be nontoxic in prolonged use and are claimed to benefit general health. In this context, the PPC-P*r* isolated from *P.rimosus* can be a suitable candidate for the treatment of inflammatory diseases.

Chapter- 8

Cytotoxic, anti-proliferative and apoptotic activities of PPC-*Pr*

Synopsis

In this study, the in vitro short-term cytotoxic activity against Dalton's lymphoma ascites (DLA) and Ehrlich's ascites carcinoma (EAC) cell lines, and long-term cytotoxic, anti-proliferative and apoptotic properties of PPC-*Pr* were examined using human colon cancer cell line HCT116 as a model system. Short-term cytotoxic activity against DLA and EAC cell lines was studied using Trypan blue exclusion method and PPC-*Pr* did not show any cytotoxicity in the short term assay. In the long-term cytotoxic assay, HCT116 cells were cultured in the presence of PPC-*Pr* at various concentrations (100-1000 µg/ml) for 24-96 h and the percentage of cell viability was evaluated by MTT (3-(4-5 dimethylthiozol-2-yl) 2-5 diphenyl tetrazolium bromide)method. The results showed that PPC-*Pr* inhibited the cells viability in time and concentration-dependent manner. We found that anti-proliferative effect of PPC-*Pr* was associated with apoptosis on HCT116 cells. The morphological changes in the PPC-*Pr* treated HCT116 cells were analyzed by fluorescent DNA-microscopy with 4', 6-Diamidino-2-phenylindole dihydrochloride hydrate (DAPI) and Acridine orange-ethidium bromide (AO/EB) staining. The oligonucleosomal DNA fragmentation in the PPC-*Pr* treated cells was studied using Comet assay. These investigations indicate that the polysaccharide-protein complex from *P.rimosus* possessed cytotoxic, anti-proliferative activity and induced marked apoptosis in tumor cells in vitro.

8.1. Introduction

Apoptosis has recently become a focus of interest in oncology and may also shed light on cancer therapy (Marx, 1993). Programmed cell death or apoptosis plays an integral role in a variety of biological events, including morphogenesis, tissue homeostasis, and removal of unwanted or harmful cells (Schultz & Harrington, 2003). Failure to accurately undergo apoptosis can cause severe anomalies in humans, either due to the accumulation or due to the deficiency of a particular cell type. Abnormal inhibition of apoptosis is a hallmark of cancer and autoimmune diseases, whereas excessive cell death has been implicated in a

number of neurodegenerative disorders (Thompson, 1995). Programmed cell death is a physiological and crucial process that is regarded as the preferred way to eliminate cancer cells (Mukhtar & Ahmad, 1999).

Polysaccharides or polysaccharide-peptide (protein) complexes are the major bioactive ingredients of various mushrooms. They exert their anti-tumor action via activation of the immune response of the host organism; hence they are regarded as biological response modifiers (BRMs) (Kima et al., 2004; Ruan et al., 2005). However, increasing experimental evidences confirm that the polysaccharides or their complexes could have cytotoxic or cytostatic effect on various tumor cell lines in vitro by inducing cell apoptosis, whereas less toxic to normal cells (Lavi et al.,2006; Lee et al., 2005; Yang et al., 2005; Li et al., 2004; Takako et al., 2004). Polysaccharide-peptide (PSP) isolated from the fruiting body of *Coriolus versicolor* (Lin et al., 2004), and polysaccharides obtained from the fruiting body of *Ganoderma lucidum* (Jiang et al., 2004) were shown to have growth inhibitory effects on cancer cells mediated by cell cycle arrest and/or induction of apoptosis. A polysaccharide-peptide complex (PSP) extracted from *Trametes versicolor* significantly reduced proliferation of MAD-MB-231 breast cancer cells by increasing p21 expression and decreasing cell-cycle protein cyclin D1 expression (Chow et al., 2003). A protein-bound polysaccharide (PBP) from *Phellinus linteus* had an anti-proliferative effect on SW480 human colon cancer cells mediated by inducing apoptosis and G_2/M cell cycle arrest with a decrease of cytochrome c release and reduced cyclin B1 expression (Li et al., 2004). In addition, a water soluble β-glucan isolated from *Poria cocos* was shown to have growth-inhibitory effects on human breast carcinoma MCF-7 cells mediated by cell-cycle arrest and apoptosis induction (Zhang et al., 2006). Therefore anti-tumor activities of mushroom polysaccharides are not only mediated by the immunopotentiation (Zaidman et al., 2005), but can also be resulted from a direct inhibition on the tumor cells. However, structure of mushroom polysaccharides are very diverse in terms of their monosaccharide composition, linkages in the main chain, degree of branching, percentage of non-carbohydrate components such as protein or peptide percentage as well as functional group modification. Thus,

the correlation of the chemical structure of mushroom polysaccharides and the mechanisms of their anti-proliferation activities is still unclear.

Development of non-invasive treatments of cancer is currently required as the traditional cancer treatments are often toxic to normal cells and can cause serious side effects (Wasser, 2002). Search for anti-cancer drugs with higher bioactivities and lower toxicity from nature has been very intensive in recent years. The protein bound polysaccharides isolated from mushrooms are shown to exhibit a variety of pharmacological properties with out causing adverse effects. In this study cytotoxic activity of PPC-*Pr* against the EAC and DLA murine cancer cell line was evaluated. Also the effect of PPC-*Pr* on the proliferation of HCT116 human colon cancer cells was studied and, ultimately, assessing the putative mechanism underlying the inhibition of colon cancer cell growth in vitro. This is the first report on the direct cytotoxic effect of PPC-*Pr* from *P. rimosus* on HCT116 human colon cancer cells through the induction of apoptosis as studied by cell morphology and DNA analysis.

8.2. Materials and methods

8.2.1. Isolation of PPC-*Pr* from *P.rimosus*

PPC-*Pr* was isolated from the hot water extract of *P.rimosus* as described in section 4.2.1.

8.2.2. Cell lines

Ehrlich's ascites carcinoma (EAC) and Dalton's lymphoma ascites (DLA) cells diluted to $1x10^6$ viable cells/0.1 ml as described in 5.2.2., were used for the Trypan blue exclusion method. HCT 116 cells (human colon cancer cell line) were maintained as described in section 3.1.2. PPC-*Pr* dissolved in the basal medium as a stock solution of 10 mg/ml, was further diluted with the basal medium to the desired concentrations and used for MTT assay.

8.2.3. Short-term cytotoxicity assay using Trypan blue exclusion method

Tumor cell lines (EAC and DLA) in 0.1 ml of Phosphate buffered saline (PBS) (0.2 M, pH 7.4); various concentrations of PPC-*Pr* (50µg/ml-2mg/ml) and PBS in a final volume of 1ml were incubated in clean sterile tubes for 3h at 37^0 c. After incubation, 100µl of 1% trypan blue solution was added and the survival rate

was assayed using a haemocytometer by counting living cells that excluded the dye. Dead cells take up the stain and appear in blue color. Percentage of cytotoxicity was calculated as:

% of cytotoxicity = $(T_{Dead} - C_{Dead} / T_{Total}) \times 100$

where, T_{Dead} is the number of dead cells in the PPC-*Pr* treated tube, C_{Dead} is the number of dead cells in the control tube and T_{Total} is the number of dead and live cells in PPC-*Pr* treated tube (Ajith and Janardhanan 2003).

8.2.4. Long-term cytotoxicity and cell proliferation assay

Cell density of HCT 116 was determined following exposure to various concentrations of PPC-*Pr* for evaluating the effect of PPC-*Pr* to modulate cell proliferation (Anto et al., 2000). Briefly, cells were seeded at a density of 5×10^3 cells/well into 96-well plates. After 24 h, PPC-*Pr* was added to the medium at the concentrations of 100, 500 and 1000 µg/ml, incubated for 24 to 96 h as indicated. At the end of the incubation, 100 µl of 3-(4-5 dimethylthiozol-2-yl) 2-5 diphenyl-tetrazolium bromide (MTT) (2mg/ml) per well was added to each well and incubated for another 2 h. The formazan crystals formed were solubilized in acidified isopropanol after aspirating the medium. The extent of MTT reduction was measured spectrophotometrically at 570 nm using an ELISA reader (Bio-Tek, Winooski, VT). All determinations were conducted in triplicate. The cell survival was calculated by following formula:

CS (%) = (OD /Mean OD_0) x 100, where OD and OD_0 indicated the absorbance of treated cells and untreated cells, respectively. A graph was plotted with percentage viability in the Y-axis and concentrations of PPC-*Pr* in the X-axis. The value of IC_{50}, which is the concentration of PPC-*Pr* required to inhibit the colon cancer cells by 50% of the control level, was estimated from the plot.

8.2.5. Morphological studies of apoptotic cell

8.2.5.1. DAPI (4', 6-Diamidino-2-phenylindole dihydrochloride hydrate) staining

Chromatin changes in the PPC-*Pr* treated cell line were studied by DAPI staining procedure. HCT 116 cells were cultured on cover slips at seeding density of 5×10^5 cells in a 12- well plate for 72 h with various concentrations of PPC-*Pr*.

The cells were washed with 1X phosphate-buffered saline (1X PBS), fixation and permeabilisation of cells were done with acetone: methanol (1:1) for 7 min. Following 3 washes with 1X PBS, the cells were stained with DAPI (0.5µg/ml) in dark for 2 minutes. The cover slips were removed from the 12-well plates, washed with PBS, mounted on glass slides to view under a Nikon inverted fluorescent microscope (TE-Eclipse 300) attached with a camera and photographed under phase contrast and fluorescent conditions. Apoptotic cells were morphologically defined by cytoplasmic and nuclear shrinkage, and by chromatin condensation or fragmentation.

8.2.5.2. Acridine orange-ethidium bromide (AO/EB) staining

The frequency of apoptotic induction of HCT 116 cells following exposure to PPC-*Pr* was determined by fluorescent microscopy of nuclear stains, acridine orange and ethidium bromide. Cells were cultured and treated with PPC-*Pr* as in the above experiment. After treatment, washing once with 1X PBS, the cells were stained with 100 µl of a mixture (1:1) of acridine orange-ethidium bromide (4µg/ml) solutions for 3 min. The cells were immediately washed with PBS, viewed under inverted fluorescent microscope equipped with a red filter and photographed as above. Two hundred cells per sample were counted in triplicate for each dose point. Four types of cells were distinguished according to the fluorescence emission and the morphological pattern of chromatin condensation in the stained nuclei; (1) Viable normal cells have uniform bright green nuclei with organized structure; (2) early apoptotic cells (which still have intact membranes but have started to undergo DNA cleavage) have bright green nuclei, but with highly condensed or fragmented chromatin visible as bright green patches or fragments; (3) late apoptotic cells have bright orange to red nuclei with highly condensed or fragmented chromatin; (4) Necrotic cells have a uniformly orange to red nuclei with organized structure.

8.2.6. Analysis of DNA fragmentation by Comet assay

For the comet (single cell gel electrophoresis) assay, initially 100 µl of 0.5% normal melting agarose (NMA) in PBS was added to the frosted microscope slides, then covered with 22 mm x 50 mm parafilm cover slips and allowed to

solidify for 3-4 min at room temperature. The cover slips were gently removed after the agarose had set. HCT 116 cells were grown in culture bottles and treated with PPC-*Pr* in the same conditions as described above. After treatment, cells were scraped off into the ice-cold 1X PBS to yield approximately $1x10^6$ cells/ml. 5 µl of the cell suspension was added to 75 µl of 0.5% low melting point agarose (LMPA) in PBS, mixed gently at 37^0C by repeated pipetting using a cut micropipette tip, and added onto NMA coated slides. Cover slips were replaced on the mixture to obtain a uniform layer. Slides were placed on a tray resting on the ice packs until the agarose layer hardened (~ 3 to 5 min). Cover slips were gently removed and a final layer of 0.5% LMPA (75 µl) was placed on the slides, covered with the cover slips and returned to the slide tray to solidify. The parafilms were removed after the agarose had set and the slides were immersed in cold lysis solution (2.5 M NaCl, 100mM EDTA, 10Mm Tris, pH 10, with freshly added 1 % Triton X-100 and 10% DMSO) followed by incubation at 4^0 C for at least 1 h. The electrophoresis in weak alkali (0.03 M NaOH, 1 mM EDTA, pH 12.1) at 25 V and 300 mA for 20 min was preceded by a 20 min immersion of the slides in the electrophoresis buffer to promote chromatin unwinding. After electrophoresis, the slides were neutralized by drop wise addition of 0.4 M Tris buffer (pH 7.5), allowed to sit for at least 5 min and repeated twice. Drained slides were stained with Ethidium Bromide (10 µg/ml) for 5 min and rinsed with cold water to remove the excess stain. The slides were covered with fresh cover slips and examined under fluorescence microscope (TE-Eclipse 300) equipped with an excitation filter of 515-560 nm and a barrier filter of 590 nm.

Apoptosis was examined by analyzing 100 nuclei from each treatment. Nuclear phenotypes identified according to their tail length: type 1- no tail or DNA diffusion halo, type 2- no tail but with a DNA diffusion halo, type 3- a tail and a DNA diffusion halo, type 4- a tail and not a DNA diffusion halo. Type 3 and 4 were markers for the early and late apoptotic stages, respectively.

8.3. Results

The PPC-*Pr* was not found to possess cytotoxic activity against the murine cell lines DLA and EAC up to a tested concentration of 2mg/ml in the short-term

Trypan blue exclusion assay. MTT assay revealed the metabolic activity of the cells and reflected its growth potential indicating the balance between proliferation and death. The HCT 116 cells were incubated with 100, 500 and 1000µg/ml of PPC-*Pr* for 24, 48, 72 and 96 h. As shown in Figure 8.1., PPC-*Pr* inhibited colon cancer cells growth in time and dose-dependent manner ($P<0.001$). After 48 h treatment, PPC-*Pr* demonstrated distinct inhibition at concentrations of 500 and 1000µg/ml; whereas the growth inhibitory effects for 24 h treatment were not significant (Figure 8.2). The concentrations of PPC-*Pr* with 50 % inhibition on the cancer cell growth, i.e., IC_{50}, were found to be 852, 456 and 420 µg/ml for 48, 72 and 96 h, respectively. The mechanism of action for the growth inhibition was further investigated after the HCT 116 cells had been incubated with various concentrations of PPC-*Pr* for 72 h. As the percentage of inhibition did not increase for 100 µg/ml of PPC-*Pr*, 500 and 1000µg/ml concentrations were chosen for further experiments.

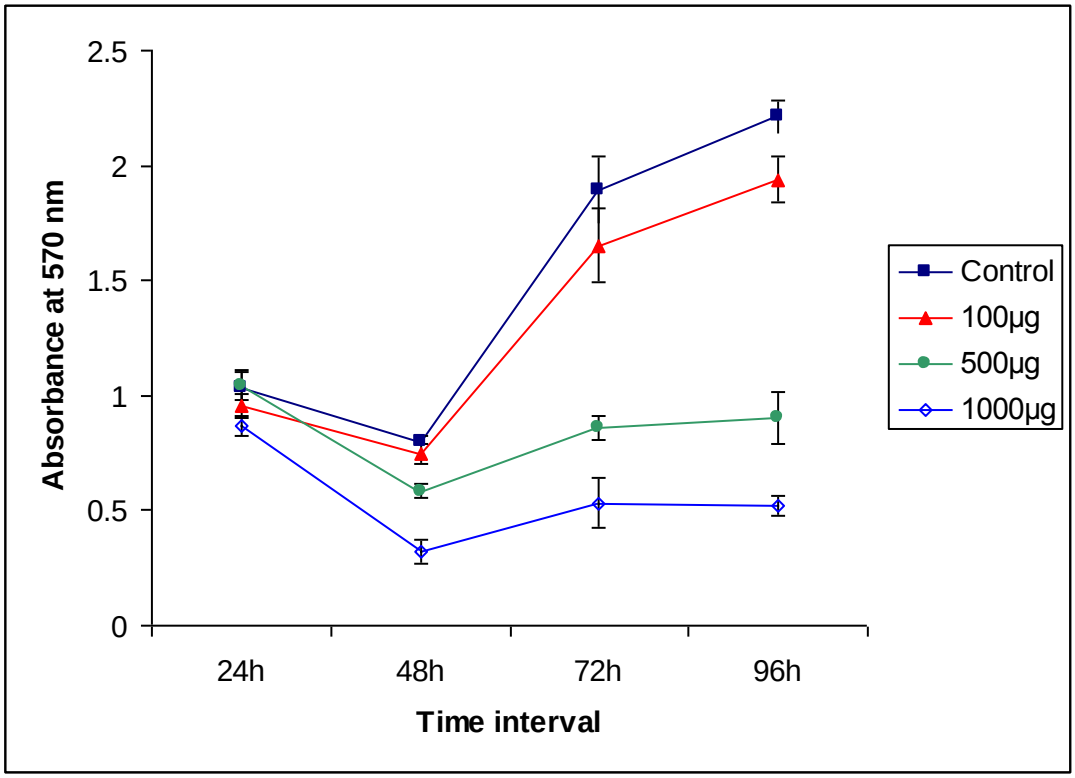

Figure 8.1. Cytotoxic effect of PPC-*Pr* on HCT 116 cells. Cells were incubated with 100, 500 and 1000µg/ml of PPC-*Pr* for 24, 48, 72 and 96 h. Cell viability was measured by the MTT assay. Data are averages ± S.D (bars) of triplicate determinations.

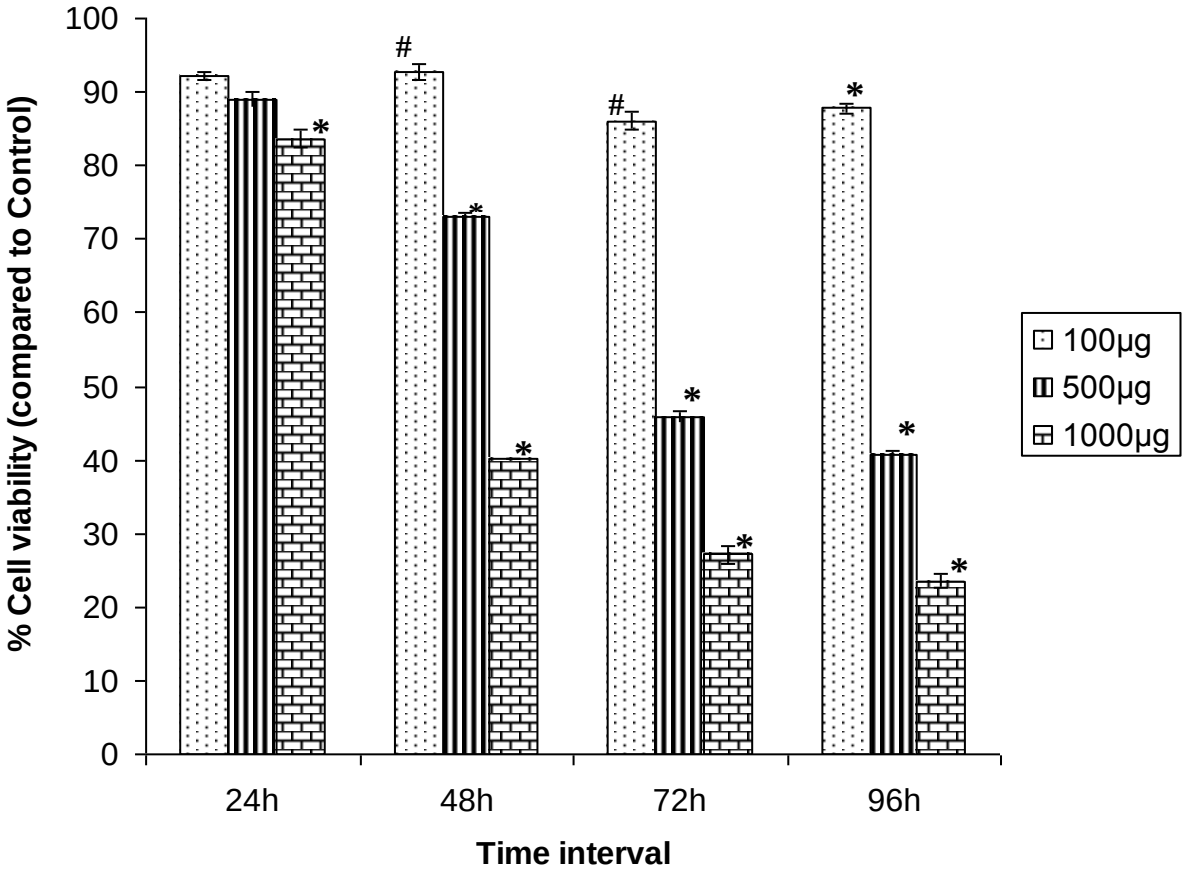

Figure 8.2. Effect of PPC-*Pr* on HCT 116 cell proliferation. Viable HCT116 cells were determined using the MTT method as described in Materials and methods and expressed as a percentage of the untreated control cell samples. Samples were incubated for 24, 48, 76 and 92 h in the presence or absence of various concentrations of PPC-*Pr.* Values are the mean ± S.D. of data from at least three independent experiments; * $p<0.001$, # $p<0.01$ compared with control group.

The characteristic changes of apoptosis were observed in the morphology of PPC-*Pr* treated cells. While the untreated HCT 116 cells (control) were well spread with flattened morphology, many round-shaped cells poorly adhered to the culture plates were noticed in those treated with PPC-*Pr* for 72 h. Cultures contained abundant particles and debris with reduced number of HCT 116 cells remaining intact. Significant decrease of the number of HCT 116 cells treated with 500 and 1000µg/ml of PPC-*Pr* was observed compared to the control group.

The morphological changes observed consequent to DAPI staining are represented in Figure 8.3. The untreated cells (control) showed uniform staining of nucleus and chromatin network (Figure 8.3a. & 8.3b.). The cells treated withPPC-*Pr* showed nuclear shrinkage and chromatin condensation. Marginalisation of chromatin network was also observed in the treated cells (Figure 8.3c.-8.3f). The apoptotic morphology in cellular bodies and the chromatin condensation were also confirmed by Acridine orange-ethidium bromide double staining. The compact masses of chromatin aggregated along the nuclear membrane. Round, compact granular masses appeared nearer the center of the nucleus and there was a reduction in nuclear volume. Cytoplasm displayed condensation and some of the nucleus degenerated into discrete spherical or ovoid fragments of highly condensed chromatin (Figure 8.a-8.4f). Compared with the spontaneous apoptosis observed in control cells (early apoptotic 0.77 %; late apoptotic 4.64 %), PPC-*Pr* treated cells showed highly increased percentages of early apoptotic (500 µg/ml: 6.11 %; 1000 µg/ml: 3.64 %), and late apoptotic (500 µg/ml: 43.65 %; 1000 µg/ml: 65.39 %) cells, with concomitant increase in the total number of apoptotic cells (Figure 8.4g).

Chromatin degradation into multiple internucleosomal fragments of 180-200 base pairs is a distinct biochemical hallmark for apoptosis. In order to investigate whether the morphological changes observed in PPC-*Pr* treated HCT 116 were consistent with chromatin degradation, the DNA damage in individual cells was studied by comet assay (Figure 8.5a, 8.5b& 8.5c). The comet assay showed that control cells had few nuclei with fragmented chromatin whereas a high number of nuclei had no detectable tail (type 1 & 2). Apoptotic nuclei (type 3 & 4) were more frequent in PPC-*Pr* treated cells (Figure 8.5d). These results support the observations derived from DAPI and AO/EB staining, suggesting the excessive chromatin fragmentation in PPC-*Pr* treated cells was the result of apoptosis.

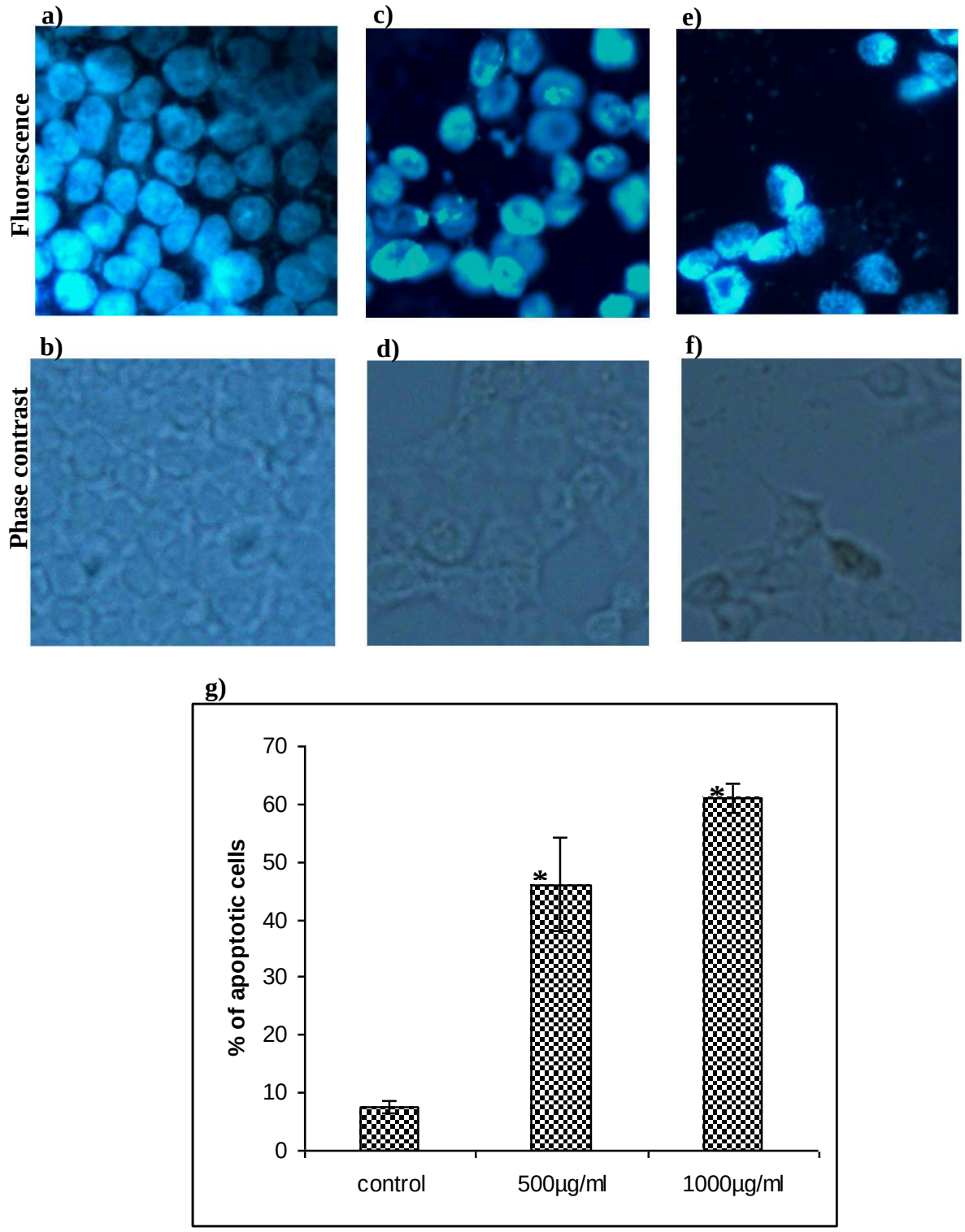

Figure 8.3. Fluorescent microscopic analysis of apoptotic cells stained with DAPI. Cells were treated for 72 hrs without PPC-*Pr* (a & b), with PPC-*Pr* at a dose of 500µg/ml (c & d) and 1000 µg/ml (e & f). The representative cells were counted as apoptosis in fluorescence microscopy with x 200 magnification (g). Values are ± S.D; *p<0.001 compared with control group.

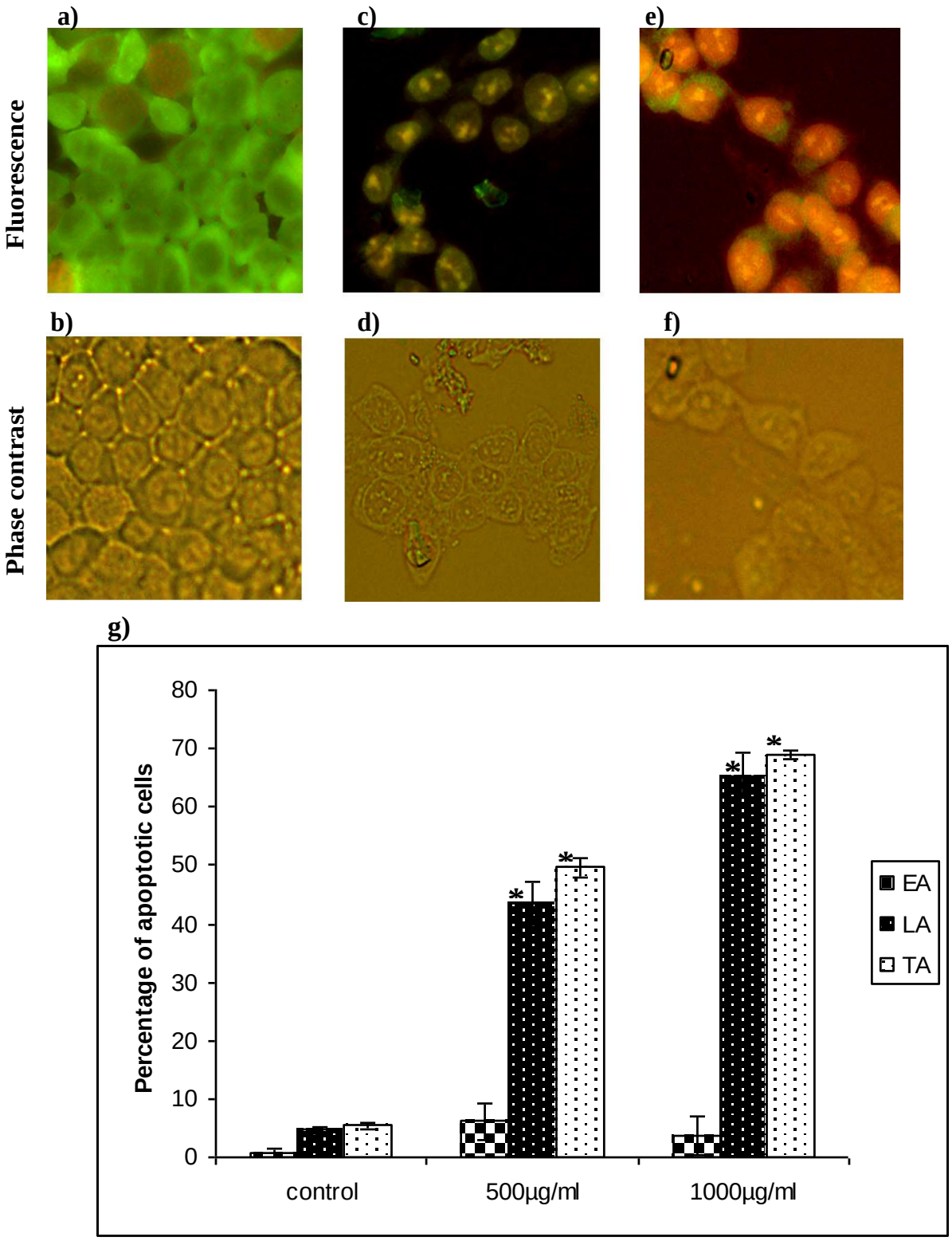

Figure 8.4. Fluorescent microscopic analysis of apoptotic cells stained with AO/EB. Cells were treated for 72 hrs without PPC-*Pr* (a & b), with PPC-*Pr* at a dose of 500µg/ml (c & d) and 1000 µg/ml (e & f). The representative cells were counted as apoptosis in fluorescence microscopy with x 200 magnification (g). Values are ± S.D; *p<0.001 compared with control group.

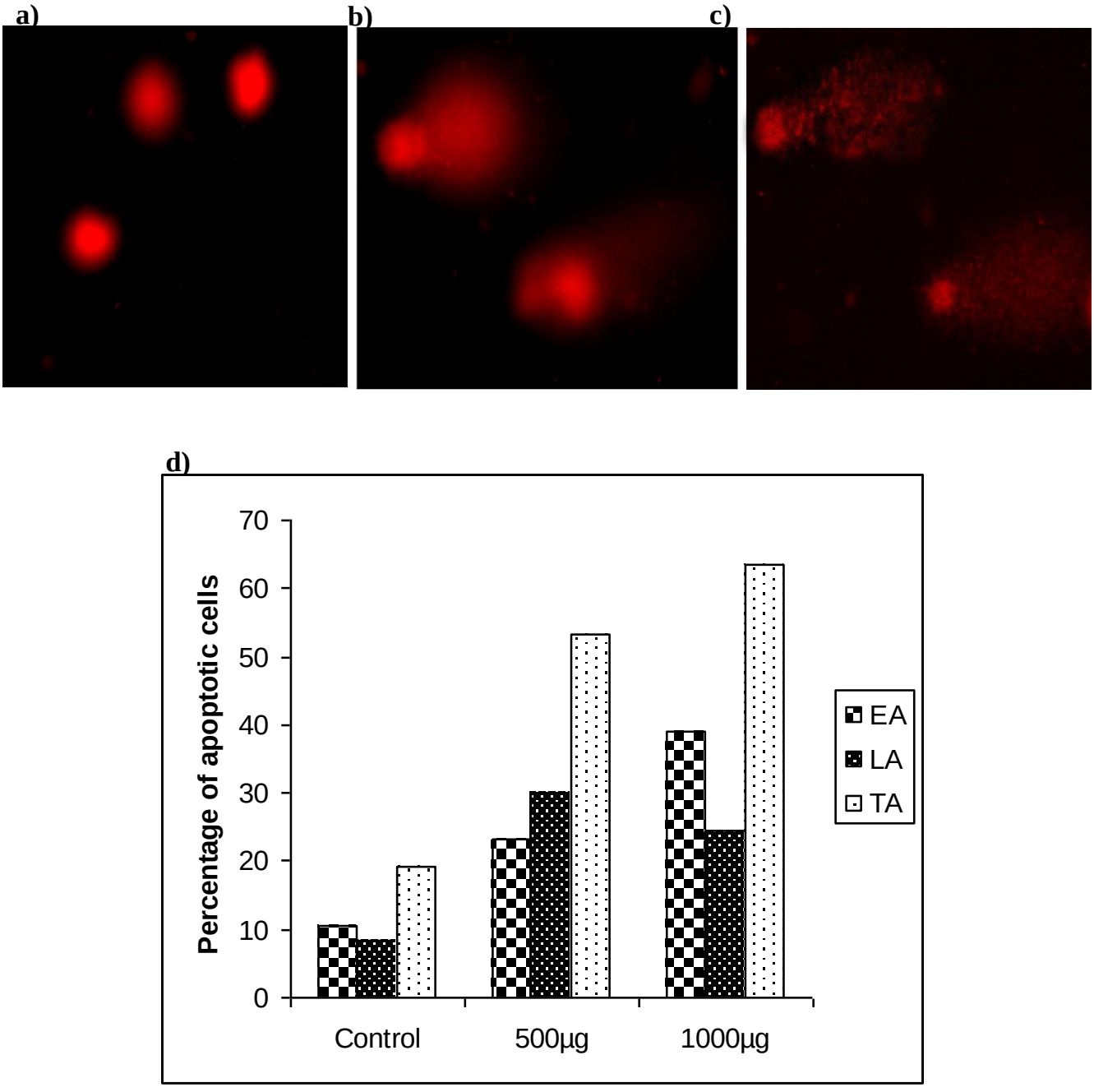

Figure 8.5. DNA fragmentation analysis of apoptotic cells. Cells were treated for 72 hrs without PPC-*Pr* (a), with PPC-*Pr* at a dose of 500µg/ml (b) and 1000 µg/ml (c). The treated cells were subjected to comet test, stained with EB and classified by phenotypes. Control group showed a low frequency of apoptotic nuclei (type 3 & 4), in contrast to PPC-*Pr* treated cells (d).

8.4. Discussion

The ability to induce cell apoptosis is an important property of anti-cancer drugs (Frankfurt and Krishnan, 2003). In the past years, increasing number of reports revealed that polysaccharides, proteo-polysaccharides originated from mushrooms and other natural products exhibited direct action on the proliferation of the tumor cells in vitro at certain concentrations by modulating cell-cycle progression and inducing apoptosis. PSP, PSK, *Lycium barbarum* polysaccharide (LBP) and *Phellinus linteus* polysaccharide showed the inhibition of tumor cells proliferation by inducing the cell apoptosis (Lee et al., 2005; Li et al., 2004; Lin et al., 2003; Takako et al., 2004; Zhang et al., 2005; Yang et al., 2005).

Apoptosis modulates cell cycle and finally leads to cell death (Paulovich et al., 1997). Unlike necrosis in which dying cells fall apart releasing their contents and provoking macrophage activation and inflammation, apoptotic cells are rapidly taken up by phagocytosis and degraded in phagolysomes (Roitt et al., 2001). Various tumors undergoing the process of apoptosis were induced by treatments of radiation, chemotherapeutic agents and mild hyperthermia (Kerr et al., 1994). Cell death in tumors, whether spontaneous or treatment-induced, occurs predominantly via apoptosis rather than necrosis (Wyllie et al., 1980; Arend et al., 1990). The characteristic morphological changes of apoptosis include membrane blebbing, chromatin condensation and the formation of apoptotic bodies. In addition, apoptotic cells undergo DNA double strand cleavage into fragments of multiples of about 185-200 base pairs (Wyllie et al., 1980; Gong et al., 1994).

Apoptosis is a tightly regulated process and its mechanisms involve mainly two signaling pathways, including cell death receptor pathway and mitochondrial pathway (Reed, 2001). In the mitochondrial pathway, a variety of death signals triggers the release of several pro-apoptosis proteins. Among the numerous factors known to modulate cancer-related apoptosis, Bcl-2 family and p53 are the most extensively characterized mechanistically. Bax is a crucial mediator and as a tumor suppressor (Yamaguchi et al., 2003), it mediates the p53-induced apoptosis and increases sensitivity to the drug-induced apoptosis. Meanwhile, Bcl-2 is an anti-apoptosis protein. When it is activated or prevalent, apoptosis is prohibited

(Tsujimimoto and Shimizu, 2000). And relative expression levels of Bcl-2/Bax were reported to determine the sensitivity to apoptosis (Danial and Korsmeyer, 2004). Caspase have been shown to be activated during apoptosis in many tumor cells. Related references revealed that whatever the mitochondrial pathway and cell death receptor pathway, both finally activate caspase-3, which is essential for DNA fragmentation, the morphological change associated with apoptosis, and its activation represents a key and irreversible point in the development of apoptosis (Cui et al., 2007).

DAPI is a DNA specific dye, which forms a fluorescent complex by attaching in the minor groove of consecutive (3-4 base pairs) A-T rich sequences of DNA. DAPI can penetrate through the intact plasma membrane to stain DNA and makes visible the changes in the chromatin more clearly. Staining of apoptotic cells with fluorescent dyes such as AO and EB is considered the correct method for evaluating the changed nuclear morphology. AO is used to determine the number of cells within the given population, which has undergone apoptosis, but it cannot distinguish viable from non-viable cells. To achieve this, a mixture of AO and EB was used. The differential uptake of these two dyes allows the identification of viable and non-viable cells and also the visualization of aberrant chromatin organization. Among the methods used to study apoptosis, microscopic examination of AO/EB stained cells is recommended as the most reliable, since it makes it possible to perform high-quality studies of cell morphology, nuclear and chromatin disintegration as well as to distinguish viable, early or late apoptotic and necrotic cells (Baskic et al., 2006).

Double stranded DNA fragmentation giving the characteristic "ladder" pattern on agarose gel electrophoresis is on of the most widely accepted criteria used to characterize apoptotic cell death because it is discernible from the random single stranded DNA breaks produced by necrosis (Yaoita et al., 2000). However, this technique is difficult to quantify and not particularly sensitive as an extensive number of cells (> 10^6 cells/sample) is required in synchronous fragmentation to produce oligonucleosomal ladders (Page et al., 2000). At present, enzymatic techniques are used for detecting DNA fragmentation at the individual cell level,

the most common being the terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) assay. But this assay also suffers from lack of specificity and can not distinguish between apoptotic and non apoptotic cell, as it labels DNA strand breaks from any insult (Singh, 2000). With heterogeneous cell population, DNA ladder formation tends to miss contributions from small sub-populations and obtained results can not be quantified. The detection of apoptosis at the single cell level requires an assay that allows analyses of individual cells for the biological changes induced by apoptosis. Comet assay or single cell gel electrophoresis (SCGE) is one such technique that can detect the fragmented DNA on a cell-by-cell basis, thereby readily allowing the detection of apoptotic cells. By virtue of its ability to detect DNA fragmentation at the level of individual cell comet assay allows analysis of sub-populations of cells (Bacso et al., 2000). The "apoptotic comet" appears usually as a ring with less DNA in the center of the head (the "pin" head) followed by a large cloud (the "puffy" tail) representing randomly cleaved DNA of different chain lengths. This is characteristic of apoptotic DNA fragmentation. Necrotic DNA damage, on the other hand, shows a comet with considerable DNA both in the head and in the tail as a result of non-random DNA cleavage. The comet assay thus can be used to detect a cell undergoing apoptosis and also for the quantitative estimation of apoptosis.

The current experimental results indicate that PPC-*Pr*, a novel polysaccharide-protein complex isolated from the fruiting body of *P.rimosus*, is able to significantly inhibit the growth of HCT116 colon cancer cell line in a time and concentration-dependent manner. The proliferation inhibition of HCT116 was the result of apoptosis induction, as evidenced by the cell morphological analyses of the PPC-*Pr* treated HCT 116 cells by DAPI and AO/EB staining methods. The typical DNA fragmentation of apoptosis in the treated cells is obtained in comet assay, which further indicates that PPC-*Pr* is able to induce the apoptosis of HCT116 cells. The mechanisms underlying these effects depend on the initiating stimulus, but a common feature is the activation of certain endonucleotidases leading to DNA fragmentation (Barry and Eastman, 1992; Wyllie et al. 1984). Endonucleotidase activation may result from disruption of DNA supercoil

structure (Murakami et al., 1995), interference with DNA repair mechanisms (Darzynkiewicz, 1995) or interference with normal cellular signaling pathways (Bergamaschi et al., 1993). It has been suggested that endonucleotidase activation depends upon the disruption of the interaction of histones with DNA. The apoptosis induced by PPC-*Pr* on HCT116 cells might be mediated via membrane polysaccharide receptor on cell surface, since PPC-*Pr* cannot penetrate cells due to its large molecular mass. Since the PPC-*Pr* treated HCT116 cells showed significant DNA fragmentation, the possible mechanism of PPC-*Pr* induced apoptosis might involve caspase-3 activation and the following cascades of reactions. However, further detailed studies are required to clarify the cellular signaling process by which PPC-*Pr* induces apoptosis in HCT116 cells. In conclusion, PPC-*Pr* inhibited the proliferation of HCT116 cells by triggering apoptosis in addition to causing DNA damage. The findings thus suggest the potential use of PPC-*Pr* in cancer therapy.

Chapter- 9

Isolation and characterization of β-D-(1→6) glucan from the crude polysaccharide-protein complex (PPC-*Pr)* of *P.rimosus*

Synopsis

Neutral fraction was separated from the crude polysaccharide- protein complex (PPC-*Pr*) isolated from the fruiting body of *P. rimosus*. The isolation of neutral fraction from PPC-*Pr* was achieved by fractionation using CTA-OH, solvent precipitation by chilled ethanol, deproteinization, DEAE-cellulose chromatography, dialysis and freeze-drying. The proximate analysis showed that neutral fraction was composed mainly of polysaccharides (92.3 %) and only traces of protein (7.5 %) were present. The paper chromatography (PC) and thin layer chromatography (TLC) analysis of neutral fraction after acid hydrolysis with trifluroacetic acid showed that it was composed of D-glucose as the only monosaccharide unit. Further characterization of neutral fraction was done by IR as well as ^{1}H and ^{13}C NMR spectroscopy. IR spectrum showed absorption characteristic of β-glucan at 910 cm^{-1}. ^{1}H and ^{13}C NMR spectroscopy showed that the neutral fraction was a (1→6)-D-glucan with β- linkages. The molecular weight determination of the compound was accomplished by gel filtration chromatography using Sephadex G 100. The molecular weight of neutral fraction was approximately 170,000 Da. MALDI-TOF analysis revealed that the molecular weight of neutral fraction range between 17,264 to 178,326 Da and the degree of polymerization was estimated to be in the range of 96 to 990. Results thus, suggested that neutral fraction of PPC-*Pr* is a high molecular weight noble biomolecule, β-D-(1→6) glucan.

9.1. Introduction

A variety of polysaccharides having different structures has been derived from various sources and by different extraction processes (Bohn and BeMiller, 1995; Kollar et al., 1997). A number of mushroom polysaccharides with anti-tumor activities, such as lentinan from *Lentinus edodes* (Chihara, 1992a; Ochiai et al., 1992), grifolan from *Grifola frontosa* (Adachi et al., 1987; Suzuki et al., 1989), Schizophyllan from *Schyzophyllum commune* (Jong et al., 1991; Mizuno, 1999) have attracted much attention and are now used in clinics. An analysis of structural features of the various anti-tumor mushroom extracts revealed that high molecular weight glucans linked in 1-3 and 1-6 linkages that would provide

solubility were the compounds responsible for anti-tumor activity in mushrooms (Mascarenhas, 1994).

β-D-glucan is a carbohydrate polymer with chains of glucose molecules linked together by β-glycosidic linkages (Wang et al., 2002; Usui et al., 1983; Sone et al., 1985). Protocols for the structural elucidation of mushroom polysaccharides are well established. The building blocks of β-glucan have been studied by degradation of the polysaccharides to oligosaccharides with a specific D-glucanase enzyme (Wood et al., 1991). The released oligosaccharides have then been analyzed by gel chromatography (Bock et al., 1991) and HPLC methods (Izydorczyk et al., 1998). Rydlund (1995) used capillary electophoresis (CE) for analyzing mono and oligosaccharides from the various samples. CE provided a sensitive analysis method also for the structural components of β-glucan. Matrix-assisted laser desorption/ionization (MALDI) has been developed as a successful tool for the analysis of biopolymers and macromolecules (Karas et al., 1978; Bahr et al., 1994; Fitzgerald and Smith, 1995). MALDI-TOF mass spectroscopy is an emerging technique offering promise for the fast and accurate determination of a number of polymer characteristics. MALDI is a "soft" ionization method and so results predominantly in the generation of singly charged molecular-related ions regardless of the molecular mass, hence the spectra are relatively easy to interpret. Fragmentation of the sample ions does not usually occur. NMR spectroscopy is nowadays a primary tool for structural analysis (Mizuno, 1999; Wasser, 2002) and widely used in the structural elucidation of β-glucan.

Numerous mushroom polysaccharides have been studied for their anti-tumor activities, based essentially on mouse transplanted Sarcoma 180 tests. However, most of these polysaccharides have not been adequately confirmed as to their purities and chemical properties. No work relating to the structural features of the polysaccharide from *P.rimosus* has been reported. In the present study, a water soluble glucan consisting of (1→6) linked D-glucose moieties with β linkages have been isolated for the first time. Detailed structural studies of this glucan were carried out and are presented herein.

9.2. Materials and methods

9.2.1. Isolation of neutral polysaccharide from the crude polysaccharide-protein complex PPC-*Pr*

Neutral polysaccharide was isolated from the crude polysaccharide- protein complex PPC-P*r* by the method of Mizuno (2000) with slight modifications (Figure 9.1.). The PPC-P*r* was dissolved in deionized water, 0.2M Cetyltrimethylammonium hydroxide (CTA-OH) was added drop wise with continuous stirring until the pH exceeded 12 and the mixture was allowed to stand at 4^{o} C for 12 hr. Centrifugation at 10,000 rpm for 20 min was done; the supernatant obtained was precipitated with X5 volume of chilled ethanol and allowed to stand for 48 hr at 4^{o} C. The precipitate obtained after centrifugation was deproteinized by Sevag method (Staub, 1965) as described in section 4.2.1. The aqueous phase obtained was again precipitated with chilled ethanol and the precipitate obtained was passed through DEAE – cellulose column. The precipitate was loaded on to the DEAE - cellulose column of bed volume 50 ml, and the column was eluted with deionized water. Each 10 ml fractions collected were tested with Anthrone method (Yemm and Wills, 1954) described in Section 3.2.3., to detect the presence of polysaccharides. The fractions showing positive anthrone test were combined, concentrated to 10 ml volume and then dialyzed against deionized water using dialysis baging of M_r cut-off 12,000 for 3 days to remove small molecules in the solution, followed by lyophilisation to obtain the neutral fraction.

9.2.2. Estimation of total carbohydrate content

Quantitative examination of carbohydrate content was done by Phenol sulphuric acid method using glucose as the standard, as described in section 3.2.2.

9.2.3. Estimation of total protein content

Total protein content was determined by the Bradford method using bovine serum albumin as the standard, as described in section 3.2.4.

9.2.4. Determination of monosaccharide components by Partition (Paper and Thin layer) chromatography

In order to detect the monosaccharide profile, 1 mg of neutral fraction was

hydrolyzed with TFA, paper and thin layer chromatography were carried out as described in section 4.2.3.

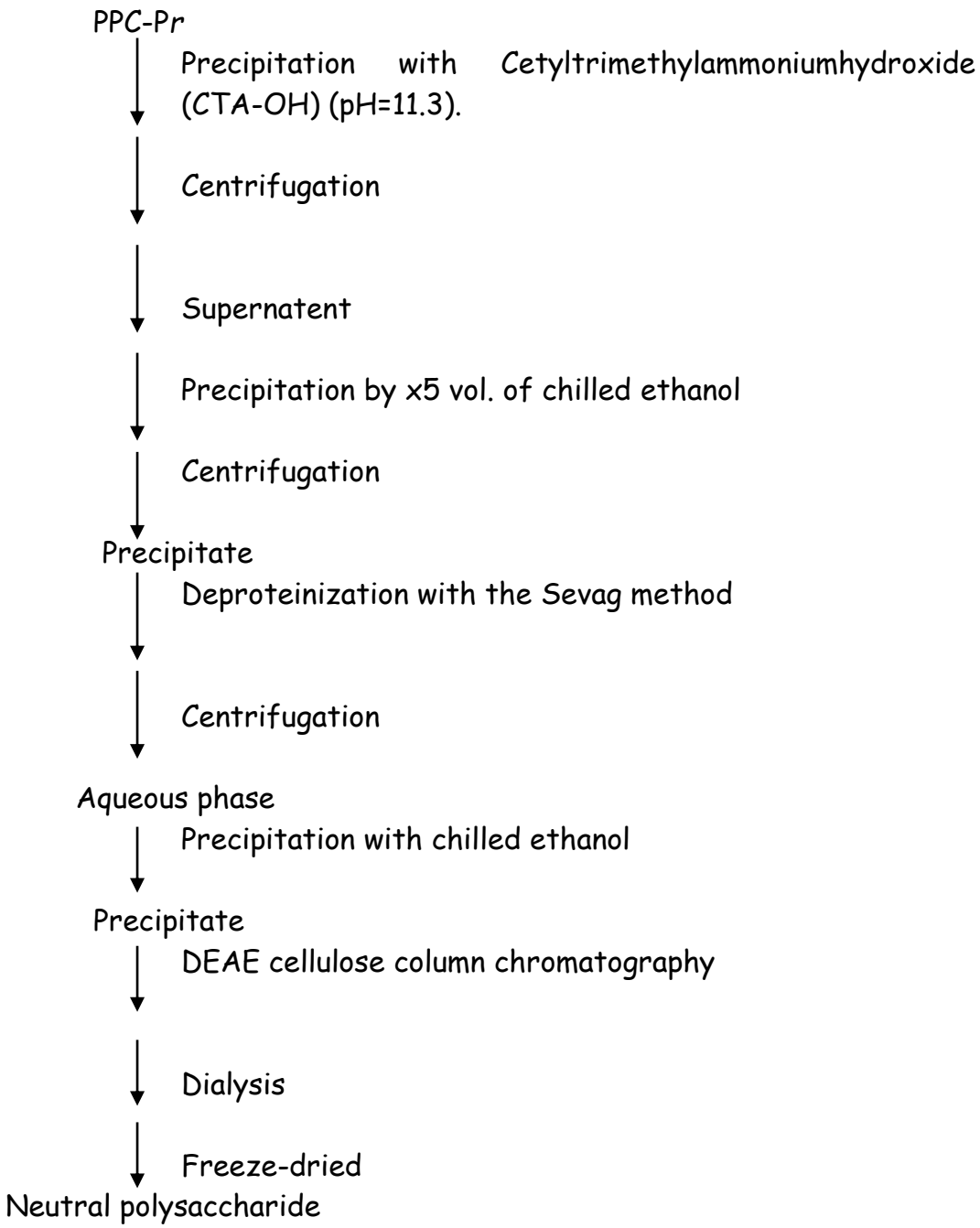

Figure 9.1. Procedure for the isolation of neutral polysaccharide from the crude polysaccharide-protein complex (PPC-*Pr*) of *P. rimosus*.

9.2.5. Fourier Transform-Infra Red (FT-IR) Spectrometry

Principle

Infrared Spectroscopy gives information on the vibrational and rotational modes of motion of a molecule and hence an important technique for identification and characterization of a substance. Chemical bonds in different environments will absorb varying intensities and at varying frequencies. Thus IR spectroscopy involves collecting absorption information and analyzing it in the form of a spectrum. When exposed to IR radiation, a minimum of one vibrational motion must alter the net dipole moment of the molecule in order for absorption to be observed. In simple terms, IR spectra are obtained by detecting changes in transmittance (or absorption) intensity as a function of frequency. Detectors measure the heating effect produced by infrared radiation. The frequencies at which there are absorptions of IR radiation ('peaks' or 'signals') can be correlated directly bonds within the compound in question.

Procedure

FT-IR (Magna- IR 550) was analysed using the KBr disc for detecting functional groups. Neutral polysaccharide was grind into KBr matrix. Radiation from a broad-band source was passed through neutral polysaccharide and was dispersed by a monchromator into component frequencies. Then the beams fall on the detector, which generated an electrical signal which was recorded.

9.2.6. C^{13} and H^{1} Nuclear magnetic resonance (NMR) spectroscopy

Principle

NMR spectroscopy is a method of absorption spectroscopy. In NMR, a molecular sample, usually dissolved in a liquid solvent, is placed in a magnetic field and the absorption of radio-frequency waves by certain nuclei (protons and others) is measured. The relative positions and intensity of absorption signals provide detailed information from which chemical structures may be elucidated. All nuclei possess a positive charge. For some nuclei, this charge confers the property of spin, which causes the nuclei to act like tiny magnets. The angular momentum of the spin is described by the quantum spin number *I*. If *I* is an integral number (I = 0, 1, 2, etc), then there is no net spin and no NMR signal for

that nucleus. However, if *I* is half-integral (*I* = 1/2, 3/2, 5/2, etc), the nuclei have spins, and when placed in a magnetic field, the spins orient themselves with (parallel) or opposed (antiparallel) to the external magnetic field. The nuclei aligned with the magnetic field have lower energy (are more stable) than those opposed. Energy in the radio-frequency range is sufficient to flip the nuclei from the parallel to the antiparallel alignment. The NMR instrument is designed to measure the energy difference between the nuclear spin states. Absorption of energy may be detected by scanning the radio frequency range and measuring the absorption that causes spin state transition (resonance). The point of resonance for a nucleus is dependent upon the electronic environment of that nucleus, so an NMR spectrum provides information that helps to elucidate biochemical structures.

Procedure

C^{13} and H^{1} NMR spectroscopy were performed with a Mercury Plus 300 NMR spectrometer (Varian, USA) at room temperature. Neutral polysaccharide was dissolved in D_2O for C^{13} and H^{1} NMR analysis and the chemical shifts were expressed in ppm.

9.2.7. Determination of molecular weight by Gel exclusion chromatography using Sephadex G 100

The molecular weight of the neutral polysaccharide was determined by Gel filtration chromatography using Sephadex G-100 column as described in section 4.2.6.

9.2.8. Matrix Assisted Laser Desorption / Ionization-Time of Flight (MALDI-TOF) Mass Spectrometry

Principle

The ionization/desorption of biomolecules in MALDI requires, as the name implies, a matrix. The analyte is embedded in the matrix which absorbs the UV-light emitted from a laser, often a nitrogen laser with 337 nm wavelength, thereby aiding the analyte to desorb in intact form. A fraction of the analyte molecule is ionized (Quist et al., 1994). Different matrices suite different biomolecules and for carbohydrates the most frequently used matrix is 2, 5-dihydroxybenzoic acid

(DHB) (Strupat et al., 1991). Since MALDI is a pulsed ion source it is often coupled to a discontinuous mass analyzer such as TOF analyzer. In a MALDI-TOF instrument the ions formed in the ion source are accelerated in an electric field and thereafter propagate through a field free region before reaching the detector. When entering the field free region all ions of the same charge state have obtained approximately the same kinetic energy, which is defined by the acceleration voltage. Hence, low mass ions will travel faster and hit the detector before heavier ions. A TOF spectrum is obtained by measuring the time elapsed from the laser pulse to the detection of the various ions having propagated through the field free region and by measuring the intensity of the detector signal at each time. Summation of TOF- spectra from several laser shots is typically performed. A mass spectrum can be obtained by converting the TOF to *m/z* using the fundamental relations between these quantities via a calibration procedure.

Procedure

The MALDI-TOF Mass Spectrometry was performed on a Micromass TofSpec 2E instrument using a nitrogen 337 nm laser (4 ns pulse). At least 40-50 shots were summed up. The matrix used is 2, 5-dihydroxy benzoic acid (DHB) dissolved in H_2O. The sample dissolved in H_2O and the matrix spotted on MALDI target and allowed to dry before introducing into the mass spectrometer.

9.3. Results

The neutral fraction was isolated from crude polysaccharide-protein complex (PPC-*Pr*) of *P. rimosus* by fractionation with CTA-OH, repeated solvent precipitation, deproteinization, DEAE cellulose column chromatography, dialysis and freeze-drying. The neutral fraction was obtained as brown colored powder form by a yield of 0.252%. On reaction with Phenol sulphuric acid reagent, neutral fraction showed characteristic color reaction indicating the presence of polysaccharides. The total polysaccharide present in neutral fraction was 92.3%. Neutral fraction also produced characteristic color reaction with Bradford's reagent, indicating the presence of protein, but only trace amount of protein (7.5%) was found (Table 9.1.).

Table 9.1. Total polysaccharide and protein content of the neutral fraction

Contents	Conc. in mg/ml	Percent (%)
Polysaccharide	0.923	92.3
Protein	0.075	7.5

For the determination of monosaccharide constituents of neutral polysaccharide, the R_f value of hydrolysate was compared with the R_f values of standard monosaccharides obtained by Paper and Thin layer chromatography. The R_f values of standards on Paper chromatography were: D-Glucose 0.479, D-Fructose 0.351, D-galactose 0.404, D-mannose 0.447 and D-xylose 0.528. On TLC analysis, the R_f values produced by standards were: D-Glucose 0.457, D-Fructose 0.436, D-galactose 0.447, D-mannose 0.500 and D-xylose 0.553. Paper chromatography analysis showed that the hydrolysis product of neutral polysaccharide had only single spot with R_f value 0.479, which coincided with the R_f value of glucose (Figure 9.2.). This result was confirmed by TLC analysis in which also neutral polysaccharide produced only single spot with R_f value 0.457, which coincided with the R_f value of glucose (Figure 9.3.).

In order to determine the functional groups of the purified neutral polysaccharide, the FT-IR spectra were measured in KBr pellets. The spectrum of FT-IR is shown in Figure 9.4. Absorption peak at 910 cm^{-1} is characteristic of β-glucan. The band corresponding to the ν (C =O) vibration in the carboxyl group at 1651 cm^{-1} indicates that this carbonyl group was hydrogen bonded. Furthermore, the banding like structure in the region of 2939.30 cm^{-1} as well as a continuous absorption beginning at approximately the region of 3400 cm^{-1} are characteristic of a carbohydrate ring. Absorption at 1076.20 also indicated a pyranose form of the glycosyl residue. These results indicate that neutral polysaccharide is consisted of cyclic glucose with β glycosidic bonds i.e.; β-glucopyranose.

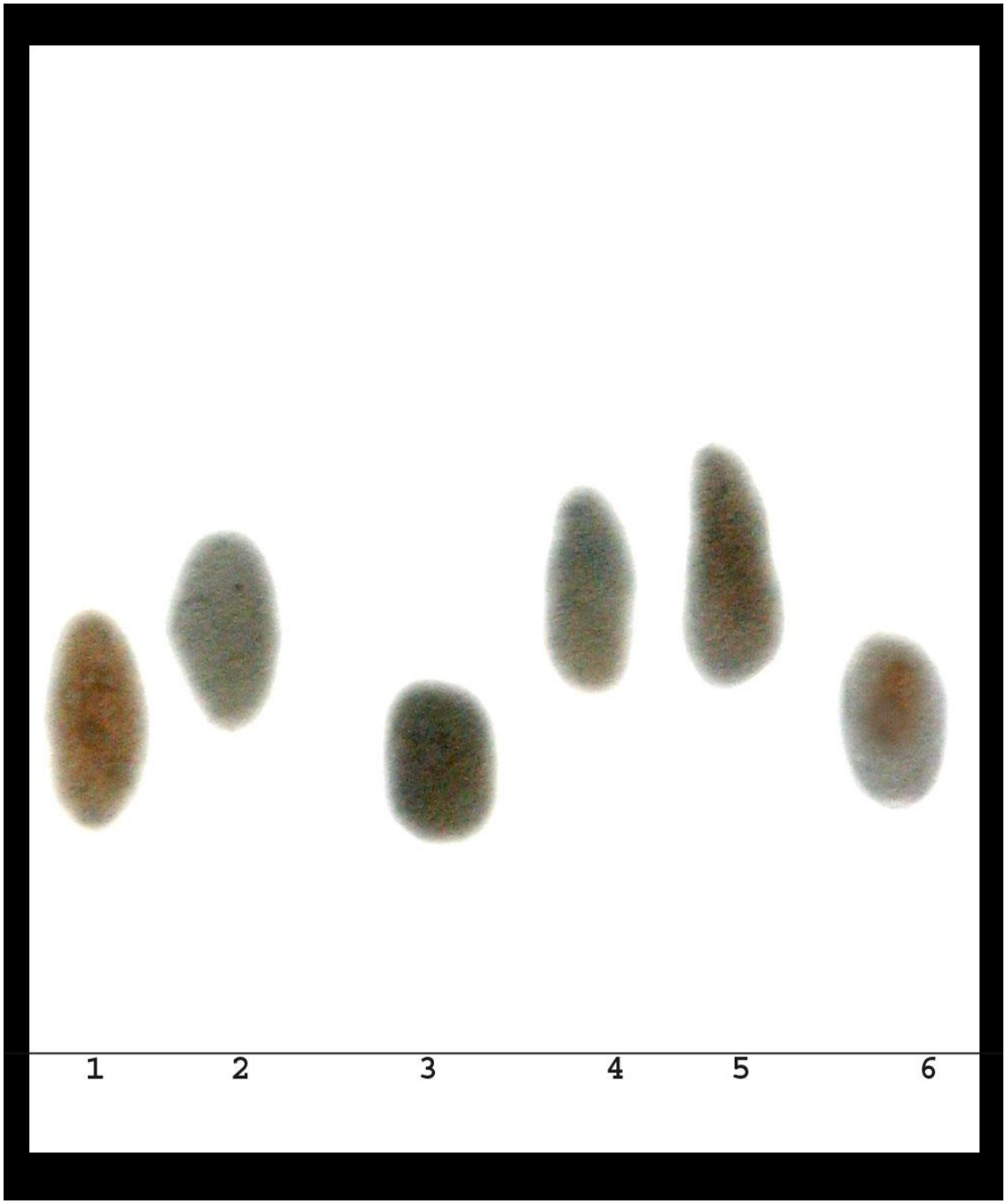

Figure 9.2. Paper chromatographic pattern of monomer forms of neutral polysaccharide using Whatman No:1 filter paper. Solvent system-butanol: acetic acid: water (2: 1: 1), Spray reagent-aniline diphenylamine phosphoric acid reagent. spot (1), (2), (3), (4) and (5) Glucose, Fructose, Galactose, Mannose and Xylose respectively, used as standards; Spot (6) Neutral fraction.

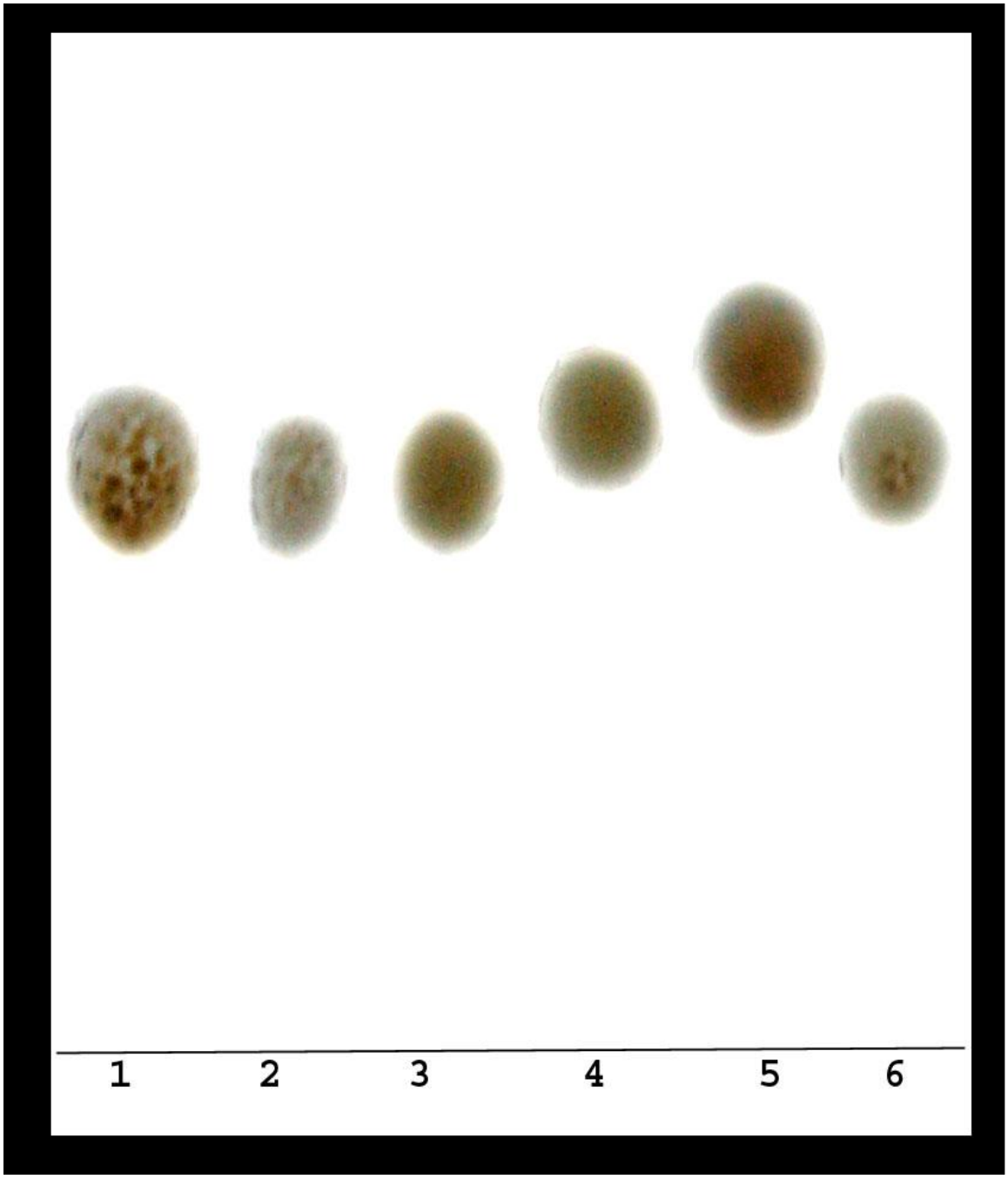

Figure 9.3. TLC pattern of monomer forms of neutral polysaccharide from PPC-*Pr* using silica gel G. Solvent system-butanol: acetic acid: water (2: 1: 1), Spray reagent-aniline diphenylamine phosphoric acid reagent. Spot (1), (2), (3), (4) and (5) Glucose, Fructose, Galactose, Mannose, Xylose respectively, used as standards; Spot (6)neutral fraction.

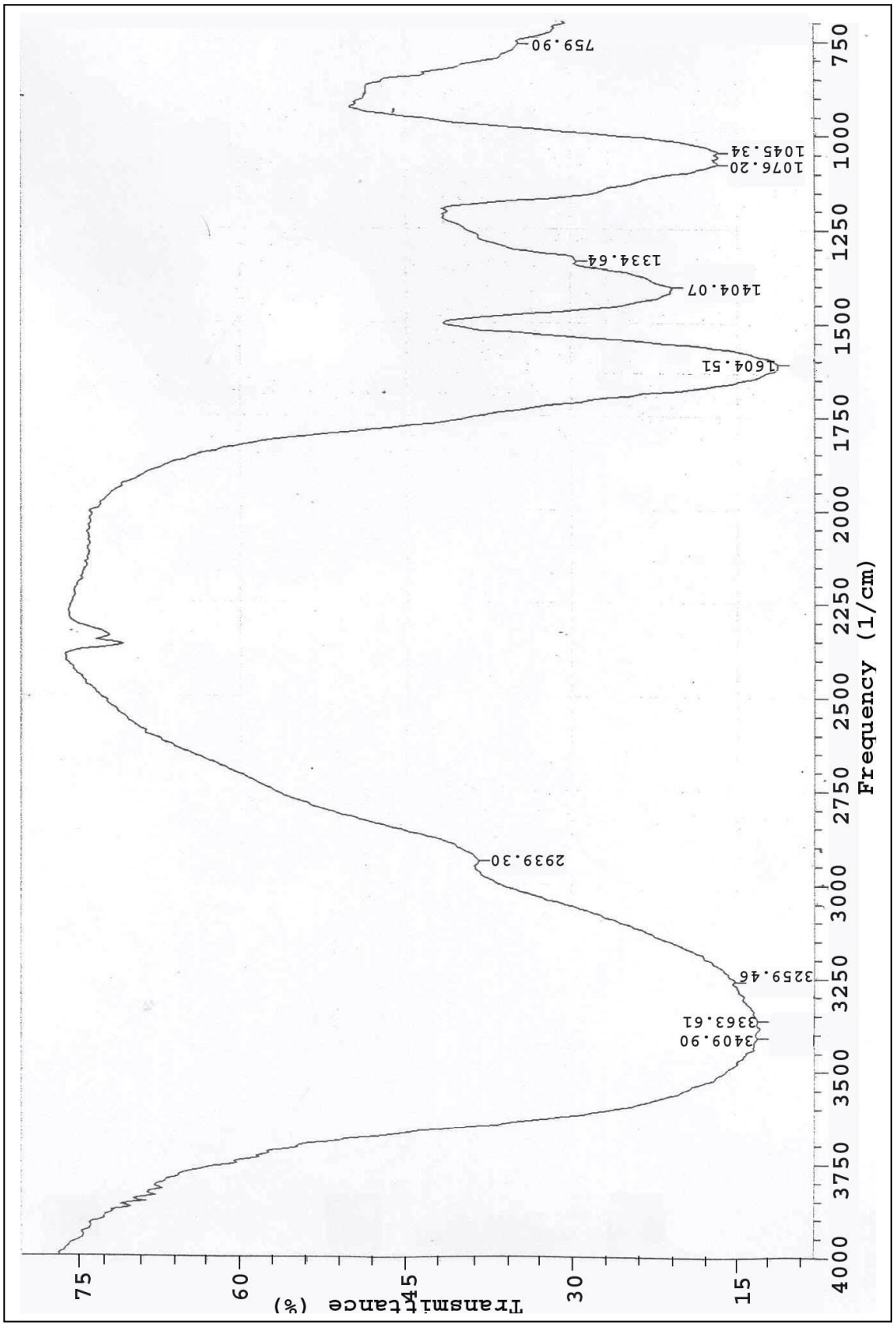

Figure 9.4. FT-IR spectrum of neutral polysaccharide isolated from PPC-P*r*

The ^{13}C NMR spectrum of neutral polysaccharide given in Figure 9.5 was similar to that of mushroom β-D-glucans. The cyclization of a monosaccharide renders the carbonyl carbon asymmetric. The ^{13}C spectrum of neutral polysaccharide showed exactly the number of lines equal to the number of carbons indicating that the molecule has no symmetry. This again confirmed the cyclic or pyranose form of glucose residues in neutral polysaccharide as well as the purity of the compound. The β configuration of the neutral polysaccharide was confirmed by peak in the region of 103.0 ppm. There was only one anomeric peak at 103.0 ppm in the ^{13}C spectrum; this suggested that the neutral polysaccharide contained one kind of configuration for the glucose residues, namely the β-configuration. Signal at 103.0 ppm also showed the presence of (1→6) β glycosidic bond. The presence of the D-(1→6) linked residues was also evidenced by low-intensity signal at 69.4 ppm. The peaks of C-1, C-2, C-3, C-4, C-5 and C-6 were 103.0, 73.0, 75.5, 69.4, 74.9 and 60.7 respectively. The signals of ^{1}H NMR were 4.46 ppm (β-C-1), 3.32 ppm (C-2), 3.59 ppm (C-3), 3.43 ppm (C-4), 3.68 ppm (C-5), and 3.83 ppm (C-6) and are shown in Figure 9.6. In the ^{1}H spectrum, signals at 1.2339 and 1.1716 were due to D_2O which was used as the solvent. Single large peak in the ^{1}H spectrum indicated the purity of the compound. Results thus, suggested that neutral fraction is a β-D (1→6) glucan. Result was also confirmed by Computer-assisted structural analysis of oligo- and polysaccharides (*An extension of CASPER to multibranched structures*). The assignments of the ^{1}H and ^{13}C NMR signals were based on comparisons with literature (Table 9.2.).

The elution profile of gel filtration chromatography is shown in Figure 9.7. The elution volume of standard size markers was in 60 ml (Blue Dextran), 140 ml (Dextran T-500), and 300 ml (Dextran-M.W. 60,000). The elution volume of neutral polysaccharide was 260 ml. Sephadex G 100 gel chromatography gave a value of 170,000 Da for the molecular mass of neutral polysaccharide, when compared to dextrans of known molecular weight.

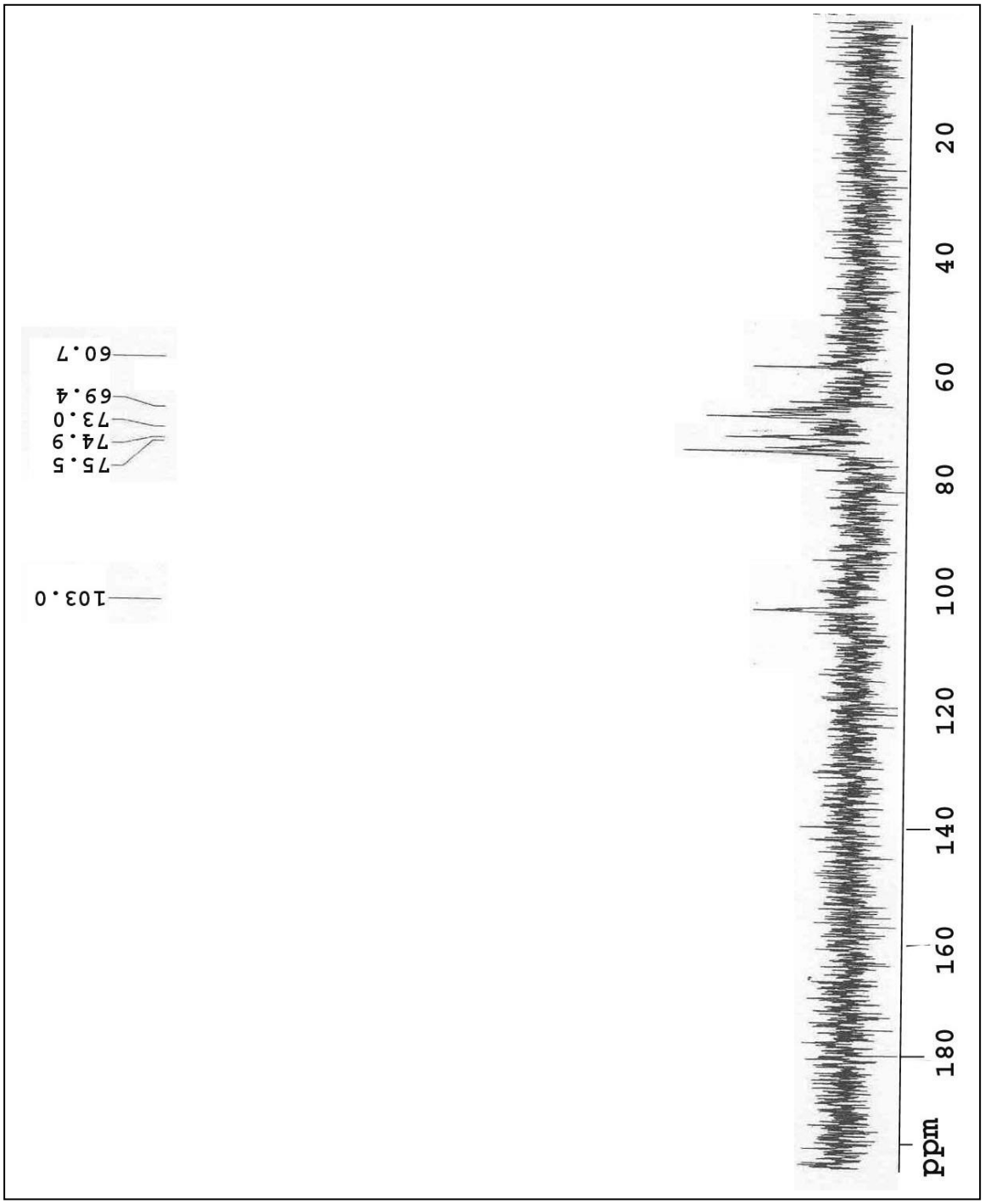

Figure 9.5. ^{13}C NMR spectrum of neutral polysaccharide in D_2O.

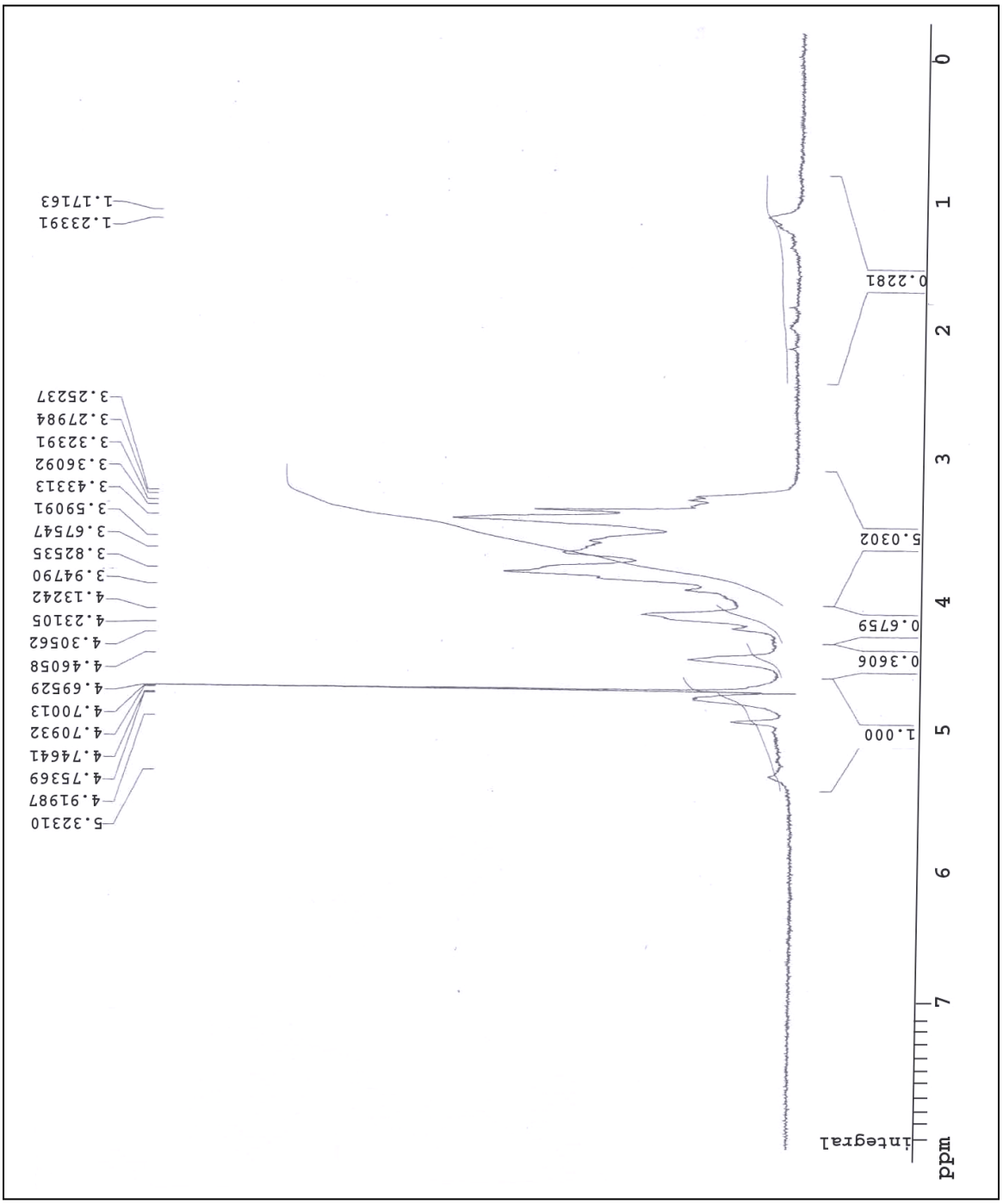

Figure 9.6. ^{1}H NMR spectrum of neutral polysaccharide in D_2O.

Table 9.2. Computer assisted structural analysis of neutral polysaccharide of PPC-Pr (Experimental results obtained were compared with literature data by Stenutz et al., 1998).

beta glucan

Simulated structure

→6)-β-D-Glc^{i}-(1→

→6)-β-D-Glc^{i}-(1→	103.69	73.96	76.66	70.57	75.84	69.53	
	4.53	3.34	3.51	3.47	3.62	3.88	4.20

Assignment of ^{1}H resonances

Experimental	Simulated	Exp-Sim	Assignment
4.46	4.53	-0.07	β-D-Glc^{i} - 1
4.23	4.20	0.03	β-D-Glc^{i} - 6'
3.83	3.88	-0.05	β-D-Glc^{i} - 6
3.68	3.62	0.06	β-D-Glc^{i} - 5
3.59	3.51	0.08	β-D-Glc^{i} - 3
3.43	3.47	-0.04	β-D-Glc^{i} - 4
3.32	3.34	-0.02	β-D-Glc^{i} - 2

Error 0.34 ppm (0.01/shift), Systematic error -0.00 ppm, RMS error=0.03 ppm

Assignment of ^{13}C resonances

Experimental	Simulated	Exp-Sim	Assignment
103.00	103.69	-0.69	β-D-Glc^{i} - 1
75.50	76.66	-1.16	β-D-Glc^{i} - 3
74.90	75.84	-0.94	β-D-Glc^{i} - 5
73.00	73.96	-0.96	β-D-Glc^{i} - 2
69.40	70.57	-1.17	β-D-Glc^{i} - 4
60.70	69.53	-8.83	β-D-Glc^{i} - 6

Error=13.75 ppm (2.29/shift), Systematic error=-2.29 ppm, RMS error=3.72 ppm

Experimental structure

→6)-β-D-Glc^{i}-(1→

→6)-β-D-Glc^{i}-(1→	103.00	73.00	75.50	69.40	74.90	60.70	
	4.46	3.32	3.59	3.43	3.68	3.83	4.23

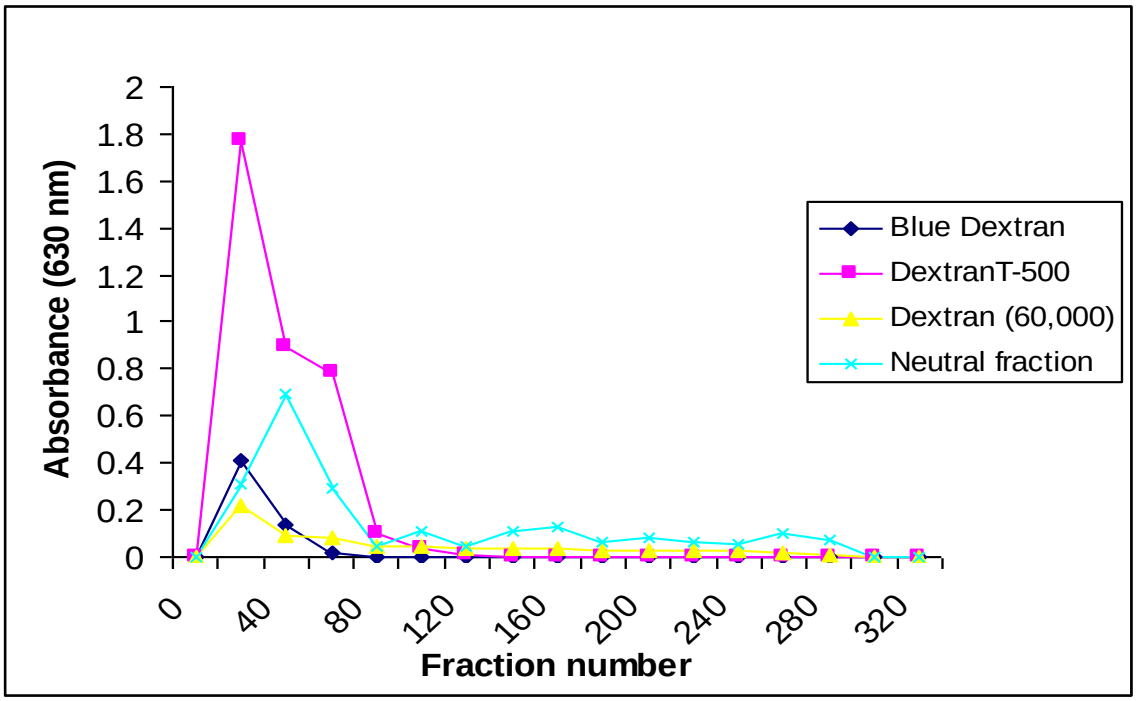

Figure 9.7. Gel filtration of neutral polysaccharide isolated from PPC-*Pr* on Sephadex G-100 column (2.2 x 50 cm). The column was eluted with deionized water and O.D. of collected fractions was measured at 630 nm by the Anthrone method.

The range of molecular mass of the neutral polysaccharide was determined by positive-ion MALDI-TOF spectrum. This analysis revealed the presence of several protonated molecular ions [Glc n + H] $^{+}$ and the corresponding mass spectrum is given in Figure 9.8. The mass of the singly charged molecular ion were calculated as 162.14n + 1.008 Da and 162.14n + 1.008 + 18.015 (mass of reducing end residue) Da respectively where n is the number of glucose units. For example, if n = 10, the mass of the corresponding glucan should be 1640.4 Da respectively. The MALDI-TOF mass spectrum showed that the neutral polysaccharide was composed of a mixture of glucose polymers with molecular weights around 17,264 ~178,326 Da. This pseudomolecular ion species had masses identical to those expected for a cyclic form of glucan composed of 96 to 990 glucose residues. Among the obtained signals, the ions at m/z 37135, 43267, 72483 and 141661 were most intense. These signals represented the main glucan species containing 206, 240, 402 and 786 glucose residues respectively with in the ring. The theoretically calculated mass for these native glucans were 33400, 38913, 65180 and 127442 Da respectively.

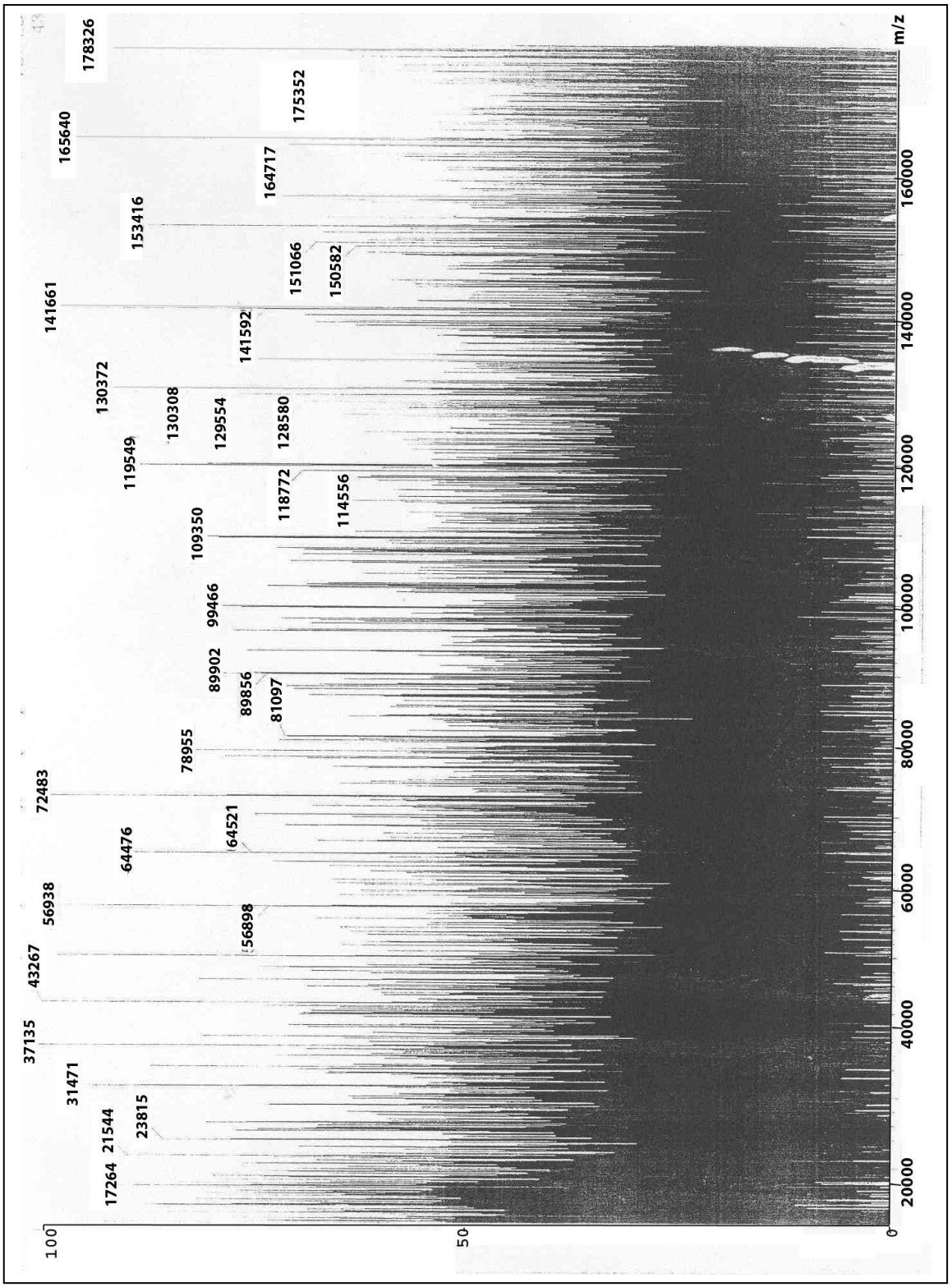

Figure 9.8. MALDI-TOF mass spectrum of neutral polysaccharide using 2, 5-DHB as matrix. The m/z values are reported as the nominal mass of the protonated molecular ions $[Glc\ n + H]^+$

9.4. Discussion

After two decades of intensive research on medicinal mushrooms, Mizuno and his co-workers in Japan developed reliable procedures for successful extraction, fractionation and purification of polysaccharides from fruiting bodies or mycelia. Polysaccharides extracted are further purified using a combination of techniques, such as ethanol concentration, fractional precipitation, acidic precipitation with acetic acid, ion-exchange chromatography, gel filtration and affinity chromatography. Basically, ion-exchange chromatography through DEAE-cellulose columns separates neutral polysaccharides from acidic ones (Mizuno, 1999). Neutral fraction isolated from the PPC-P*r* showed the presence of both polysaccharide and protein indicating it to be a protein bound polysaccharide. However, it predominantly consisted of polysaccharides and compared to the crude PPC-P*r*, it showed higher polysaccharide/ peptide ratios. The presence of D-glucose as the only monomer was detected by PC as well as TLC analysis. The results showed that neutral polysaccharide is a D-glucan with protein bound to it.

Characteristic absorption in the IR spectrum at 910 cm^{-1} was typical for β-glycosidic linkage between individual glycosyl residues and at 860 cm^{-1} for α-glycosidic linkage (Kim et al., 2003d). Neutral polysaccharide had the characteristics of IR spectrum absorption for β-glucan at 910 cm^{-1}. Absorption peak indicating the α- glycosidic linkage was absent, thus all the D-glucopyranose units of neutral polysaccharides were linked by β-glycosidic bonds.

NMR spectroscopy has proved to be an excellent method to retrieve structural information about β-glucan. One-dimensional ^{1}H and ^{13}C NMR spectra have been used in investigations of anomeric protons (Westerlund et al., 1993) and for comparison of β-glucans of different origins (Dais and Perlin, 1982). With two-dimensional NMR experiments more detailed information of the structural features of β-glucan can be obtained (Ensley et al., 1994). The presence of signals with a chemical shift above 102 ppm indicates β configuration of glucose (Usui et al., 1983). Signals at 92 to 96 ppm are characteristic for the C1 resonances of reducing glucose residues (Choma and Komaniecka, 2003). Neutral

polysaccharide produced a peak of C1 resonance at 103.0 in the ^{13}C NMR spectra and no signals were found at 92-96 ppm. These results confirmed the previous observation that the glucan molecules are cyclic and all glucose residues are β-linked. NMR signal around 100.5 indicate α – conformation of polysaccharide. Such a signal was absent in the NMR spectra of neutral polysaccharide.

The resonances at 82-83 ppm are due to C2 implicated in β-(1, 2) glycosidic linkages. The absence of a signal at 73 to 74 ppm indicates that all C2 carbons are involved in glycosidic bonds (Dell, 1983). Neutral polysaccharide produced no signal around 82-83 ppm, indicating the absence of β-(1, 2) glycosidic linkages and also the presence of resonance between 73-74 ppm showed that C2 carbons are not involved in glycosidic bonds. Based on literature data, the resonance due to C1 of Glc residues substituted at O-3 is observed at 101.7 ppm and signals at 100.0 ppm correspond to C1 Glc*p* (glucopyranose) residues linked by substitution at O-4 (Cui et al., 2006). Neutral polysaccharide produced no signals around 101.7 or 100.0 ppm, showing that the glucose residues are not connected by (1, 3) or (1, 4) glycosidic bonds. The broad C3 signal in the region of 81.4 ppm indicates the presence of D-(1, 3) linked glucan with branched D-(1, 6) residues. The presence of the D-(1, 6) linked residues is evidenced by signal around 103.0 ppm and the low intensity signal at 69.5 ppm (Kim et al., 2003d). No resonance was found in the region of 81.4 ppm in the NMR spectra of neutral polysaccharide showing that it does not belong to (1, 3) linked glucan with (1, 6) branches. The NMR spectra of neutral polysaccharide produced characteristic signal at 103.0 ppm and also low intensity signal at 69.4 ppm, hence the glucose residues of neutral polysaccharide are linked by β-(1, 6) glycosidic bonds. ^{13}C NMR spectrum displayed a simple pattern with six signals that could be assigned unambiguously to a β- (1→6)-linked D-glucan by reference with literature data (Table 9.3.). From all the analyses, the structure of the neutral polysaccharide was determined as that shown in Figure 9.9.

Table 9.3. Comparative ^{13}C & ^{1}H NMR data for neutral polysaccharide and other mushroom β-D (1→6) glucan from literature (Chauveau et al., 1996).

	C-1	**C-2**	**C-3**	**C-4**	**C-5**	**C-6**
N1 Glucan (from *Boletus erythropus*)	102.6	73.2	76.2	69.8	75.8	60.6
Neutral polysaccharide (from *Phellinus rimosus*)	103.0	73.0	75.5	69.4	74.9	60.7
	H-1	**H-2**	**H-3**	**H-4**	**H-5**	**H-6,6'**
N1 Glucan (from *Boletus erythropus*)	4.26	3.05	3.14	3.11	3.30	3.48 3.71
Neutral polysaccharide (from *Phellinus rimosus*)	4.46	3.32	3.59	3.43	3.68	3.83 4.23

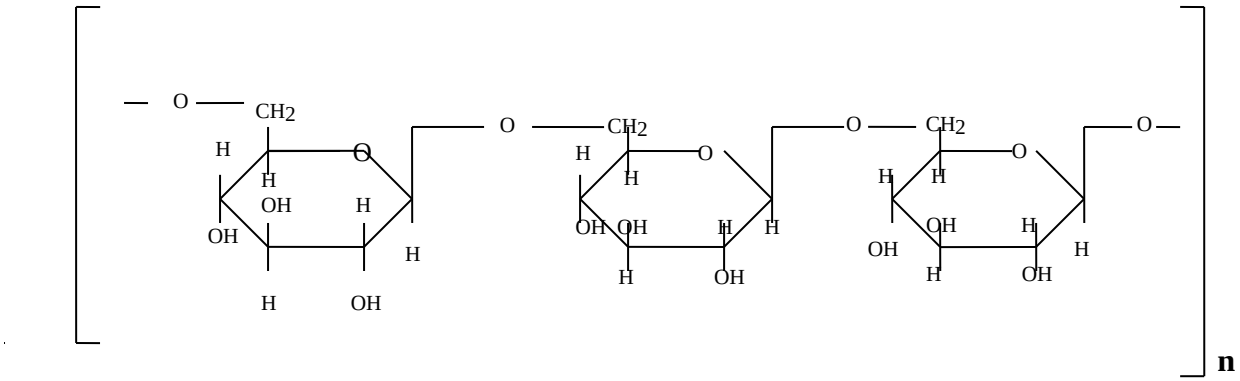

Figure 9.9. The possible structure of β-D-(1→6) glucan isolated from *P.rimosus*

In recent years, several mass spectroscopic studies have been focused on the analysis of polysaccharide materials or oligosaccharides. The ions produced by MALDI are predominantly singly charged molecular ions so that simple mass spectra can be used for distribution analysis of neutral polysaccharides (Harvey,

2008; Harvey, 2006; Ojima et al., 2005). Direct detection of saccharides in sulphuric acid/ phenol has been commonly used, but it can not derive information on molecular weights of different saccharides and the differences among pentoses, hexoses and unusual sugars. Gel permeation chromatography (GPC) has been used for measurements of the size of polysaccharides by comparison of results with appropriate polysaccharide standards (Deery et al., 2001). Nevertheless, the resolution of GPC is generally poor and the process is very time consuming. Since most polysaccharides are polydispersible to molecular weight, GPC is not a direct method for accurate mass measurements. On the other hand, MALDI-MS is practical for more qualitative mass determination (Hung et al., 2008). The gel permeation chromatography showed the molecular weight of neutral polysaccharide approximately as 170,000 Da. MALDI-TOF MS revealed that the molecular weight of neutral polysaccharide range up to 178,326 Da. This is in agreement with the molecular weight obtained by Sephadex G100 chromatography. The molecular weight of neutral polysaccharide was found to be 7 times smaller than that of the crude PPC-P*r*.

According to the above results, it was concluded that the neutral polysaccharide of *P.rimosus* contained predominantly water soluble β-D-glucan linked together by (1→6) glycosidic bonds and traces of protein bound to it. Their repeating units of structure were [→6)-β-D-Glc*p* (1→] $_n$. According to their estimated molecular weight, it is possible to assume that neutral polysaccharide contained 96 to 990 repeating units.

Chapter - 10

Biological properties of β-D-(1→6) glucan of *P.rimosus*

Synopsis

Biological properties like anti-tumor, anti-inflammatory and anti-oxidant activities of the β-D-(1→ 6) glucan of *P.rimosus* were studied. Anti-tumor activity was studied using EAC induced ascites and DLA induced solid tumor models. The compound was able to increase the life span of EAC induced ascetic tumor mice significantly. At a dose of 5 and 10 mg/kg, β-D-(1→ 6) glucan prevented the development of DLA induced solid tumor by 54.61 and 79.67 % respectively. Anti-inflammatory activity was studied using carrageenan, dextran induced acute and formalin induced chronic inflammatory models. β-D-(1→ 6) glucan significantly reduced the acute and chronic inflammation in a dose dependent manner. In both the acute and chronic inflammatory models, the activity of β-D-(1→ 6) glucan was found to be higher than the standard reference drug, diclofenac. Anti-oxidant activity was studied by evaluating superoxide radical, hydroxyl radical, nitric oxide, and 1,1-diphenyl 2-picryl hydrazyl (DPPH) radical scavenging activities, inhibition of lipid peroxidation and ferric reducing antioxidant power (FRAP). However, β-D-(1→ 6) glucan showed no significant anti-oxidant activity.

10.1. Introduction

The healing and immunostimulating properties of mushrooms have been known for thousands of years. These mushrooms contain biologically active polysaccharides that mostly belong to the group of β-glucans. β -D-glucan is a polysaccharide yielding exclusively D-glucose upon acid hydrolysis (Mizuno 1996; 1999). Glucans are present with different types of glycosidic linkages, such as (1→3), (1→6) – β-glucans and (1→3)-α-glucans, but some are true heteroglycans. The others mostly bind to protein residues as PSP complexes (PSPC) (Gorin and Barreto-Berger, 1983).

Wide varities of applications of β-glucan have been reported, including thickening and stabilizing agents in chemical industries, and immunostimulating

and anti-tumor agents in clinical uses (Czop and Kay, 1991; Yanaki and Kojima, 1981; Yanaki and Kojima, 1983). Apart from these applications, β-glucan has been used as a substance that enhances the skin's natural ability to heal and protect itself against infection (Mansell, 1994). β-glucan is now in use as a component of various cosmetics as a water-soluble particulate or its chemically modified soluble forms such as carboxymethyl or phospholyated glucan (Czop and Kay, 1991). Several different kinds of polysaccharides have been produced from the mushrooms and their diverse physiological activities have been elucidated. However, their applications have been concentrated on mainly medicinal uses such as anti-tumor and immunostimulating agents (Kim et al., 2001; Kuo et al., 1996; Shinha et al., 2001; Sone et al., 1985; Yang et al., 2000).

Carcinostatic mechanism of β-D-glucan is somewhat different from that of conventional chemotherapeutics for cancers, belonging to immunotherapeutics to inhibit or eliminate growth of cancer cells by activating and reinforcing immunological functions of the host. It means that anti-tumor polysaccharides are positioned in a sort of Biological Response Modifiers (BRM) based on their action mechanism, hardly showing adverse effects and drug induced sufferings. β-glucan can prevent oncogenesis due to the protective effect against potent genotoxic carcinogens. As immunostimulating agent, which acts through the activation of macrophages and Natural Killer (NK) cell cytotoxicity, β-glucan can inhibit tumor growth in promotion stage too. Anti-angiogenesis can be one of the pathways through which β-glucan can reduce tumor proliferation, prevent tumor metastasis. β-glucan as adjuvant to cancer chemotherapy and radiotherapy demonstrated the positive role in the restoration of hematopiesis following by bone marrow injury. Immunotherapy using monoclonal antibodies is a novel strategy of cancer treatment. These antibodies activate complement system and opsonize tumor cells with iC3b fragment. In contrast to microorganisms, tumor cells, as well as other host cells, lack β-glucan as a surface component and can not trigger complement receptor 3-dependent cellular cytotoxicity and initiate tumor-killing activity. This mechanism could be induced in the presence of β-glucans (Akramiene et al., 2007).

Biological properties of water soluble β-D-(1→ 6) glucan isolated from the crude polysaccharide protein complex of *P.rimosus* were studied. Murine cell lines EAC and DLA were used for the anti-tumor studies. Anti-inflammatory activity was studied using carrageenan, dextran induced acute and formalin induced chronic oedema models. Anti-oxidant activity was studied using various in vitro systems. β-D-(1→ 6) glucan of *P.rimosus* was found to possess significant anti-tumor and anti-inflammatory activities in a dose dependent manner, however, the anti-oxidant activity was very poor. The findings of the present study are given in this chapter.

10.2. Materials and methods

10.2.1. Test animals

Female Swiss albino mice (6-8 weeks old) of weight 20 ± 5g were used for the anti-tumor and anti-inflammatory studies.

10.2.2. Cell lines

Ehrlich's ascites carcinoma (EAC) and Dalton's lymphoma ascites (DLA) cell lines as described in section 5.2.2 were used for the anti-tumor studies.

10.2.3. Isolation of β-D-(1→ 6) glucan from PPC-*Pr*

β-D-(1→ 6) glucan was isolated from PPC-*Pr* as described in section 9.2.1.

10.2.4. Determination of anti-tumor activity

The test compound β-D-(1→ 6) glucan at a dose of 5 and 10 mg/kg body weight as i.p. was used for the anti-tumor studies.

10.2.4.1. Ascites tumor model

The ability of β-D-(1→ 6) glucan to inhibit ascites tumor was studied using the method described in section 5.2.4

10.2.4.2. Solid tumor model (Preventive effect)

Effect of β-D-(1→ 6) glucan on solid tumor induced by DLA cell line was studied using the method described in section 5.2.5.

10.2.5. Determination of anti-inflammatory activity

Above mentioned doses of β-D-(1→ 6) glucan as i.p. was used for the anti-inflammatory experiments.

10.2.5.1. Carrageenan induced acute paw oedema model

Effect of β-D-(1→ 6) glucan on carrageenan induced acute inflammation was studied using the method described in section 7.2.3.1.

10.2.5.2. Dextran induced acute paw oedema model

Effect of β-D-(1→ 6) glucan on dextran induced acute inflammation was studied as described in section 7.2.3.2.

10.2.5.3. Formalin induced chronic paw oedema model

Effect of β-D-(1→ 6) glucan on formalin induced chronic inflammation was studied as described in section 7.2.3.3.

10.2.6. Determination of anti-oxidant activity

The anti-oxidant activity of β-D-(1→ 6) glucan was determined by evaluating superoxide radical, hydroxyl radical, nitric oxide, and 1,1-diphenyl 2-picryl hydrazyl (DPPH) radical scavenging activities, inhibition of lipid peroxidation and ferric reducing antioxidant power (FRAP) as described in section 6.2.2. to 6.2.7.

10.3. Results

In the ascites tumor model, β-D-(1→ 6) glucan significantly increased the life span of EAC induced ascetic mice. β-D-(1→ 6) glucan administration at a dose of 5 and 10 mg/kg increased the life span of animals by 41.86 % and 52.71 % ($P<0.001$) respectively (Table 10.1.). The β-D-(1→ 6) glucan showed significant preventive activity against DLA induced solid tumor model in a dose-dependent manner (Table 10.2.). The β-D-(1→ 6) glucan at a dose of 5 mg/kg and 10 mg/kg could prevent 54.61 % and 79.67 % of solid tumor growth respectively in tumor induced mice. The weight and volume of tumor in β-D-(1→ 6) glucan treated groups at the end of fifth week was significantly lower ($P<0.001$) than the control group. It was found that the ablility of β-D-(1→ 6) glucan to prevent the tumor proliferation at a dose of 10 mg/kg was comparable to the activity of the standard reference drug cisplatin (Figure 10.1.).

The β-D-(1→ 6) glucan showed significant anti-inflammatory activity against acute and chronic models of inflammation in a dose dependent manner (Table 10.3.). In the carrageenan acute inflammatory model, the activity of β-D-

(1→ 6) glucan at a dose of 5 & 10 mg/kg was found to be higher than that of the reference drug, diclofenac at a dose of 10 mg/kg (Figure 10.2.). In the dextran model, the activity of β-D-(1→ 6) glucan at a dose of 5 & 10 mg/kg was equal or higher than the reference drug, diclofenac (Figure 10.3.). The β-D-(1→ 6) glucan was also effective in ameliorating formalin induced chronic inflammation (P<0.001). The inhibitory activity of β-D-(1→ 6) glucan at a dose of 5 and 10 mg/kg was found to be higher than the activity of reference drug, diclofenac (Figure 10.4.). β-D-(1→ 6) glucan showed no significant anti-oxidant or free radical scavenging activity up to a tested concentration of 2mg/ml.

Table 10.1. Effect of β-D-(1→ 6) glucan on EAC induced Ascites tumor model

Group	**Treatments (mg/kg)**	**Survival time (days)**	**% increase in life span**	**Mortalilty at 30th day**
Control	Normal Saline	21.5±1.38		6/6
Cisplatin	2	35.0*	62.97	0/6
β-D-(1→ 6) glucan	5	30.5 ± 2.94*	41.86	2/6
β-D-(1→ 6) glucan	10	32.8 ± 2.40*	52.71	1/6

Values are mean ± SD, n = 6 ; *P<0.001 significant with respect to control.

Table 10.2. Effect of β-D-(1→ 6) glucan on DLA induced preventive solid tumor model

Group	**Treatments (mg/kg)**	**Vol on 5th week (cm^3)**	**Weight of tumor (g)**	**% of inhibition**
Control	Normal Saline	4.67	5.07	
Cisplatin	4	0.12	0.54 ± 0.34*	89.28
β-D-(1→ 6) glucan	5	1.57	2.30 ± 0.39*	54.61
β-D-(1→ 6) glucan	10	0.21	1.03 ± 0.35*	79.67

Values are mean ± SD, n = 6; *P<0.001 significant with respect to control.

Table 10.3. Effect of β-D-(1→ 6) glucan on carrageenan, dextran and formalin induced paw oedema models

Groups	**Treatment** (mg/kg) i.p.	**Initial paw thickness** (cm)	**Final paw thickness** (cm)	**Increase in paw thickness** (cm)	**Percent inhibition (%)**
Carrageenan model [a]					
Control	Normal saline	0.210± 0	0.317±0.015	0.107± 0.015	
Diclofenac	10	0.215± 0.005	0.272± 0.015	0.057± 0.016	47.04
β-D- glucan	5	0.212± 0.004	0.247 ±0.010	0.035±0.010	67.29
β-D- glucan	10	0.208 ±0.004	0.237 ±0.005	0.028 ±0.008	73.52
Dextran model [b]					
Control	Normal saline	0.215±0.005	0.335± 0.013	0.012± 0.012	
Diclofenac	10	0.215±0.006	0.283± 0.015	0.068± 0.010	43.75
β-D- glucan	5	0.215±0.006	0.282± 0.005	0.068± 0.005	43.75
β-D- glucan	10	0.230±0.008	0.280± 0.008	0.050± 0.014	58.33
Formalin model [c]					
Control	Normal Saline	0.200±0.025	0.410±0.014	0.210± 0.018	
Diclofenac	10	0.200± 0.023	0.323± 0.005	0.123± 0.021	41.67
β-D- glucan	5	0.180± 0	0.287±0.005	0.107±0.005	48.81
β-D- glucan	10	0.180 ±0	0.260 ±0.008	0.080 ±0.008	61.90

Values are mean ± SD, n = 6

[a, b] Paw thickness was measured after 3 hrs

[c] Paw thickness was measured after 6 days

* p values <0.001 compared to control

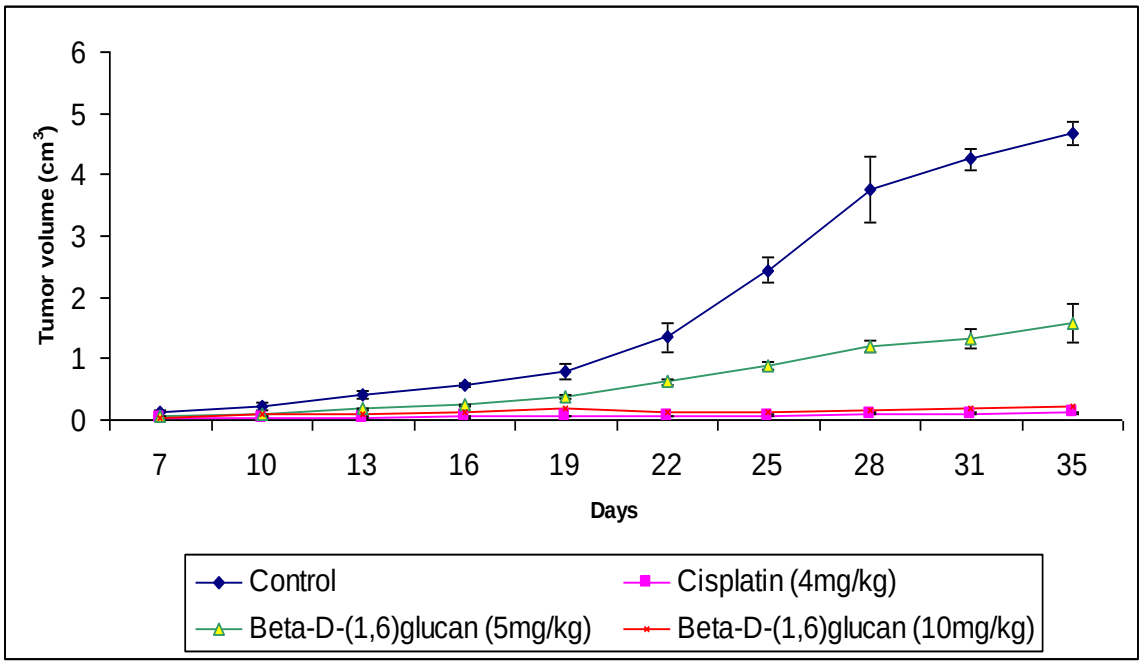

Figure 10.1. Inhibition of tumor development by β-D-(1→ 6) glucan at dose of 5 and 10 mg/kg in DLA induced preventive solid tumor model. Control group received vehicle (normal saline) only. Cisplatin at a dose of 4 mg/kg was used as standard. Drug was administered as i.p. for 10 consecutive days starting 24 h after tumor implantation. Values are mean ± SD, n=6.

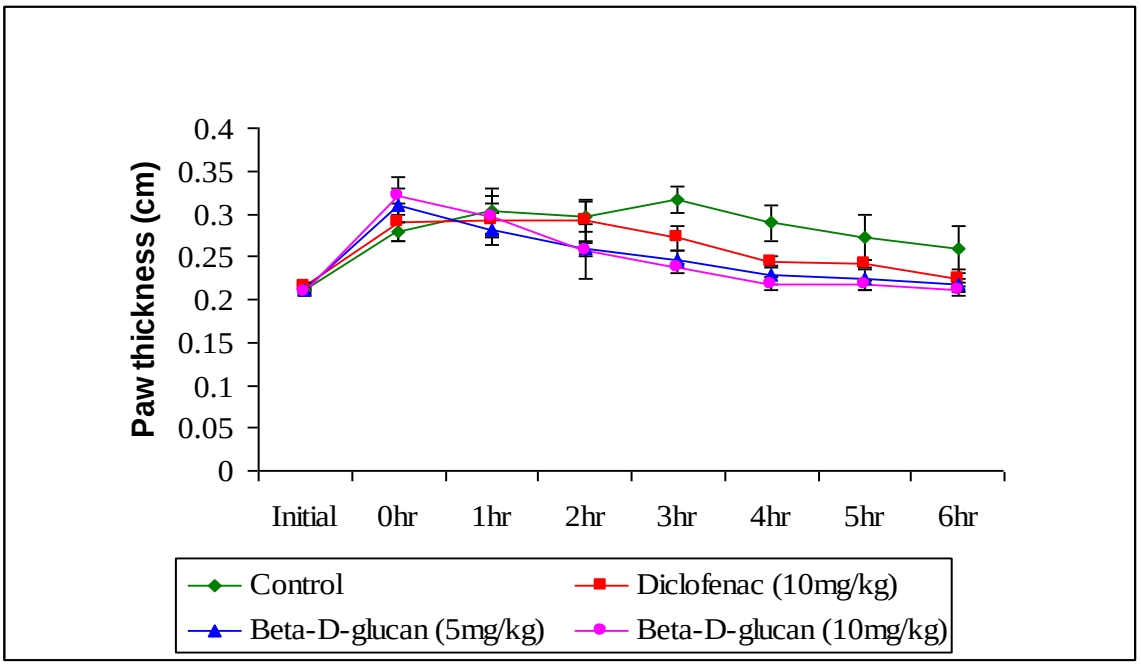

Figure 10.2. Inhibition of carrageenan induced acute inflammation by β-D-(1→ 6) glucan. Control group received vehicle (normal saline) only. Diclofenac at a dose of 10 mg/kg was used as standard. B-glucan at dose of 5 and 10 mg/kg was administered as i.p. 1 hr before the induction of oedema. Values are mean ± SD, n=6.

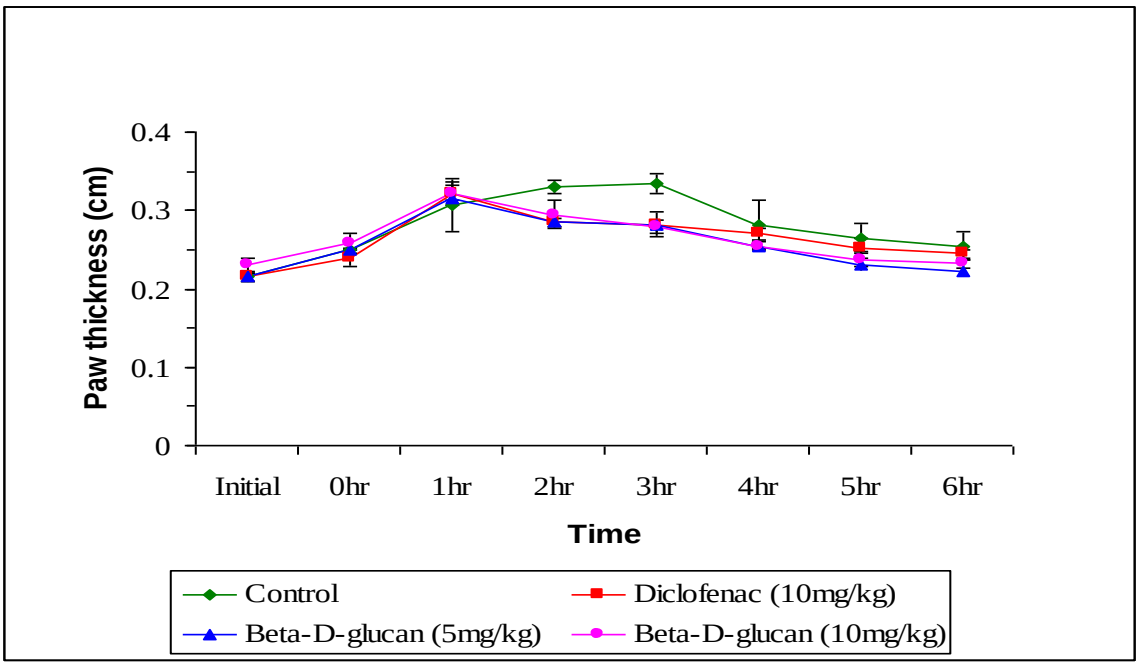

Figure 10.3. Inhibition of dextran induced acute inflammation by β-D-(1→ 6) glucan. Control group received vehicle (normal saline) only. Diclofenac at a dose of 10 mg/kg was used as standard. β-D-(1→ 6) glucan at dose of 5 and 10 mg/kg was administered as i.p. 1 hr before the induction of oedema. Values are mean ± SD, n=6.

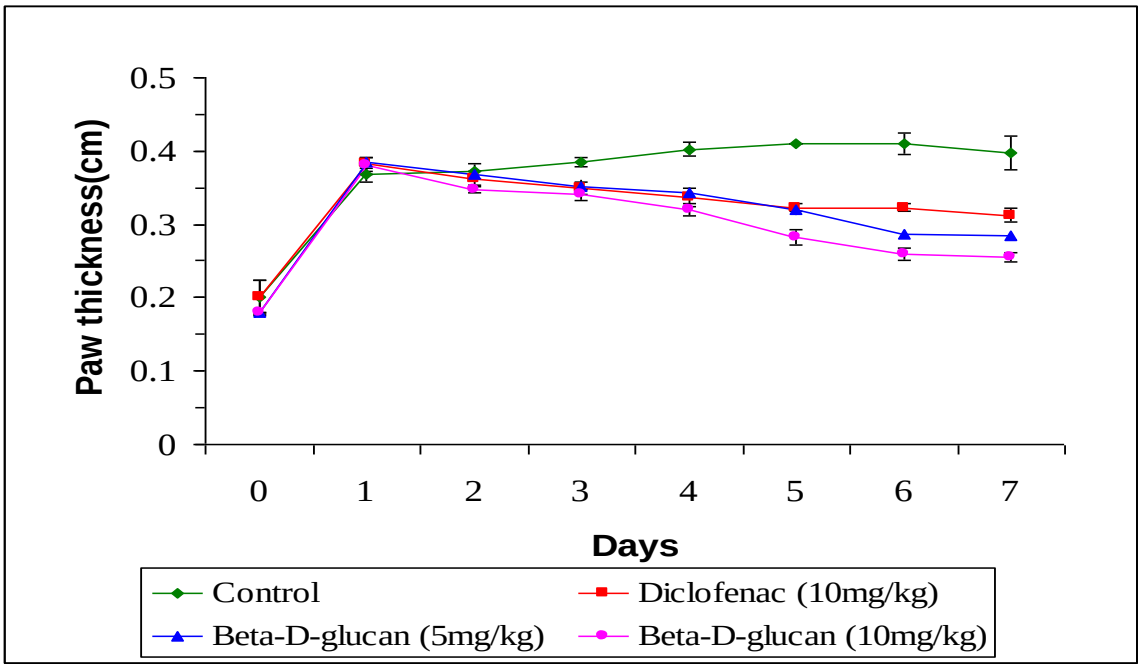

Figure 10.4. Inhibition of formalin induced chronic inflammation by β-D-(1→ 6) glucan. Control group received vehicle (normal saline) only. Diclofenac at a dose of 10 mg/kg was used as standard. β-D-(1→ 6) glucan at dose of 5 and 10 mg/kg was administered as i.p. 1 hr before the induction of oedema and continued for 6 consecutive days. Values are mean ± SD, n=6.

10.4. Discussion

Mushrooms contain biologically active polysaccharides in fruit bodies and cultured mycelium. These polysaccharides are of different chemical composition, with most belonging to the group of β-glucans and they have attracted the most attention (Wasser, 2002). Based on their mode of action, β-glucans are grouped under Biological Response Modifiers (BRM). The induction of cellular responses by mushroom and other β-glucans is likely to involve their specific interaction with one or more cell surface receptors. This has been the focus of intense research in recent years. Previously reviewed studies suggested that the complement receptor type 3 (CR3 = CD11b/CD18) was a prime candidate for the β-glucan receptor on human monocytes, neutrophils, and NK cells. The β-glucan receptor site was localized to a lectin site C-terminal of the I-domain of CD11b (Thornton et al., 1996). The same site was identified as the β-glucan receptor on mouse leukocytes, which exhibited functional characteristics comparable with those of the human equivalent (Xia et al., 1999). The incubation of NK cells or neutrophils with small soluble β-glucans primed the CR3 receptor in such a way to enhance the cytotoxicity against iC3b-opsonized target cells that were otherwise resistant to CR3-mediated cytotoxicity (Xia et al., 1999; Vetvicka et al., 1997). Thus, CR3 plays a dual role, and its role as a receptor for certain β-glucans primes it for its function as an iC3b-receptor for iC3b- coated target cells. This mechanism was subsequently shown to be involved in the anti-tumor activity of soluble polysaccharides. Several lines of evidence suggest that there is more than one β-glucan receptor and that, whereas some cells express only one type, others have atleast two (Mueller et al., 2000; Lowe et al., 2001). In recent years several candidates in addition to CR3 have been identified. These include dectin-1 and its human homologue, sometimes referred to as the β-glucan receptor (βGR) (Gantner et al., 2003), a scavenger receptor (Pearson et al., 1995) and glycosphingolipid lactosylceramide (Wakshull et al., 1999; Zimmerman et al., 1998).

Many mushroom polysaccharides with biological activities have been reported (Peng et al., 2003), most of which are composed of β- (1 → 3) (1 → 6)-linked-glucan or β- (1 → 6)-linked-glucan. Polysaccharides with anti-tumor action

differ greatly in their chemical composition and configuration, as well as their physical properties. Differences in activity can be correlated with solubility in water, size of molecules, branching rate and form. High molecular weight glucans appear to be more effective than those of low molecular weight (Mizuno, 1996, 1999). Present study revealed the significant anti-tumor activity of β-D-(1→ 6) glucan of *P.rimosus*. From the stand point of structure, the anti-tumor activity of the neutral fraction seemed to correlate positively with amount of β-D-(1→ 6) glucan bound with protein. In addition, relatively high molecular mass and better water solubility seemed to increase the anti-tumor activities of this polysaccharide.

Wang et al. (2005) correlated the anti-tumor activity with the total polysaccharide content. The study reports that among various polysaccharide fractions isolated from *Cordyceps Sinensis*, the best anti-tumor activity was displayed by the fraction with highest total polysaccharide content. This result was similar to various published data. By analyzing the compositions of the crude polysaccharide protein complex (PPC-*Pr*) and neutral fraction of *P.rimosus*, it was discovered that total polysaccharide content displayed a strong correlation with the anti-tumor efficacy. Among the polysaccharides isolated from *P.rimosus*, the neutral fraction had the highest polysaccharide content (92.3 %) compared to the crude PPC-*Pr* (54.8 %) and even at low dose (5 &10 mg/kg), it displayed the best anti-tumor activity almost comparable to the activity of crude PPC-*Pr* at a higher dose of 25 & 50 mg/kg. Therefore, the total polysaccharide content of β-D-(1→ 6) glucan also seemed to potentiate its anti-tumor activity.

According to the literature, several glucan type polysaccharides were shown to possess anti-inflammatory activity (Czarnecki and Grzybek, 1995). The immunostimulatory or anti-oxidant activities are mainly responsible for the anti-inflammatory activities of polysaccharides. Several studies suggested that the mechanisms of action of the glucan type polysaccharides might be due to their action on the reticuloendothelial system, through phagocytosis stimulation, along with an effect on vascular reactions, affecting capillary permeability (Whistler et al., 1976). Since β-D-(1→ 6) glucan of *P.rimosus* showed no significant anti-oxidant activity, its immunomodulatory properties may be responsible for the anti-

inflammatory activity.

The association of protein with the polysaccharide molecules is known to potentiate their anti-oxidant activity. Protein bound polysaccharides have higher free radical scavenging activity than polysaccharides alone (Liu et al., 1997). Present study showed that the β-D-(1→ 6) glucan possessed no significant anti-oxidant activity. On the other hand, the crude PPC-*Pr* of *P.rimosus* showed significant anti-oxidant activity in a dose dependent manner. The protein content of β-D-(1→ 6) glucan was very less (7.5%) compared to the crude PPC-*Pr* (28.6 %). This may be the reason for lack of anti-oxidant activity of β-D-(1→ 6) glucan of *P.rimosus.*

In conclusion, β-D-(1→ 6) glucan of *P.rimosus* showed significant anti-tumor and anti-inflammatory activities in a dose dependent manner. High carbohydrate content, high molecular weight and water solubility may be responsible for these profound activities. The β-D-(1→ 6) glucan showed no anti-oxidant activity which may be due to its less protein content. Since β-D-(1→ 6) glucan had no anti-oxidant property; immunostimulation may be the possible mechanism behind its potent biological activities.

Chapter- 11

Summary and conclusions

The goal of the present study was to isolate and characterize the crude as well as the neutral polysaccharides from the fruiting bodies of the medicinal mushroom *Phellinus rimosus* (Berk) Pilat and to study its antineoplastic properties. An attempt has been also made to elucidate the possible mechanisms behind the anti-tumor activities of these polysaccharides. In Chapter 1, general introduction to cancer and role of mushrooms in cancer treatment are briefly covered. In Chapter 2, epidemiology and etiology of cancer as well as the relationship of free radicals, inflammation and apoptosis to neoplasia are discussed in detail. This chapter also covers the pharmacological properties of mushrooms, important mushroom products used in cancer treatment and also the medicinal properties of *Phellinus* species. In Chapter 3, the materials and methods of the present study are described.

Recent improvements in chemical technology have allowed the isolation and purification of the relevant compounds, especially the polysaccharides, which contain demonstrable anti-cancer activities. The anti-tumor polysaccharides isolated from mushrooms are either water-soluble β-D-glucans, β-D-glucans with heterosaccharide chains of xylose, mannose, galactose or uronic acid or β-D-glucan-protein complexes. A crude polysaccharide fraction was isolated from the fruiting bodies of *P. rimosus* and priliminary characterization was done. Details are given in Chapter-4. Saprocarps of *P.rimosus* growing on the jackfruit tree trunks were collected from the outskirts of Thrissur, Kerala, India. The isolation of crude polysaccharide was achieved by hot water extraction, filtration, solvent precipitation, deproteinization, dialysis and freeze-drying. The quantitative analysis showed that the isolated compound contained not only polysaccharides (54.8%), but also the proteins (28.6 %), indicating it to be a protein – bound polysaccharide (PPC-*Pr*). However, the concentration of polysaccharide varies depending upon the development stage of the mushroom fruit body and also to the time after harvest and subsequent storage conditions. The PC and TLC analysis of

PPC-*Pr* revealed that it was made up of only D-glucose as monomers. The aminoacid profile analysis showed the presence of large amounts of aspartic acid, glutamic acid, alanine, glycine and serine. The molecular weight of PPC-*Pr* was approximately 1,200,000 Da by Gel permeation chromatography using Sephadex G -100 column and was completely soluble in water. Thus the PPC-*Pr* is a water soluble D-glucan-protein complex or proteoglycan of high molecular weight.

In Chapter 5, the anti-tumor activity of PPC-*Pr* is discussed. Anti-tumor activity of PPC-*Pr* was studied using the EAC and DLA murine cell lines. PPC-*Pr* administration considerably increased the life span of EAC induced ascetic tumor mice. PPC-*Pr* also showed significant preventive and curative effects on solid tumor induced by DLA cell line. PPC-*Pr* at a dose of 50 mg/kg was able to prevent the tumor proliferation as effectively as the standard reference drug Cisplatin. Thus, PPC-*Pr* possessed profound anti-tumor activity. The anti-tumor activity of PPC-*Pr* seemed to be correlated with its higher molecular weight, greater solubility in water and protein bound nature. There have been extensive in vivo studies demonstrating the anti-cancer activity of the glucan polysaccharides and polysaccharide-peptides in animal models. These studies strongly suggest an immunomodulating mode of action. Mushroom polysaccharides act as nonspecific immune system stimulants. Hence, anti-tumor activity exerted by PPC-*Pr* might be mainly through the stimulation of the host's defense mechanism. To elucidate the possible mode of action of PPC-*Pr* other than immunostimulation, the properties like anti-oxidant and free radical scavenging, anti-inflammatory, cytotoxic, anti-proliferative and apoptotic activities of PPC-*Pr* were studied.

The cumulative production of ROS/RNS through either endogenous or exogenous insults is termed oxidative stress and is common for many types of cancer cell that are linked with altered redox regulation of cellular signalling pathways. Metabolic activation of carcinogen is a free radical-dependent reaction. Many anti-oxidants are being identified as anti-carcinogens. Characterizing and optimizing such defence systems may be an important part of a strategy of minimizing cancer incidence and progression. In Chapter 6, anti-oxidant as well as free radical scavenging properties of PPC-*Pr* is described in detail. The anti-

oxidant activity of PPC- P*r* was determined by evaluating superoxide radical, hydroxyl radical, nitric oxide, and 1,1-diphenyl 2-picryl hydrazyl (DPPH) radical scavenging activities, inhibition of lipid peroxidation and ferric reducing antioxidant power (FRAP). PPC-*Pr* was found to be effective in scavenging superoxide, hydroxyl, nitric oxide radicals and also the DPPH free radical. It significantly inhibited the lipid peroxidation and FRAP assay showed the reducing ability of PPC-*Pr*. The association of protein with the polysaccharide molecules is known to potentiate their free radical scavenging activity. Hence, the protein bound nature of PPC-P*r* might be contributing significantly for its profound free radical scavenging and anti-oxidant activities.

Chronic inflammation has long been recognized as a risk factor for human cancer at various sites. Free radicals are closely associated with the damages of connective tissues in inflammation. It is becoming apparent that in addition to promoting direct toxicity, reactive oxygen metabolites may also initiate or amplify inflammation via the upregulation of several different genes involved in the inflammatory response. Development of agents with the ability to scavenge these deleterious oxyradicals present in the chronic inflammation sites is a necessary basis to develop new strategies for cancer prevention by modulating the process of inflammation. The anti inflammatory activity as well as the in vivo anti-oxidant activity of PPC-*Pr* is covered in Chapter 7. The anti-inflammatory activity of the PPC-*Pr* was determined by carrageenan, dextran induced acute and formalin, FCA induced chronic inflammatory models. The PPC- P*r* showed strong anti-inflammatory activity in both the acute and chronic inflammatory models in a dose dependent manner. The activity of PPC- P*r* was comparable to the activity of clinically used standard drug, diclofenac. The in vivo anti-oxidant activity of PPC-*Pr* was evaluated in FCA induced chronic rats. Lipid peroxides formation as well as altered levels of endogenous scavengers like superoxide dismutase, glutathione peroxidase and reduced glutathione were taken as an indirect *in vivo* evidence for the participation of free radicals in the progression of chronic inflammation. The increased lipid peroxide level in plasma and the elevated level of antioxidant enzymes superoxide dismutase and glutathione peroxidase were found in FCA

chronic rats. Blood glutathione was decreased in FCA control group. The above alterations produced in FCA induced animals were brought to normal by PPC-*Pr* in a dose dependent manner.

Apoptosis plays a necessary role as a protective mechanism against carcinogenesis by eliminating damaged cells or abnormal excess cells proliferated owing to various chemical agents' induction. Emerging evidence has demonstrated that the anti-cancer activities of certain chemotherapeutic agents are involved in the induction of apoptosis, which is regarded as the preferred way to manage cancer. In Chapter 8, the cytotoxic, anti-proliferative and apoptotic activities of PPC-*Pr* are discussed. PPC-*Pr* produced no direct cytotoxicity in the short- term assay against EAC and DLA murine cell lines. However, PPC-*Pr* was able to induce significant cytotoxicity against HCT 116 colon cancer cell line by long- term incubation. PPC-*Pr* inhibited the proliferation of HCT 116 cells considerably at a dose of 500 and 1000μg/ml. The characteristic changes of apoptosis were observed in the morphology of PPC-*Pr* treated cells by DAPI and AO/EB staining methods. Chromatin degradation into multiple internucleosomal fragments is a distinct biochemical hallmark for apoptosis and can be easily detected by Comet assay. Comet assay revealed that the apoptotic nuclei were more frequent in PPC-*Pr* treated cells than the control. Thus, by long term incubation, PPC-*Pr* induced cytotoxicity and inhibited the tumor cell proliferation in vitro by the induction of apoptosis.

In Chapter 9, the isolation of neutral polysaccharide from the PPC-*Pr* and its chemical characterization are explained. A thorough analysis of the chemical composition of the extract under investigation would not only enhance reproducibility, but would eventually make it possible to correlate specific chemical constituents or combinations of constituents with particular biological activities. Neutral polysaccharide was isolated from PPC-*Pr* by fractionation using CTA-OH, solvent precipitation by chilled ethanol, deproteinization, DEAE-cellulose chromatography, dialysis and freeze-drying. Neutral polysaccharide was composed of 92.3 % polysaccharide and 7.5 % protein. The PC and TLC analysis showed that D-glucose was only present as the monomers. IR spectrum showed

absorption characteristic of β-glucan at 910 cm $^{-1}$. ^{1}H and ^{13}C NMR spectroscopy showed that the neutral fraction was a (1→6)-D-glucan with β- linkages. The molecular weight determination of the compound by gel filtration chromatography using Sephadex G 100 showed molecular weight of neutral fraction as approximately 170,000 Da. MALDI-TOF analysis revealed that the molecular weight of neutral fraction range between 17,264 to 178,326 Da and the degree of polymerization was estimated to be in the range of 96 to 990. Thus, neutral polysaccharide of *P.rimosus* is a water soluble, high molecular weight noble molecule, β-D-(1→6) glucan bound to protein.

Biological properties like anti-tumor, anti-inflammatory and anti-oxidant activities of the β-D-(1→ 6) glucan of *P.rimosus* are described in Chapter 10. The β-D-(1→ 6) glucan showed significant anti-tumor activity in a dose dependent manner. The compound was able to increase the life span of EAC induced ascetic tumor mice significantly. At a dose of 5 and 10 mg/kg, β-D-(1→ 6) glucan prevented the development of DLA induced solid tumor by 54.61 and 79.67 % respectively. The β-D-(1→ 6) glucan displayed significant anti-tumor activity even at lower doses (5 &10 mg/kg), almost comparable to the activity of PPC-*Pr* at a higher dose of 25 & 50 mg/kg. The β-D-(1→ 6) glucan had the highest polysaccharide content (92.3 %) compared to the crude PPC-*Pr* (54.8 %) and the profound anti-tumor activity seemed to be correlated to its high total polysaccharide content. β-D-(1→ 6) glucan also significantly reduced the acute and chronic inflammation in a dose dependent manner. In both the acute and chronic inflammatory models, the activity of β-D-(1→ 6) glucan was found to be higher than the standard reference drug, diclofenac. However, β-D-(1→ 6) glucan showed no significant anti-oxidant activity.

Protein bound polysaccharides appear to be nontoxic in prolonged use and are claimed to benefit general health. PPC-P*r* and β-D-(1→ 6) glucan of *P.rimosus* produced no toxic effects on the experimental animals and can be safe compounds for prolonged use. A wide range of biologically active polysaccharides is found among higher Basidiomycetes mushrooms and their practical application is dependent not only on their unique properties but also on

biotechnological availability. Isolation of PPC-P*r* and β-D-(1→ 6) glucan from *P.rimosus* is relatively simple and straight forward, and can be carried out with minimal effort.

In conclusion, the PPC-P*r* of *P.rimosus* showed significant anti-tumor activity. The anti-oxidant, anti-inflammatory, cytotoxic, anti-proliferative and apoptotic activities of this compound are seemed to the mechanisms behind its profound anti-neoplastic property. β-D-(1→ 6) glucan of *P.rimosus* also significantly inhibited the tumor development and inflammation even at lower doses. In this context, the polysaccharides isolated from *P.rimosus* can be a suitable candidate for the prevention and treatment cancer.

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List of Papers published/communicated

1. **Meera C.R.,** Smina T.P., Nitha B., Mathew John, Janardhanan K.K., 2009. Anti-arthritic activity of a polysaccharide-protein complex isolated from *Phellinus rimosus* (Berk) Pilat (Aphyllophoromycetideae), in Freund's complete adjuvant induced arthritic rats. *International Journal of Medicinal Mushrooms 11,* 21-28.
2. **Meera C.R.,** Nitha B., Janardhanan K.K., Viswakarma R.A. Anti-inflammatory and free radical scavenging activities of polysaccharide-protein complex isolated from *Phellinus rimosus* (Berk) Pilat. *International Journal of Medicinal Mushrooms* (in press).
3. **Meera C.R,** Janardhanan K.K., 2009. Antitumor activity of a polysaccharide-protein complex isolated from a wood-rotting macro fungus *Phellinus rimosus* (Berk) Pilat. *Life Sciences* (communicated).
4. **Meera C.R.,** Janardhanan K.K., Mani S.B., Karunagaran D., 2009. A polysaccharide-protein complex isolated from the mushroom *Phellinus rimosus* (Berk) Pilat inhibits the proliferation of HCT-116 human colon cancer cells by apoptotic induction. *Cancer Letters* (communicated).
5. Nitha B., **Meera C.R.**, Janardhanan K.K., 2007. Anti-inflammatory and antitumour activities of cultured mycelium of morel mushroom, *Morchella esculenta. Current Science* **92,** 235-239.
6. **Meera C.R.,** Janardhanan K.K., 2006. Anti-inflammatory activity of neutral and acidic extracellular polysaccharides isolated from *saccharomyces cerevisiae*. *International Conference on Ethnopharamacology and alternative Medicine* (Abstract), p16.
7. **Meera C.R.,** Janardhanan K.K., 2005. Anti-inflammatory and antitumor activities of polysaccharides isolated from a wood-rotting macro fungus *Phellinus rimosus* (BERK) PILAT. *Indian Journal of Medical Research (Abstract)* **121,** 131-132.