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Characterization of Immunostimulation and Anti-cancer Activity of Polysaccharide Peptide (PSP) with Special Reference to a Small Molecular Weight Fraction (SMF)

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Characterization of Immunostimulation and Anti-cancer Activity of Polysaccharide Peptide (PSP) with Special Reference to a Small Molecular Weight Fraction (SMF)

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Abstract

Polysaccharide peptide (PSP), isolated from COV-1 strain of *Coriolus versicolor*, is commonly used as an adjunct in cancer chemo-and radio-therapy in Asia. Many clinical trials using PSP have been done in China. The most promising pharmacological activities of PSP are their immunomodulatory and anti-cancer effects. In this study, water soluble PSP extract (crude PSP) was prepared from a commercially available source. This crude PSP was shown to be an immunostimulant on the basis of its upregulation of nitric oxide, and prostaglandin E_2 production in RAW264.7 cells. The anticancer effects included induction of apoptosis of B16 cells and inhibition of angiogenesis as determined by its reduction of matrigel-based tubulization of EA hy 926 cells. It also suppressed the mouse liver metastases induced by injection of melanoma cells via the portal vein. However, the active components of PSP are not known.

Fractionation of crude PSP by Sephadex G-75 chromatography has yielded two major fractions: high molecular weight fraction (HMF, 16% by weight) and small molecular weight fraction (SMF, 50% by weight). Immunostimulatory activity of these fractions was examined in the stimulation of RAW264.7 cells. SMF was found to be 10 times more effective than crude PSP in the up-regulation of NO (EC_{50} values of 9.71 μg/mL versus 158.8 μg/mL), PGE_2 as well as interferon-β (IFN-β). However, SMF was less active than crude PSP on

induction of melanoma cells apoptosis. On the other hand, HMF was found to be primarily responsible for the anti-angiogenic effect of crude PSP.

In vivo study in mice showed that SMF treatment (14 days) was more effective than crude PSP as an inhibitor of liver metastasis induced by injection of melanoma cells. SMF also upregulated immune response *in vivo* and *ex vivo* (macrophage function, proliferation of lymphocyte and elevation of serum IFN-β level); and these immunostimulatory effects may contribute to the anti-metastasis effect of SMF.

Our data suggested that SMF was more potent than crude PSP on immunostimulation as well as inhibition of tumor liver metastases. The application of these products may be important in treatment of some tumors and cancer metastases.

Keywords

Polysaccharide peptide (PSP), *Coriolus verscicolor*, anticancer, immunostimulatory, small molecular weight fraction.

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Dedication

I would like to dedicate this thesis to my son Isaac Tianyi Song and my husband Guangtian Song. My dear son lets me know the responsibility of a mother and understand my parents. My husband has continuously supported my study .God bless!

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List of abbreviations

Abbreviation	Full Name
ADCC	Antibody-dependent cellular cytotoxicity
ANOVA	analysis of variance
AP-1	activator protein-1
B16	mouse Melanoma cell line
bFGF	basic fibroblast growth factor
BRM	biological response modifier
CAM	chick allantoic membrane
CD4	cluster of differentiation antigen 4
cGMP	cyclic guanosine monophosphate
cm	centimeter
CO_2	carbon dioxide
Con A	concanavalin A
COX	cyclooxygenase
CTL cells	cytotoxicity T lymphocytes
CV	*Corious versicolor*
DR	death receptor
EA. hy 926 cell	human endothelial cell
ECM	extracellular matrix
EGR1	early growth response gene-1
ELISA	enzyme-linked immunosorbent assay
FAP	Familial adenomatous polyposis
g	gram

G-CSF	granulocyte colony- stimulating factor
GM-CSF	granulocyte monocyte colony stimulating factor
HAT	hypoxathine-aminopterin-thymidine
HL-60	human promyelocytic leukemia cells
HMF	high molecular weight fraction
HPLC	high performance liquid chromatography
HUVECs	human umbilical vein endothelial cells
IC_{50}	50% of inhibitory concentration
IFN	interferon
IGF	insulin-like growth factor
IL	interleukin
iNOS	inducible nitrite oxide synthesis
IP-10	IFN-induced protein 10
IRF-1	interferon response factor-1
KC	kuppfer cell
kDa	kilo-dalton
LAK cells	lymphokine-activated-killer cells
LD50	50% lethal dosage
LPS	lipopolysaccharide
LS174-T	human epithelial colon cell line
µL	microliter
MEM	minimum essential medium
Mg	milligram

Min	minute
mL	milliliter
mm^2	square millimeter
MMP	matrix metalloproteinase
MTC	macrophage-mediated tumor cytotoxicity
MTT	methyl thiazolyl tetrazolium
N	n value
NaCl	sodium chloride
NADPH	reduced form of nicotinamide-adenine dinucleotide phosphate
NIH	national institute of health
NF-κB	nuclear factor – κB
ng	nanogram
NK cell	nature killer cell
nm	nanometer
NO	nitric oxide
NOS	nitric oxide synthase
°C	degree celsius
OD	optical density
PBL	peripheral blood lymphocytes
PBS	phosphate-buffered saline
PCD	programmed cell death
pg	pictogram
PGE_2	prostaglandin E2

PSK	polysaccharide-K, Krestin
PSP	polysaccharide peptide
RAW 264.7 cell	mouse monocytic-macrophage cell line
rpm	revolutions per minute
SCG 7901	human stomach adenocarcinoma
SEM	standard error of the mean
SLY	human colon adenocarcinoma
SMF	small molecular weight fraction
SMMU-7721	human hepatoma cell line
SPC	human lung adenocacinoma
STAT	signal transducer and activator transcription
TCM	traditional Chinese medicine
TIL	tumor infiltrating lymphocytes
TNF-α	tumor necrosis factor-α
TNP-470	angiogenesis inhibitor
TRAIL	tumor necrosis factor-related apoptosis-inducing ligand
V_0	void volume
VEGF	vascular endothelial growth factor
WBC	white blood cell

Chapter 1 Literature review

Introduction

This research was concerned with the anticancer and anti-metastatic effects of polysaccharide-peptide (PSP) and its sub-fractions isolated from the medicinal mushroom *Coriolus versicolor*. Before a discussion of the current knowledge relevant to this topic, a general review of the clinical strategy used in the treatment and management of cancer, as well as the basic biological mechanisms relevant to the pharmacological activities of PSP, will be presented.

1.1 General Background

1.1.1 Biological process of cancer, types of cancer, treatment strategy and their limiting side effects.

Cancer has been viewed as a rapid proliferation of abnormal cells which grow beyond their usual boundaries, and have the ability to invade adjoining parts of the body and spread to other organs in a process referred to as metastasis. Metastases are the major cause of death from cancer. These malignant properties of cancers differ from benign tumors, which are self-limited and do not metastasize. There are more than a hundred different types of cancer. They are usually classified according to the tissue from which the cancerous cells originate (the primary tumor), as well as the normal cell type of close resemblance. As an example, melanoma originates from melanocytes. Cancer is a leading cause of death worldwide. From a total of 58 million deaths worldwide in 2005, cancer accounts for 7.6 million (or 13%) of all deaths (see http://www.who.int/mediacentre/factsheets/fs297/en/index.html).

Most often, cancer is treated with a surgical procedure, radiotherapy, or chemotherapy depending on the type of cancer, its location, whether it has spread, the patient's age, and the overall health status. A physician may use one treatment method or a combination of treatment protocols. Aims of the treatments are as follows: to either remove the tumor via surgery, kill the cancerous cells by cytotoxic effect of chemo- or radio- therapy, to induce apoptosis of the cancer cells, or inhibit their metastases at later stages. Traditional cancer treatment is effective in some cases, however most treatments had severe adverse side effects, such as: fever, bone marrow depression, renal and lung toxicity, neutropenia, hemorrhage, superior vena cava syndrome, airway obstruction, electrolyte abnormality and so on (Lobrich and Kiefer, 2006). Recently, significant effort in the research and development of therapeutic agents for cancer has targeted anti-angiogenic agent and immunotherapy. The use of anti-angiogenic agents in combination with chemotherapy has significantly improved the life span of cancer patients (Rüegga *et al.*, 2006). Harnessing the immune system to treat cancer is a major goal of immunotherapy.

1.1.2 Role of apoptosis in control of cancer

Apoptosis or programmed cell death (PCD) is a process of suicide by a cell in a multi-cellular organism. It involves an orchestrated series of biochemical events leading to a characteristic cell morphology and death. The apoptotic process is executed in such a way as to safely dispose of cellular debris without influencing the microenvironment homeostasis. It is well established that most chemo- and radio-therapeutic agents eliminate cells by triggering apoptosis (Kaufmann and Earnshaw, 2000).

The apoptotic cell is characterized by a loss of cell volume and plasma, membrane blebbing, nuclear condensation, chromatin aggregation and endonucleocytic degradation of DNA into nucleosomal fragments (Zhang *et al.*, 2004-05). These changes may be triggered by two major pathways: the death receptor (DR)-induced pathway (the extrinsic pathway) and the mitochondria-apoptosome-mediated apoptotic pathway (the intrinsic pathway). Both pathways lead to caspase activation and cleavage of specific cellular substrates. The first apoptotic pathway involves ligands and their receptors, such as Fas, tumor necrosis factor (TNF), tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), and downstream molecules (i.e., caspase and Bcl-2 family members). The second pathway is the apoptotic stimuli induced by chemo-or radio-therapy, mitochondria, apoptosome, and key effector caspases.

1.1.3 Role of angiogenesis in cancer

1.1.3.1 Anti-angiogenesis: a new therapeutic approach for cancer

The solid tumors that grow beyond microscopic sizes of 1-2 mm in diameter require induced growth of new blood vessels from the host vasculature to support further proliferation. The process of new blood vessels sprouting from an existing one is known as angiogenesis (Cristofanilli *et al.*, 2002). Angiogenesis and the development of metastases are intrinsically connected (Kirsch *et al.*, 2000). Metastases, rather than primary tumors, are responsible for most cancer deaths (Chambers *et al.*, 2002). Without angiogenesis, the tumor growth stops. The new blood vessel growth is governed by local balance between stimulatory and inhibitory factors. Cancer cells that gained the angiogenic phenotype are potentially angiogenic because of a combination of increased production of inducers and a down regulation of inhibitors. The angiogenic factors may

come from the tumor cells themselves, or from proteins mobilized from the extracellular matrix, or from cells recruited by the tumor - such as macrophages or fibroblasts, or a combination of these mechanisms (Cristofanilli *et al.*, 2002). Tumor cells and their supporting endothelial cells may exist in a symbiotic relationship. Tumor cells can produce basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) to stimulate endothelial cell proliferation and migration (Kim *et al.*, 2004). VEGF and bFGF appear to be the most important proteins for sustaining tumor growth. Endothelial cells in turn can produce tumor cell growth factors such as granulocyte colony- stimulating factor (G-CSF) and insulin-like growth factor (IGF).

Angiogenesis is a therapeutic target for cancer biologists, and clinical trials of agents that inhibit angiogenesis at various steps are underway. A number of inhibitors of angiogenesis have been discovered, two of the well-studied inhibitors are angiostatin and endostatin (Jiang *et al.*, 2001). Endostatin may inhibit endothelial cell migration and endothelial cell proliferation via G1 arrest. On the other hand, angiostatin inhibits cell migration. The discovery that angiogenesis inhibitors such as endostatin can restrain the growth of primary tumors raises the possibility that such inhibitors might also be able to slow tumor metastasis. Several anti-angiogenesis agents such as angiostatin, TNP-470 have been studied clinically for potential effects on cancer metastases (O'Byrne and Steward, 2001). However, most new anti-angigenic agents have failed in clinical trials. The primary reason is that there are multiple pathways that could lead to angiogenesis. Hence, the inhibition of limited pathways is not expected to be effective because alternative pathways could overcome the inhibition and lead to development of resistance.

On the other hand, design of agents that suppress too many proangiogenic pathways may be of limited success because such agents may be too toxic.

1.1.3.2 The major approaches for investigating suppression of angiogenesis

There are many models, including *in vitro* and *in vivo*, for investigating angiogenesis. The *in vitro* angiogenesis models have been designed mimicking many of the basic steps of the *in vivo* process including endothelial cell proliferation, and migration and tube formation assay (Plank *et al.*, 2003). The matrigel tumor plug assay, Chick Chorioallantoic Membrane (CAM) assay, and corneal angiogenesis are some of the examples of *in vivo* angiogenesis models. They can reliably and accurately recreate the angiogenesis process that is essential for drug development and validation. The following review will summarize the models that are relevant to this study, define the limitations and advantages of each model.

Endothelial cell proliferation/tubulization has been used frequently to evaluate angiogenic activity. One of the most specific tests for angiogenesis is the measurement of the ability of endothelial cells to form three-dimensional structures (tube formation). When endothelial cell cultures were maintained as confluent monolayers for extended periods of time without replenishment of nutrition, it formed three dimensional structures which resembled true blood vessels. Subsequently, they were shown that some substances could enhance the tube-like formation. Matrigel or extracellular matrix (ECM) is among the most popular substances used. ECM is secreted by cells to form an interstitial basement membrane that forms the framework to which cells are attached. Usually the tube-like formation on matrigel occurs within 24 hours and methods of quantitative analysis for angiogenesis on matrigel have been established. Human

umbilical vein endothelial cells (HUVECs) are the most frequently used cells for research on angiogenesis. But the macrovascular origin and the short number of *in vitro* passages are major drawbacks of these cells. Immortalized cell lines, such as EA. hy 926 can overcome these drawbacks and, hence, have been successfully applied for the *in vitro* angiogenesis study. However, the cells derived from cell lines may undergo transformation to allow for in vitro growth and this may alter their to responsiveness to drugs and foreign chemicals (Griffioen and Molema, 2000). It is not identical to the primary cell.

In vitro tests are best viewed as providing initial information, subject to confirmation by *in vivo* assays (Auerbach *et al.*, 2003). *In vivo* tests are more difficult and time-consuming to perform, thereby limiting the number of tests that can run at any one time. Quantification is generally more difficult as well. However, *in vivo* assays are essential because of the complex nature of vascular responses to test reagents, responses that no *in vitro* model can fully achieve (Auerbach *et al.*, 2003). Chick chorio-allantoic membrane (CAM) is an extra-embryonic membrane, serving as a gas exchange apparatus by means of an extensive capillary network which is a well established model to study angiogenesis (Quagliata *et al.*, 2008). Because of its extensive vascularization, CAM has been widely used as an *in vivo* model for the evaluation of molecules promoting/contrasting angio-vasculogenesis with the advantages of low cost and ease of preparation as well as the absence of a mature immune system (Wilting *et al.*, 1991; Ribatti *et al.*, 1999; Richardson and Singh, 2003).

A mouse liver metastasis model was successfully demonstrated to reflect the characteristics of metastasis (Hamada *et al.*, 2008). Since angiogenesis is a major

contributor to metastasis, the mouse liver metastasis model has also been used to study angiogenesis (Stoeltzing *et al.*, 2003). Tumor cells are injected through the portal vein or mesenteric vein to induce the liver metastasis. As a practical and efficient tool, this approach forms a significant number of metastases and includes all of the steps involved in the metastatic process. It also allows the detection and counting of the number of metastatic cells as well as the formation of new blood vessels neighboring the metastatic tumor (Stoeltzing *et al.*, 2003).

1.1.4 Immunomodulation in cancer

Cancer cells and chemo-therapeutic agents are known to suppress the immune function of cancer patients (Ménard *et al.*, 2008). Recently more attention has been focused on stimulating the patient's immune system to eliminate malignant tumor cells and to reduce the side effect of the therapeutic agents (Ramakrishnan *et al.*, 2008). This can be done either through immunization of the patient, in which case the patient's own immune system is trained to recognize tumor cells as targets to be destroyed, or through the administration of therapeutic agents such as immunopotentiating agents, in which case the patient's immune system is recruited by the therapeutic agents to destroy tumor cells.

1.1.4.1 Immune system of the body

To understand immunomodulatory action, a short review of the immune system is given below. Innate and acquired immunity play a pivotal role in the host immune system. Phagocytes (i.e. neutrophils, monocytes, and macrophages) and lymphocytes are the two major groups of cells of the host defense system. Phagocytic cells are responsible for the non-specific cellular responses (innate immunity). They kill infectious microbes and

digest them with lysosomal enzymes. Macrophages can be activated by lymphokines (cytokines produced by lymphocytes), and other stimuli such as lipopolysaccharide (LPS). Activated macrophages kill microorganisms and tumor cells through the release of TNF, oxygen radicals, and reactive nitrogen intermediates (e.g. nitric oxide). When innate immunity fails, specific (acquired or adaptive) immune responses are activated. Leukocytes (i.e., white blood cells), specifically B and T lymphocytes, are agents of specific immunity. A group of T lymphocytes, called T-helper cells (which encompasses CD4 cells), produce protein messengers or effector molecules, called cytokines. Cytokines are a group of cell-derived proteins or peptides that transmit signals between cells of the immune system. Cytokines can stimulate, inhibit, up-regulate or down-regulate the immune system. They are similar to hormones in that they act at sites distant from their site of synthesis. Cytokines fall into several categories which include interferons (IFN-α, -β and -γ) and interleukins (IL-1 through IL-15). There is another process which involves antibodies that exists for killing infected or tumor cells. In this process, circulating antibodies bind to epitopes (specific antigens) exposed on the surface of the "foreign" cell (e.g. a tumor cell). These antibody-tagged cells are subsequently recognized by granular lymphocytes, called natural killer (NK) cells, which are able to eliminate them. In the case of cancer, the components of immune system will exert the anticancer effect by scavenging cancer cells.

In this thesis, macrophages and the pro-inflammatory mediators NO and PGE_2 as well as interferon were studied as a target for immunomodulatory function of PSP, these topics will be discussed in greater detail.

1.1.4.2 Role of macrophage in innate immune function

Macrophages are released from the bone marrow as immature monocytes and after circulating in the blood stream, migrate into tissues to undergo final differentiation into resident macrophages. Macrophages play an important role in the control of cancer. Macrophages are generally not tumouricidal for tumor cells unless activated by antibodies or 'classic' macrophage stimulants such as interferon-gamma (IFN-γ) or the bacterial product, LPS (Bingle *et al.*, 2002). Once activated, macrophages exert direct cytotoxicity towards tumor cells, or indirect cytotoxicity via the secretion of factors that stimulate the anti-tumor functions of other cell types. Direct cytotoxicity can be further divided into macrophage-mediated tumor cytotoxicity (MTC) and antibody-dependent cellular cytotoxicity (ADCC). The former involves the secretion of lytic factors into neoplastic cells by macrophages, resulting in their lysis. This is usually a slow process, taking up to 3 days for completion, and includes the release of such toxic factors as TNF-α, serine proteases, and reactive nitrogen intermediates (Urban *et al.*, 1986; Keller *et al.*, 1990). The latter is dependent on the presence of antibody on tumor cells, but the killing mechanism, although faster, is similar to that of MTC.

Kupffer cells (KC), the resident macrophages in the liver, which account for 10 % of all liver cells (Laskin, 1990), can kill malignant cells through macrophage mediated cytotoxicity (Klimp *et al.*, 2002).The presence of antibodies on the tumor cell surface bond by the Fc receptors of KC enhances the ability of KC tumor killing through cell death signaling pathway (Keller *et al.*, 2000). The ability to produce a broad range of cytokines allows KC to exert the anticancer activity. It had been reported that KC plays a crucial role in the prevention of liver metastases (van der Bij *et al.*, 2005).

1.1.4.3 Role of pro-inflammatory mediators

Immune cells can respond to foreign invaders (such as cancer cells) or stimuli to release various inflammatory mediators, such as nitric oxide (NO), TNF-α, prostaglandin E2 (PGE_2), IL-6 and IFN.

1.1.4.3.1 NO

NO has now been demonstrated to play a role in a variety of biological processes including neurotransmission, immune defense, regulation of cell death (apoptosis), and cell motility (Tadié *et al.*, 2008) . The biological effects of NO are mediated through the reaction of NO with a number of targets such as haemolysis groups, cysteine residues and iron and zinc clusters. The diverse range of targets of NO explains the wide range of roles that it plays. Due to the importance of NO, abnormal regulation or control of NO synthesis is capable of affecting a number of important biological processes and has been implicated in a variety of diseases. Up-regulation of NO production has been used as a marker for macrophage stimulation.

Nitric oxide is synthesized by nitric oxide synthases (NOS). Three isoforms of NOS exist, constitutively expressed neuronal NOS (nNOS) and endothelial NOS (eNOS), and an inducible isoform (iNOS) (Bian *et al.*, 2001). Nitric oxide and prostaglandins (PGs) generated by the expression of inducible cyclooxygenase (COX) are important mediators of immune and inflammatory responses (Perkins and Kniss, 1999). Experimental evidence suggested a relationship between NO biosynthesis and prostaglandin generation wherein endogenous levels of NO can influence the formation of PGs (Marnett *et al.*, 2000). The mechanism is that NO stimulates the enzymatic activity of COX-2, augmenting the production of PGE_2 (Corbett *et al.*, 1993). Both nitric oxide

(NO) and prostaglandins (PGs) are known to be important mediators of acute and chronic inflammation (Wang and Smart, 1999). iNOS and COX-2 gene expression are regulated by transcription factors, such as NF-κB transcription factor, on RAW 264.7 cell stimulated with LPS(Cho *et al.*, 2004).

1.1.4.3.2 PGE_2

Prostaglandin E_2 has a number of pro-inflammatory effects, including induction of fever and erythema due to increasing vascular permeability, vasodilation, and enhancement of pain and oedema caused by agents such as bradykinin and histamine. PGE_2 also regulates the production of monocyte-, macrophage- and lymphocyte-derived cytokines (Kobayashi and Narumiya, 2002). In chronic inflammatory conditions increased rates of PGE_2 production are found, and elevated PGE_2 production has been observed in patients suffering from infection. PGE_2 is thought to be responsible for promoting tumorigenesis such as colorectal tumour by inducing cell survival, cell growth, migration, invasion and angiogenesis (Chell *et al.*, 2006).

It has also been reported to increase in a size-dependent manner in the adenomas of Familial adenomatous polyposis (FAP) patients *in vivo* and in the adenomas of ApcMin mice (Hull *et al.*, 2004; Giardiello *et al.*, 2004; Stolina *et al.*, 2000). Cyclooxygenase 1 and 2 are rate-limiting enzymes in the synthesis of PGE_2. Currently, COX2 inhibitors are used clinically as anticancer agents (Chell *et al.*, 2006).

Prostaglandins (PGs) are known to be important mediators of acute and chronic inflammation (Wang and Smart, 1999). They are synthesized by COX. Two isoforms of COX exist, constitutively expressed COX-1 and inducible isoform COX-2 (Kreiss *et al.*, 2003)

1.1.4.3.3 IFN-β

1.1.4.3.3.1 General aspect of IFN

Interferons were recognized initially for their potent antiviral properties. Recently, it has been established that they may profoundly affect other vital cellular functions. The IFNs are divided into two main types, type I (included IFN-α, β), and type II (IFN- γ); and they are defined by the differences in their amino acid sequences, physicochemical properties, and induction by different agents from different cell types. The inducing agents include low molecular weight compounds, viruses, bacteria, bacterial products, polymers and antigens or mitogens. Interferons are produced by a wide variety of cells such as leukocytes, epithelial cells, and NK cells. The IFNs possess a broad spectrum of activity and are involved in complex interactions. They display antiviral activity, impact cellular metabolism and differentiation, and possess antitumor activity. The antitumor effects appear to be due to a combination of direct anti-proliferative, as well as indirect immune-mediated effects.

1.1.4.3.3.2 Anticancer activity of IFN-β and Shortcoming of IFN-β therapy

IFNs have been observed to positively impact both the quality and duration of life for hundreds of thousands of cancer patients with chronic leukemia, lymphoma, bladder carcinoma, melanoma, and renal carcinoma (Ernest, 2005). Among all IFNs, IFN-β is the most potent and has been widely used in treatment of some types of cancer, especially melanoma (Borden, 2005).

The anticancer effect of IFN has been studied extensively. IFN has shown to possess anti-proliferative and apoptotic effects (Chawla-Sarkar, 2001; Leaman, 2003;

Murata, 2006; Ikeda, 2002). IFN- β is more potent in apoptosis induction when compared to the others (Chawla-Sarkar, 2001). The ability of IFN-β induction of apoptosis *in vitro* has been extended to melanoma xenografts transplanted into nude mice. IFN-β was significantly more effective in reducing tumor growth as compared to IFN α-2. IFN- β may also play a pivotal role in host response to tumors (Deonarain, 2000; Deonarain, 2003). Some reports suggested that regression of tumor by IFN was due to inhibition of angiogenesis. For example, the continuous incubation of different human carcinoma cells with noncytostatic concentrations of IFNs down-regulated transcription and protein production of basic fibroblast growth factor , interleukin 8, and collagenase type IV , all of which are involved in the angiogenic response (Singh, 1995; Slaton, 1999; Chang, 1997). The inductions of chemokines, IFN-induced protein-10, and monokine induced by IFN-gamma have been shown in mouse tumor models to exert anti-angiogenic effects (Lindner, 2002; Angiolillo, 1995; Sgadari, 1997). Vascular endothelial growth factor (VEGF), an important angiogenic factor, can also be inhibited by interferon treatment (von Marschall, 2003). IFN-induced guanylate-binding protein was correlated with antiproliferative effects in endothelial cells (Guenzi, 2001; Guenzi, 2003). The anti-proliferative, apoptotic and angiostatic activity of IFN-β makes it an attractive anticancer agent.

Despite its potential as an anticancer agent, interferons have many shortcomings which limit their clinical application. Pharmacokinetic studies have demonstrated that the half-life of IFNs in the circulation of patients is in the order of 5 min (Salmon, 1996). The resulting lack of sustained levels may have been responsible for their failure to inhibit or eradicate tumors (Grander and Einhorn, 1996; Salmon, 1996). The results of preclinical

and clinical studies suggest that attempts to increase the antitumor efficacy by increasing the dose and the exposure to IFNs could actually result in higher toxicity and lower efficacy (Coles, 2005). It is likely that higher doses induce counter-regulatory mechanisms. More recently, IFN has been used in the context of combination therapy for the treatment of metastatic melanoma (Ryuichi *et al.*, 2003). Numerous trials have been performed combining IFN with IL-2, or with chemotherapy and have been met with some successes (Minischetti *et al.*, 2000; Ryuichi *et al.*, 2005; Damdinsuren *et al.*, 2003). Therefore, the development of new strategies that overcome the untoward effects of IFNs and bypass counter-regulatory mechanisms could be of great experimental and clinical value.

1.2 *Coriolus vesicolor*

1.2.1 Medicinal mushrooms and *Coriolus vesicolor*

The number of different mushrooms on Earth is estimated to be at 140,000 species, yet only about 10% (approximately 14,000 named species) are known species. For centuries the medicinal value of certain mushrooms has been recorded. The oldest written record of the medicinal application of mushroom is an Indian medicinal treatise from 3000 BC (Kaul, 1997). "Shen Nong Ben Cao Jing", a compendium of material *medica* in China compiled around 200BC to 200AD, states the beneficial effects of several kinds of mushrooms (Jong and Birmingham, 1992). More than a hundred species of mushrooms are documented by practitioners of Chinese medicine as treatments for a wide range of ailments. Also, many mushroom-derived medicinal products are manufactured by Japanese, Korean and Chinese pharmaceutical companies. These

medicinal mushrooms are used as anticancer agents, immune system enhancers, vascular support agents, antioxidants, and anti-inflammatory agents (Chris and Meletis, 2005).

Coriolus versicolor (CV) is one of the most popular medicinal mushrooms, which is also known as "Yun Zhi" in China and "Turkey Tail" in North America. It belongs to the *Basidiomycetes* class and *polyproraceae* family (Hobbs, 1997). More than 120 strains of CV have been found in nature. Its dried fruit body or *mycelia* has historically been used for the treatment of cancer, chronic hepatitis and infection of upper respiratory, urinary and digestive tracts in Asia (Hobbs,1995; Yang *et al.*,1993a). According to the theory of Traditional Chinese Medicine (TCM) practice, CV is considered useful for dispelling heat, removing toxins, strengthening physique, increasing energy and spirit, and enhancing the host's immune function (Yang *et al.*, 1993b). Traditionally, the fruit bodies (mushrooms) are harvested, dried, ground to powder, and made into tea to be used as a health supplement (Liu and Bau, 1980). In TCM, CV is prepared as a hot-water extraction/decoction for cancer patients.

1.2.2 Polysaccharide peptide (PSP) isolated from *Coriolus versicolor*

1.2.2.1 Production and chemical characteristics of PSP

In 1977, Krestin (PSK), an extract isolated from CM-101 strain of CV, with anticancer effects was marketed in Japan as a drug for cancer therapy. Inspired by this, Professor Qing-yao Yang extracted a protein bound polysaccharide (PSP) from the deep layer cultivated mycelia of Cov-1 strain of CV in 1983 and found that it had a promising anticancer and immunostimulatory activity. Currently, the commercially available PSP is manufactured as an extract of CV grown as a submerged culture in bioreactors. For this, mature mycelia are extracted with hot water, followed by alcoholic precipitation; and the

precipitate is freeze-dried to produce a light brown, water-soluble powder named PSP (Yang, 1993a).

Because the major active component in this product is believed to be a polypeptide or protein-bond polysaccharide, it was named commercially as "Polysaccharide-peptide" (PSP) (Yang, 1999a). Although the extract is known to contain multiple chemical components, to date, majority of work has focused on the high molecular weight component(s), which corresponds to the polysaccharide peptide fraction. Characterization studies suggested that the molecular weight of the polysaccharide-peptide is approximately 100 kDa. It is a group of polysaccharides chemically linked to a core peptide chain (Figure 1.1). The polypeptide moieties are rich in aspartic acid and glutamic acid. Monosaccharides with α-1, 4 and β-1, 3 glucosidic linkages constitute the polysaccharide moieties. In addition to glucose, PSP contains small amounts of five other monosaccharides: galactose, mannose, xylose, arabinose and rhamnose (Chu *et al.*, 2002; Jong and Yang, 1999; Yang, 1999a; Tsukagoshi, 1984).

1.2.2.2 Clinical evidence for the anti-cancer effect of PSP

Since the first development of PSP in 1983 in China, a number of clinical trials have been conducted mostly in China. Phase I clinical trials were carried out by Xu (1993) and it was shown that an oral dose of up to 6g/day was well tolerated and lacking in side-effects. Patients showed improvement in appetite and general condition with a stabilization of haematopoietic parameters. The Phase II multi-center study conducted, by the Shanghai PSP Research Group involving 8 hospitals in Shanghai, was carried out in

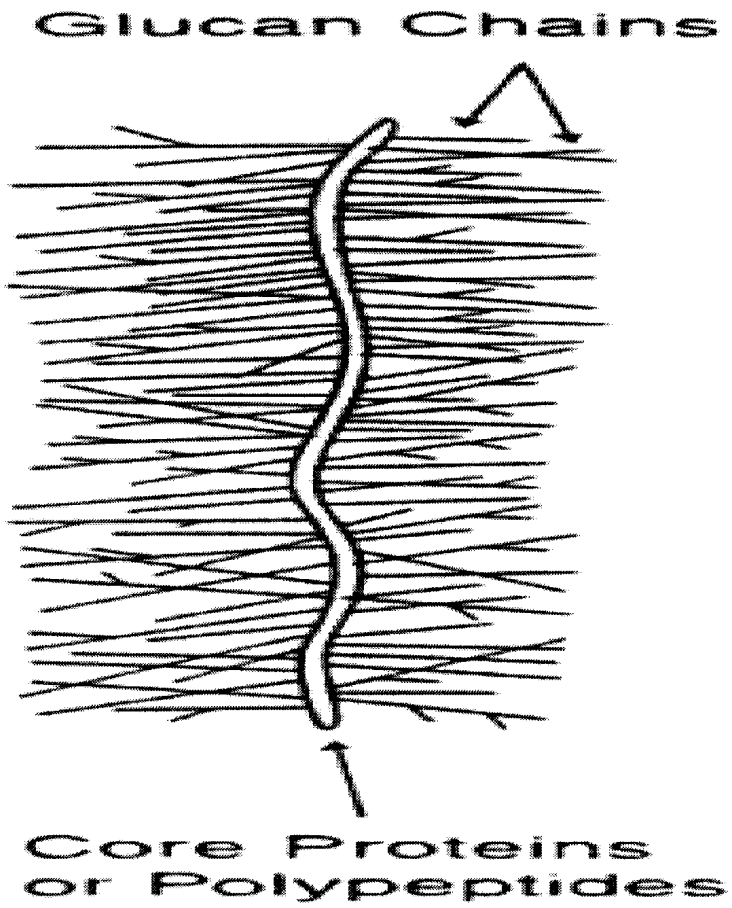

Figure 1.1 Schematic structure of polysaccharide peptide (Yang *et al.*, 1999)

patients with cancers of the stomach, lung and esophagus. The dosage was 1g three times daily with a total dosage of 190 g per treatment cycle. Results showed a significant improvement in the immunological status of the patients after surgery, radiotherapy and/or chemotherapy (Liu and Zhou, 1993). This phase II study also confirmed the role of PSP as a biological response modifier (BRM) (Li *et al.*, 1999), which was previously put forward by Yang *et al* (1993b) on the basis of its immunomodulatory activity. Results of a more recent double-blind Phase II trial in Shanghai hospitals involving approximately 300 patients suffering from gastric, esophageal or lung cancer whowere treated with conventional radiotherapy and/or chemotherapy together with PSP showed that PSP improved overall clinical symptoms (Liu and Hou, 2004). In addition, some Phase III clinical trials of PSP in combination with conventional therapies have been conducted and demonstrated significant benefits against cancers of the stomach, esophagus and lung (Jong and Yang, 1999; Yang, 1999b). Because of these promising results from the clinical trials, a NIH-sponsored clinical trial on PSP is being conducted in the United States (http://nccam.nih.gov/research/extramural/awards/2004/).

1.2.2.3 Pharmacological activity of PSP

In addition to clinical studies, many studies have been conducted to elucidate the underlying mechanism(s) of action of PSP (Kidd, 2000; Chu *et al.*, 2002; Ooi *et al.*, 2000).

1.2.3.3.1 Immunomodulatory activity

Numerous reports have documented the ability of PSP to activate cellular and humoral components of the host immune system. Spleen T-lymphocytes cultured with

various concentrations of PSP solutions showed that concentrations in excess of 100 μg/mL produced an increase in T-lymphocyte proliferation by a factor of 1.5 to 4 times. PSP also promoted the proliferation of T-lymphocytes in human peripheral blood. PSP augmented T-helper cell (CD4+) activation, and also increased the ratio of CD4+/T suppressor (CD8+) production (Li *et al.*, 1999). PSP also had a significant effect on induction of cytokines. PSP increased the secretion of IL-2 in mice (Yang *et al.*, 1993). PSP at 10-1000 μg/mL induced interferon alpha and gamma production from human peripheral leukocytes 4 and 8 times respectively higher than that of the control groups (Li *et al.*, 1990). PSP can promote the expression of the IL-6 gene of peripheral blood lymphocytes (PBL) in humans and, hence, induce the production of interleukin 6 (IL-6) (Yang *et al.*, 1993b). Cultured peritoneal macrophages obtained from mice which had received PSP in drinking water for 2 weeks showed an increase in the production of reactive nitrogen intermediates, reactive oxygen intermediates (like superoxide anions) and tumor necrosis factor (Liu *et al.*, 1993). PSP has been shown to stimulate the release of TNF-α, interleukin-1β, prostaglandin E2 and histamine in mouse peritoneal macrophages and masts cells both *in vitro* and *in vivo* (Chan and Yeung, 2006b).

Jong and Yang (1999) reported that the immunostimulatory effect of PSP involved the activation of immune cells such as macrophages, T, B lymphocytes and natural killer cells. The upregulation of the phagocytic functions of macrophages has been reported in normal mice after PSP oral feeding (0.5-1g/kg) (Yang *et al.*, 1993b). Other *in vivo* studies have revealed that PSP generally has no significant immunological effects on a normal host, but can restore a depressed immunological responsiveness caused by tumor or chemotherapy (Chu *et al.*, 2002). Research has demonstrated that PSP

can antagonize the immunosuppression caused by chemotherapeutic agents (such as Cyclophosphamide). PSP was effective in restoring cyclophosphamide-induced immunosuppressed rats' immune system by stimulating lymphocyte proliferation, NK cell functions, and the growth of spleen and thymus (Qian *et al.*, 1997). In mice with suppression of IL-2 production from cyclophosphamide toxicity, PSP restored IL-2 production to normal levels (Yang, 1999b). Lu *et al.* (1995) demonstrated that under the immunosuppressive condition of cyclophosphomidium treatment, the levels of lymphocyte maturation and antibody production in mice were maintained near normal with PSP administration. PSP was also reported to prevent thymus involution and to increase production of IgG and complement C3 in sarcoma-bearing mice (Yang *et al.*, 1993b).

1.2.2.3.2 Anticancer activity

Polysaccharide peptide has been shown to inhibit the growth of tumor cell lines *in vitro* and to have *in vivo* anti-tumor activity (Tzianabos, 2000). *In vitro* experiments of PSP were reported to inhibit the proliferation of P388 leukemia cells, HL 60 cells, Ehrlich ascites cells and some human tumor cell lines including SCG-7901, SPC, SLY as well as human breast cancer cells (Ho *et al.*, 2004a, 2005; Zeng *et al.*, 2005). Yang *et al,* (1993b) noted an inhibitory effect of PSP on the incorporation of ^{3}H-uridine and ^{3}H-thymidine into nucleic acids in Ehrlich ascites tumor cells. The anti-proliferative potencies of PSP against human gastric cancer, lung cancer, lymphoma and mononuclear leukemia cell lines were similar. PSP also induced apoptosis in HL-60 cells (Zeng *et al.*, 2005).

In vivo experiments showed that PSP inhibited the growth of murine sarcoma 180 in tumor bearing mice (Qian *et al.*, 1997). Zeng *et al.* (1993) reported that oral administration of PSP to nude mice inhibited growth of human lung adenocarcinoma by 50-70%. Wang *et al.* (1993) found that PSP administered intraperitoneally (IP) to mouse at 50 mg/kg per day for about 3 weeks produced approximately 45% inhibition of the growth of Lewis lung cancer. Oral administration of PSP (2.5 g/kg, 1.0 g/kg) daily for 4 weeks resulted in a tumor (nasopharyngeal carcinoma inoculated in mice) growth inhibition rate of 77% and 63% in treated groups with no adverse influence on blood system and body weight (Zeng *et al.*, 1999).

Angiogenesis is a key factor in tumor metastasis. More recent studies have shown PSP also possess anti-angiogenic activity both *in vitro* and *in vivo*. PSP suppressed pulmonary metastasis development from induced sarcomas and prostate cancer in mice, as well as lymphatic metastasis of mouse leukemia P388. Moreover, it could prolong the survival period in spontaneous metastasis models (Ho *et al.*, 2004b). Lui (2006) also observed the anti-angiogenic effect of PSP on matrigel-based endothelial cell tubulization and Chick Allantoic Membrane (CAM) model (Ye, 2004). Ho *et al.* (2004b) studied the anticancer effect of PSP with S180 tumor-bearing mouse model by oral PSP feeding and confirmed the conclusion of Ye (2004). The PSP-treated animals showed significant anti-angiogenic features (such as dense sinusoids and hot spots). Immunostaining of tumor tissues with antibody against the endothelial cell marker (Factor VIII) demonstrated a positive correlation in that both the vascular density and tumor weight were lower in mice treated with PSP. Based on those results the authors concluded that anti-angiogenesis may be one of the pathways through which PSP mediated its anti-tumor activity.

1.2.3.4 Identifying the bioactive components of PSP

Most researches in PSP have used crude PSP extract from commercial sources without fractionation; and it is generally believed that the high molecular weight component(s), the polysaccharide-peptides, are the major contributor of the pharmacological activity (Yang *et al.,* 1999a). In our previous study, PSP was fractionated by Sephadex G-75 chromatography into a high molecular weight fraction (HMF) and a small molecular weight fraction (SMF). The HMF showed concentration dependent anti-angiogenic potential *in vitro* in inhibiting tubulization of vascular endothelial cells in the Matrigel based assay (Ye, 2004). In addition, this fraction inhibited age-related new blood vessel formation in the fertilized chick chorioallantoic membrane (CAM) model *in vivo* following application of the test material on plastic cover-slips (Ye, 2004). In a more recent study, HMF was found effective in suppressing melanoma cell-induced metastasis in mice (Lui, 2006).

The small molecular weight components of CV are thought to be pharmacologically inactive and it is often discarded during the extraction procedure. However, our previous studies have shown that the SMF isolated from crude PSP aqueous extract with G-75 chromotography was found to possess biological activity. In fact, SMF was demonstrated to be significantly more active in immunostimulation than the crude extract and HMF (Lui, 2006). This interesting observation was limited to the up-regulation of macrophage NO production *in vitro*. Additional studies with other immunostimulatory parameters are required to fully appreciate the pharmacological significance of this observation and the role in the anticancer effectiveness of PSP.

1.2.3.5 Safety

It appears that PSP does not harm normal cells, and has the ability to distinguish between normal and cancer cells (Jong and Yang, 1999). According to the results of toxicological research concerning acute, subacute, subchronic and chronic toxicity, as well as reproductive and genetic toxicity, PSP is a very safe product (Chu *et al.*, 2002; Jiang *et al.*, 1992). Oral administration of high dose PSP did not produce death or toxic symptoms in either rats or mice (Jiang *et al.*, 1992; Jin, 1999, Chan *et al.*, 2006). Results of Phase I, II and III clinical trials for PSP showed that PSP improved the survival rate and quality of life in cancer patients without apparent toxicity (Kidd, 2000).

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Chapter 2 Rationale for studies

2.1 Rationale and Hypotheses

PSP is known to have a wide range of pharmacological activities, such as anti-angiogenesis, immunostimulation and apoptosis induction that could explain its beneficial effects in cancer patients. Few efforts have been devoted to identify the bioactive component(s) of PSP, although it is generally believed that the polysaccharide-peptides, the high molecular components, is the major – and only – bioactive component of PSP (Yang *et al.*, 1999). Our recent study has showed that in addition to the HMF, a small molecular weight fraction (SMF) isolated from crude PSP extract by Sephadex G-75 gel filtration also exerted significant stimulation of macrophage NO production (Ye, 2004; Lui, 2006). In fact, the specific activity (per unit dry weight) was far greater with the SMF. However, the significance of these observations was not clear because other immunostimulatory effects of this bioactive component have not been examined. Nitric oxide and PGE_2 are both important mediators of inflammation and are known to possess anticancer properties; and both are regulated by the same gene family, NF-κB (Wang and Smart, 1999; Cho *et al.*, 2004). It is expected that SMF will also upregulate PGE_2. PSP is known to upregulate cytokines, such as INFs (Ho *et al.*, 2004). Since INFs have been shown to modulate other immune functions it became important to assess the effect of SMF on this parameter.

We hypothesized that:

1. Both HMF and SMF are bioactive; each has its own specific pharmacological property.

2. SMF is the major immunostimulatory component of PSP and has a wide range of immunostimulatory activity *in vitro*, which contributes to its anticancer effect.

3. SMF possesses an anticancer effect *in vivo*

In view of the fact that INFs, in particular INF-β, also exert direct anticancer effects, including inhibition of angiogenesis and induction of apoptosis and immuno-stimulation, up-regulation of endogenous INFs by PSP and SMF, if it could be validated, would be expected to show synergistic interaction with therapeutically-administered INFs. Since clinically relevant levels of IFNs are associated with adverse side effects, a combinational therapy of PSP and INFs is expected to be efficacious at sub-therapeutic levels of INFs. Therefore, we also hypothesize that SMF exerts synergistic interaction with interferon-β with respect to anti-angiogenesis, apoptosis and immunostimulation *in vitro*.

2.2 Specific aims

To test the hypotheses, the following specific aims were developed:

1. To fractionate crude PSP aqueous extract by gel filtration chromatography to yield the HMF and SMF, and to determine relative potencies of these fractions as well as the crude PSP extract in anti-angiogenesis, apoptosis induction and immunostimulation *in vitro*.

2. To characterize or study the spectrum of immunostimulatory activity of the major immunostimulatory component(s) with special reference to the up-regulation of pro-inflammatory mediators (NO, PGE_2) and INF-β.

3. To study the combinational effect of major immunostimulatory components of PSP and INF-β with *in vitro* models of angiogenesis, apoptosis and immunostimulation to evaluate synergistic interactions.

4. The *in vitro* findings will be validated with the *in vivo* model of melanoma-induced mouse liver metastases to determine the contribution of imunostimulation and anti-angiogenic mechanism towards the anti-metastatic effect.

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Chapter 3

Characterization of the anti-cancer and immunomodulatory activity *in vitro* of polysaccharide-peptide (PSP) with special reference to the small molecular weight fraction (SMF).

3.1 Introduction

Polysaccharide peptide (PSP) preparations, isolated from the Chinese fungus *Coriolus versicolor* COV-1 strain, are commonly used as an adjunct to cancer chemotherapy or radiotherapy in China. The primary and purported pharmacologically active component(s) are thought to be composed of peptide-bound polysaccharides with a molecular weight of approximately 100 kDa. The polypeptide moieties are rich in aspartic acid and glutamic acid. Monosaccharides with α-1, 4 and β-1, 3 glucosidic linkages constitute the polysaccharide moieties (Yang *et al.*, 1989). Since the first development of PSP in 1983 in China by Professor Qing-yao Yang, a number of clinical trials have been conducted. The results suggested that PSP improved the quality of life by decreasing the cancer treatment-related symptoms and was effective against some forms of tumors such as stomach, lung, esophagus and breast cancers (Sun and Zhu, 1999; Sun *et al.*, 1999; Jong and Yang, 1999; Yang, 1999). However, concerns have been raised regarding the quality of these trials; a clinical trial on PSP sponsored by NIH is being conducted in the US (http://nccam.nih.gov/research/extramural/awards/2004/).

PSP has been demonstrated to possess anticancer activity (Kidd, 2000), but the underlying mechanisms are poorly understood. PSP is considered to be a biological response modifier (BRM) (Yang *et al.*, 1993b), which is defined as an agent that can modify the host's biological response by stimulating the immune system and thereby eliciting various therapeutic effects (Li, 1999). In addition, a wide range of pharmacological activities, such as anti-angiogenesis (Yoshino *et al.*, 2005; Ho, 2004), induction of apoptosis (Lau *et al.*, 2004), and activation of immune cells (Macrophage,

NK cells etc) to be cytotoxic have been demonstrated in several experimental models (Ho *et al.*, 2004; Gong *et al.*, 1998).

Our laboratory has recently reported that Sephadex G-75 chromatographic fractionation of a crude PSP aqueous extract resulted in the isolation of a high molecular weight fraction (HMF) and a small molecular weight fraction (SMF) (Ye, 2004; Lui, 2006). A preliminary study showed that both fractions possessed pharmacological activities, but their relative contribution to the anticancer effect of the crude PSP extract was not known. This question was addressed in the present study by evaluating the effectiveness of the crude PSP extract and its two sub-fractions in the stimulation of immune response, induction of apoptosis and inhibition of angiogenesis.

Our preliminary study also showed that SMF was more potent on the basis of unit dry weight than the crude PSP extract on the up-regulation of NO production from macrophages; however, the nature and characteristics of this immunostimulatory activity have not been elucidated. One of the objectives of this study was to characterize the immunostimulatory properties of SMF, with special reference to the up-regulation of proinflammatory mediators and cytokines, such as interferons.

Interferons (IFN) consist of several subtypes which includes IFN-α, -β, -ω (type I), and IFN-γ (type II) (Stark *et al.*, 1998). Interferons are known to have anticancer properties, and they have impacted positively on both quality and longevity of life for many cancer patients with chronic leukemia, lymphoma, bladder carcinoma, melanoma, and renal carcinoma (Borden, 2005). Its anticancer effect may be mediated by antiproliferative, angiostatic, and immunomodulatory mechanisms. However, its clinical

use has been limited by its shortcomings such as the relatively short half-life (5 minutes) in the circulation of patients and the resulting lack of sustained levels achieved in patients (Salmon, 1996). Attempts to increase the anti-tumor efficacy by increasing dosages have resulted in an increase in toxicity and reduced efficacy (Coles, 2005). In view of the immunostimulatory and anticancer effect of PSP and SMF, it has become important to examine the potential synergistic interaction between SMF and IFNs.

3.2 Materials and methods

3.2.1 Materials

Polysaccharide peptide of *Coriolus versicolor* was purchased from Shanghai Xinkang Pharmaceutical Company (Shanghai, China, Lot: Z10980124). Sephadex G-75 and G-25 were purchased from GE Healthcare Bio-sciences AB (Uppsala, Sweden). Cell culture medium and reagents were purchased from Gibco Laboratories (Grand Island, NY, USA). IFN-β was purchased from Cedarlane Company (CEDARLANE® Laboratories Limited, CA). The cell death ELISA kit was purchased from Roche Company (Roche Diagnostics GmbH, Mannhein, Germany). PGE_2 and IFN-β enzyme immunoassay kits were purchased from PBL Biomedical Laboratories (PBL biomedical laboratories, Piscataway, NJ). Lipopolysaccharide (LPS), colorimetric 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenltetrazolum bromide (MTT) and Griess reagent were purchased from Sigma Aldrich (St. Louis, MO, USA).

3.2.2 Preparation of crude PSP aqueous extract and its fractionation by Sephadex G-75 chromatographic technique and further isolation of SMF with G-25 chromatography.

One gram of commercial PSP product (Xin-kang Company, China) was first added to 30 mL distilled water and dissolved at room temperature for 30 minutes. The solution was centrifuged at 350 g in a centrifuge (Beckman Model TJ-6, U.S.A.) for 30 minutes to remove insoluble substances. The supernatant (25.8 mL) was lyophilized with a freeze dryer (Labconco.Corporation, Kansas City, Missouri, U.S.A.) at -50 °C. For chromatographic fractionation of the crude PSP aqueous extract, an aqueous extract was prepared by dissolving 500 mg of the lyophilized crude PSP in 5 mL of distilled water, the solution (100 mg/mL) was loaded onto a calibrated Sephadex G-75 column (2.8 × 90 cm) and eluted with distilled water (pH 6.8) at 4 °C with a flow rate of 0.8 mL/min. Fractions were collected and the absorbance was measured at 230 nm with a spectrophotometer (Multiscan, USA). Fractions were pooled and collected to yield 2 major fractions corresponding to peak 1, peak 3 (Figure 3.1). Fractions corresponding to peak 1 and 2 were designated as the high molecular weight fraction (HMF) which is known to be PSP, and small molecular weight fraction (SMF), respectively. These fractions were lyophilized and stored at -20 °C until ready for further analysis.

SMF (100 mg/mL) was further fractioned by Sephadex G-25 (1.8 × 80 cm) chromatography. It was eluted with distilled water with a flow rate 0.5 mL/min at 4 °C. The eluted solution was collected and scanned at 230 nm with a spectrophotometer.

3.2.3 Cell culture

EA. hy 926 cell culture

EA. hy 926 cell is a human cell line that expresses highly differentiated functions characteristic of human vascular endothelium and has been used in the matrigel-based tubulization assay (Edgell *et al.*, 1990; Bauer *et al.*, 1992). It was established by fusing a human umbilical vein endothelial cell with a human carcinoma cell line A 549; and it is a continuous and clonable cell line that offers many advantages over primary cultures of endothelial cells in terms of uniformity and reproducibility (Edgell, 1983). EA. hy 926 cells were kindly provided by Dr. Edgell from the University of North Carolina. They were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, 1% antibiotics (100 units/mL of penicillin-G and 100 mg/mL streptomycin), and Hypoxanthine-Aminopterin-Thymidine (HAT) media supplement (0.5 mg HAT in 100 mL culture medium) in a 75 cm^2 tissue culture flask (Becton Dickinson Labware, Franklin Lakes, NJ). The flask was maintained in a cell culture incubator (Krendro Laboratory Products, Asheville, NC) with 5% CO_2 at 37 °C. When the cells attained 90% confluence, they were detached with Trypsin-EDTA solution and sub-cultured with DMEM into new flasks.

B16F10 melanoma cell line

B16F10 is a spontaneously derived malignant melanoma cell line originating from C57BL/6 mice. This cell line was kindly provided by Dr. Anne Chambers (London Health Sciences Centre, LHSC). The cells were cultured in α-medium supplemented with 10% fetal bovine serum and 1% antibiotics (100 units/mL of penicillin-G and 100 mg/mL streptomycin) in a 75 cm^2 cell culture flask. The flask was maintained in a cell culture incubator with 5% CO_2 at 37 °C. When the cells grew to confluence, they were detached

with trypsin-EDTA solution, sub-cultured with α-medium and redistributed into new flasks at a ratio of 1:5.

RAW 264.7 macrophage cell line

The mouse monocytic-macrophage cell line RAW 264.7 was kindly provided by Dr. Jeff Dixon (Department of Physiology & Pharmacology, University of Western Ontario). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum and 1% antibiotics (100 units/mL of penicillin-G and 100 mg/mL streptomycin). RAW 264.7 cells were cultured in a 75 cm^2 tissue culture flask. The flask was maintained in a cell culture incubator with 5% CO_2 at 37 °C. The medium was replaced every two days. When the monolayers attained 90% confluence, they were subcultured by removing the growing medium and washed with 5 mL of Dulbecco's Phosphate Buffered Saline (PBS) 3 times. Cells were then scraped with cell scraper (BD Bioscience Labware, MA, USA). The cell suspension was centrifuged at 350 g for 5 minutes in a bench centrifuge. The supernatant was removed and the cells were re-suspended into new culture medium and redistributed into new flasks at a ratio of 1:3.

3.2.4 Matrigel based endothelial cells tubulization Assay

The matrigel based tubulization model is a well established angiogenesis model that reflects some aspect of angiogenesis (Bauer *et al.*, 1992). Endothelial cells were allowed to tubulize on the top of matrigel to form a tube-like structure, which could subsequently be quantified by counting the number of branching points. Matrigel (ECM gel from engelbreth-holm-Swarm murine sarcoma, Sigma) was thawed overnight at 2-8

°C and placed in the wells (75 µL per well) of a pre-chilled 96-well tissue culture plate with pre-chilled pipette tips and incubated at 37 °C for 1 hour to allow polymerization. EA. hy 926 cells suspended in DMEM medium were seeded on the matrigel at a density of $2x10^4$ cells in each well. Different concentrations of crude PSP or its fractions were added to the cell suspensions in the coated wells (100 µL/well). The plate was then put in an incubator at 37 °C for 18 hours. Digital images of the tube-like structures were observed at 4X magnification to identify the field for examination and then photographed at 10X magnification through an inverted phase-contrast microscope (Nikon Corporation, Tokyo, Japan) with a Motican 2000 digital camera (Independent Products Co., UK).

Quantification of the tubulization response on Matrigel was performed as follows: the points at which at least 3 branches converged were counted for each picture by using Adobe Photoshop software program (Amazon Co. UK). The density of branching points was calculated by the following formula: total number of branching points / total area of the well (0.32 cm^2).

3. 2.5 Cytotoxicity assay (MTT assay)

The cytotoxicity of the PSP compounds for the EA. hy 926 cells were based on changes in cell viability measured by MTT assay. This assay was based on the activity of the mitochondrial succunate dehydrogenase to reduce the substrate, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenltetrazolum brominde, to a colored product formazan. The concentration of this formazan dye developed in the assay is directly related to the dehydrogenase activity of the cell preparation. Therefore the MTT assay could be use to measure changes in number of viable cells (Wang *et al.*, 2006). Briefly, EA. hy 926 cells

(1×10^5 cells/mL, 100 μL) were treated with 100 μL of different concentrations of crude PSP or its sub-fractions, which were then plated into 96-well cell culture plate in triplicate and incubated at 37 °C for 20 hours. MTT (final concentration 0.5 mg/mL) was added into each well and incubated for 4 hours. The medium was removed and dried in the incubator for 30 minutes. Thereafter, stop solution (100 μL) was added to each well and incubated at 37 °C for 1 hour. The absorbance of the resulting solution was read at 570 nm with a spectrophotometer.

3.2.6 Apoptosis of B16 melanoma cells analysis by Cell death Detection ELISA kit

Induction of apoptosis is one of the reported anticancer mechanisms of PSP (Hsieh *et al.*, 2006; Hui *et al.*, 2005). The B16 (melanoma) cell line was used to study the anti-apoptotic effect of PSP. B16 cells (1×10^5 cells/mL, 500 μL) were cultured in 24-well tissue culture plate in the presence or absence of different concentration of crude PSP or its sub-fractions and incubated for 24 hours. Cells were collected and the cell death ELISA kit manual protocol was followed to test the apoptosis. The cell solution was centrifuged at 1500 g for 5 minutes, and the resulting pellet was resuspended with 500 μL incubation buffer and incubated at 20 °C for 30 minutes. The lysate was centrifuged with a bench-top centrifuge (Eppendorf centrifuge 5403, USA) at 20,000 g for 10 minutes. The supernatant was carefully removed. Then the resulting supernatant was diluted (1:10) with incubation buffer (approximately 1×10^4 cell equivalents/mL) to detect the nucleosomes in the sample by immunoassay. One hundred microlitre mixtures were added to the coated plate wells and incubated for 90 minutes. The wells were washed three times with washing buffer prior to the addition of 100 microlitre conjugate solution

in the wells and then incubated for 90 minutes. Substrate solution subsequently was added to the well, which was mixed for 15 minutes with a plate shaker (Precision Scientific Inc., USA). The optical density was determined with a spectrophotometer at 405 nm. According to the Cell death detection ELISA kit recommendations, hypertonic buffer (Tris 10 mM, NaCl 400 mM, $CaCl_2$ 5 mM and $MgCl_2$ 10 mM; pH 7.4) was used as a positive control to induce the cellular apoptosis.

3.2.7 Measurement of NO production by RAW 264.7 macrophages

Synthesis of NO was determined by assay of culture supernatants for NO_2^-, a stable reaction product of NO with molecular oxygen. RAW 264.7 cells were seeded into the 96-well tissue culture plates at a density of 10^5 cells per well. Cells were treated with different concentrations of crude PSP and its sub-fractions as well as the positive control, lipopolisaccharide (LPS, 1 μg/mL). NO_2^- production was measured after 24 hours (Stuehr and Marletta, 1987). Briefly, 50 μL of culture supernatant was incubated with an equal volume of Griess reagent (0.5% sulfanilamide, 0.05% N-(1-naphthyl)-ethylenediamine dihydrochloride in 2.5% H_3PO_4) in 96-well tissue culture plates for 10 minutes at room temperature. The absorbance at 550 nm was measured in a spectrophotometer along with $NaNO_2$ standards.

3.2.8 Measurement of PGE_2 and IFN-β

The commercial enzyme immunoassay kits were used to determine PGE_2 and IFN-β concentrations in culture medium according to the manufacturer's protocols. Briefly, 50 μL of collected culture medium was added to the well and incubated for 1 hour. The wells were washed with washing buffer - three times - prior to the addition of

100 µL labeled secondary antibody. The plate was incubated at room temperature for 60 minutes. Then, it was washed and 100 µL of substrate solution was added. Thereafter it was incubated for 60 minutes. Stop solution was added to stop the reaction before optical density was determined on a spectrophotometer at 450 nm. Standards were run with each assay in the same plate.

3.2.9 Data analysis

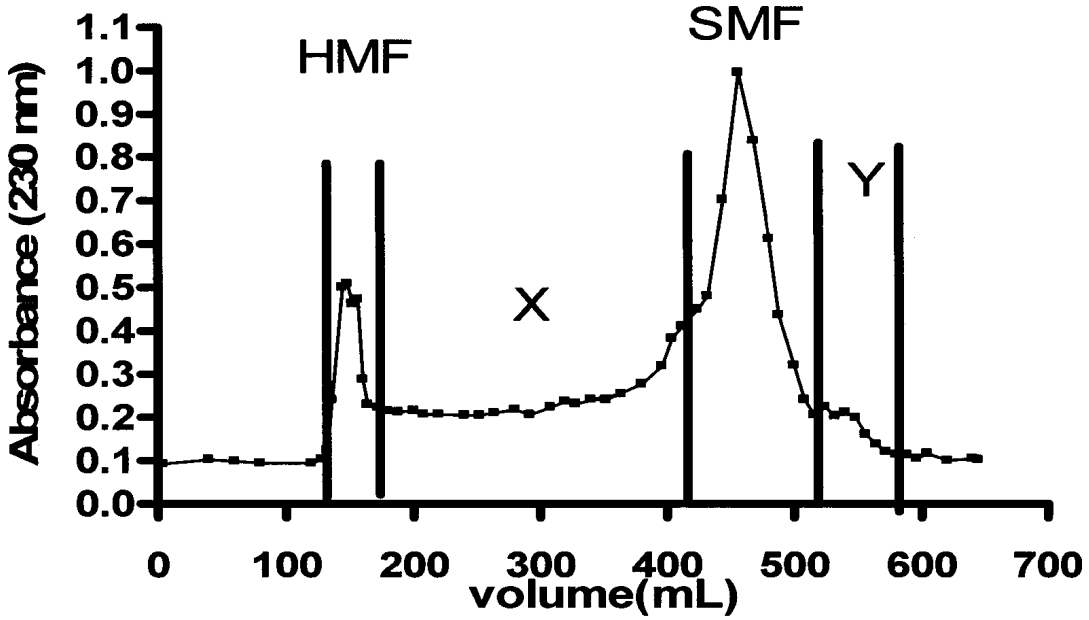
Data was expressed as Mean ± standard error of the mean (S.E.M). For comparing multiple means, the overall difference between groups was determined by one-way ANOVA followed by Tukey's post hoc test to determine the statistical significance between individual groups. p value < 0.05 was considered to be statistically significant. All statistical analysis was calculated with the Graph-pad Prism computer program (Graphpad Software, version 4.1, San Diego, CA).

3.3 Results

3.3.1 Chromatography fractionation of crude PSP water extract

The gel filtration profile of crude PSP is shown in Figure 3.1. There were three major peaks on the chromatogram. Fractions with elution volumes of 140 mL to 180 mL, which corresponded to the void volume of the column, were pooled. This 40 mL fraction was designated as HMF. This HMF was consistent with the elution of PSP fraction in the void volume of Sephadex G-75 chromatography (Yang *et al.*, 1999; Ye, 2004). The SMF was obtained from fractions with elution volumes of 408 mL to 512 mL. Two other fractions were also collected with respective elution volumes of 180 to 408 mL and 512 to 548 mL, which have been designated as fraction "X" and "Y", respectively. Data

Figure 3.1 Sephadex G-75 gel filtration chromatography of water-soluble PSP crude extract. Sephadex G-75 column was calibrated with blue dextran to give a void volume value of 40 mL (from 124 mL to 148mL). Five mL of crude PSP water-soluble extract (100 mg/mL) was loaded on the column and eluted with distilled water at flow rate of 0.8 mL/min at 4 °C. Total volume of 460 mL fractions was collected, the absorbance of each fraction was measured at 230 nm. Four fractions (HMF, SMF, X and Y) were pooled and lyophilized and stored at – 20 °C.


HMF
SMF
X
Y
Absorbance (230 nm)
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
100
200
300
400
500
600
700
volume(mL)

presented in Table 3.1 showed the % yields (based on dry weight of the four fractions isolated from crude water-soluble PSP) as: 15.85, 29.06, 52.98 and 1.71% for HMF, X, SMF and Y, respectively. The yield of crude PSP extract from the commercial product was around 72.5% (data not shown), the overall recovery of the gel filtered fractions amounted to 11.25, 20.6, 37.61 and 0.83% respectively for the 4 fractions. SMF constituted the largest PSP fraction on the basis of dry weight.

3.3.2 Effects of crude PSP and its sub-fractions

3.3.2.1 Effect of crude PSP and its sub-fractions on EA. hy 926 cells tubulization

The representative photographs of matrigel-induced tubulization and the inhibitory effects of different concentrations of crude PSP are shown in Figure 3.2. The density of branching points was significantly attenuated in crude PSP-treated groups. Quantitative analysis of treatment effects is shown in Figure 3.3A. Treatment with 100 and 1000 μg/mL of crude PSP significantly decreased branching point density ($p<0.05$). As shown Figure 3.3B and C, HMF, but not SMF, exerted an inhibitory effect on tubulization. The inhibitory potency (based on maximum inhibitory response) was in the order of crude PSP (86%) > HMF (40%).

In order to distinguish the treatment-induced inhibitory effects of tubulization from their cytotoxicity on EA. hy 926 cells, the treatment effect was also examined on standard endothelial cell monolayers concurrently with the matrigel-based assay. Cytotoxicity on EA. hy 926 cells based on the MTT assay was performed with the highest dose of each treatment group. The results showed that crude PSP and its fractions did not inhibit the viability of endothelial cells grown in monolayers (Figure 3.4).

3.3.2.2 Induction of apoptosis in B16 melanoma cells

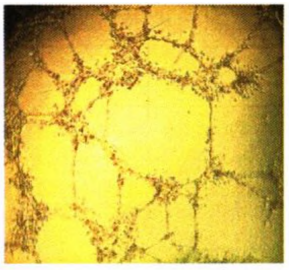
Table 3.1 Percent yields of four fractions recovered from the crude PSP extract following Sephadex G-75 gel filtration.

Fraction	% yield from crude PSP extract*	% yield from commercial PSP#	N value
HMF	15.85±1.3	11.25±0.9	10
X	29.06±2.9	20.6±2.0	10
SMF	52.98±2.1	37.61±1.5	10
Y	1.71±0.5	0.83±0.4	10

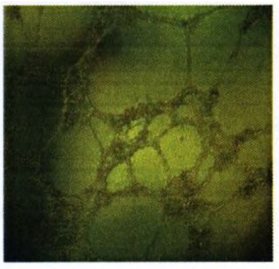
* Based on the water soluble crude PSP extract.

Based on the dry weight of commercial PSP powder used for making the original solution.

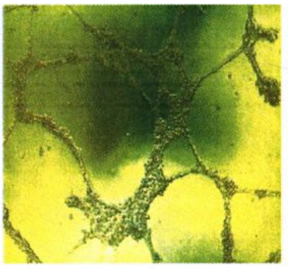
Figure 3.2 Representive photographs of Matrigel-based EA. hy 926 cells tubulization in absence and presence of different concentration of crude PSP waster extract. EA.hy 926 cells were plated on Matrigel (75 μL/well) on 96-well cell culture plate at a density of 1.8×10^4 cells per well in the absence (A) and presence of 1 μg/mL (B), 10 μg/mL (C), 100 μg/mL (D) and 1000 μg/mL (E) of crude PSP water extract. Photographs showed the tube-like structure on Matrigel after 18 hours incubation. The experiment was carried out six times (n=6).



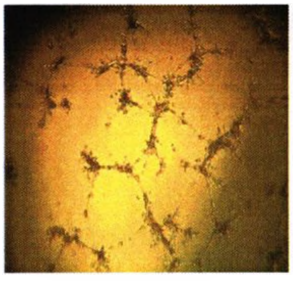
A: control



B: crude PSP 1 µg/mL



C: crude PSP 10 µg/mL

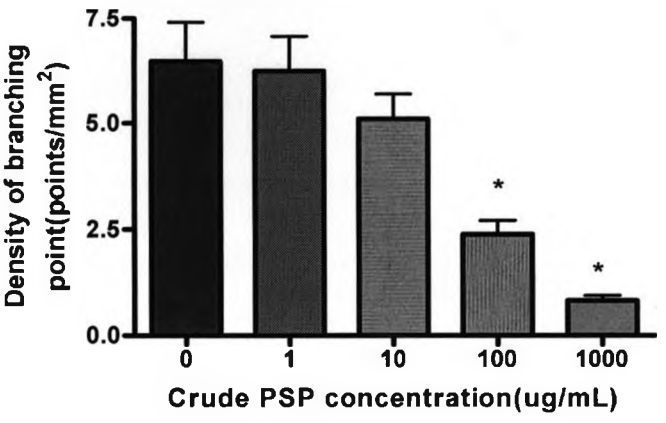


D: crude PSP 100 µg/mL

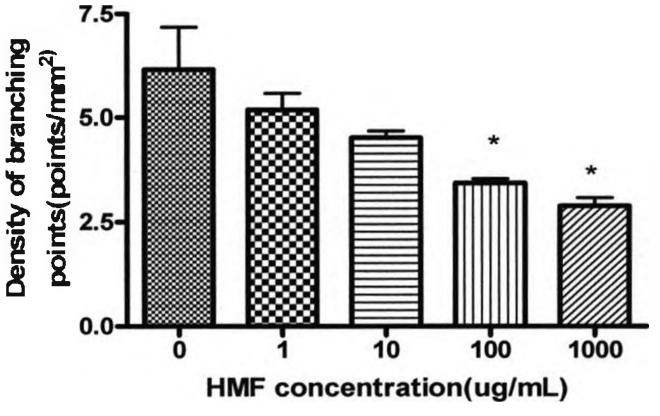
E: crude PSP 1000 µg/mL

Figure 3.3 Dose-related inhibition of tubulization of EA. hy 926 cells by crude PSP extract, HMF and SMF. EA. hy 926 cells were plated on the matrigel (75 μg/mL) in the absence and presence different concentration of crude PSP (A), HMF (B) and SMF (C). After 18 hours of incubation, photographs of tube-like structures developed on the matrigel were taken. Quantitative analysis was performed by calculating the density of branching point of each treatment group and control. Each treatment group and control group was performed in duplicate in each experiment and the experiment was repeated six times (n=6). Data are expressed as mean ± S.E.M and analyzed using one-way ANOVA. From 100 μg/mL to 1000 μg/mL, crude PSP significantly decreased the density of branching point ($p<0.05$). From 100 μg/mL to 1000 μg/mL HMF significantly inhibited the branching points ($p<0.05$). SMF did not have a significant effect on the density of branching point ($p>0.05$). * indicated significant difference from control at $p<0.05$.

A


Density of branching point(points/mm2)
7.5
5.0
2.5
0.0
*
*
0
1
10
100
1000
Crude PSP concentration(ug/mL)

B


Density of branching points(points/mm2)
7.5
5.0
2.5
0.0
*
*
0
1
10
100
1000
HMF concentration(ug/mL)

C

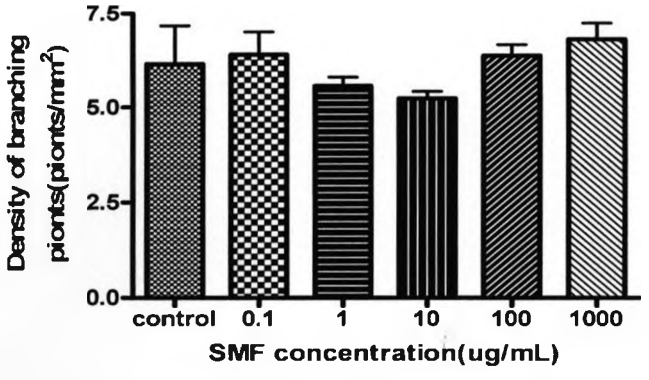
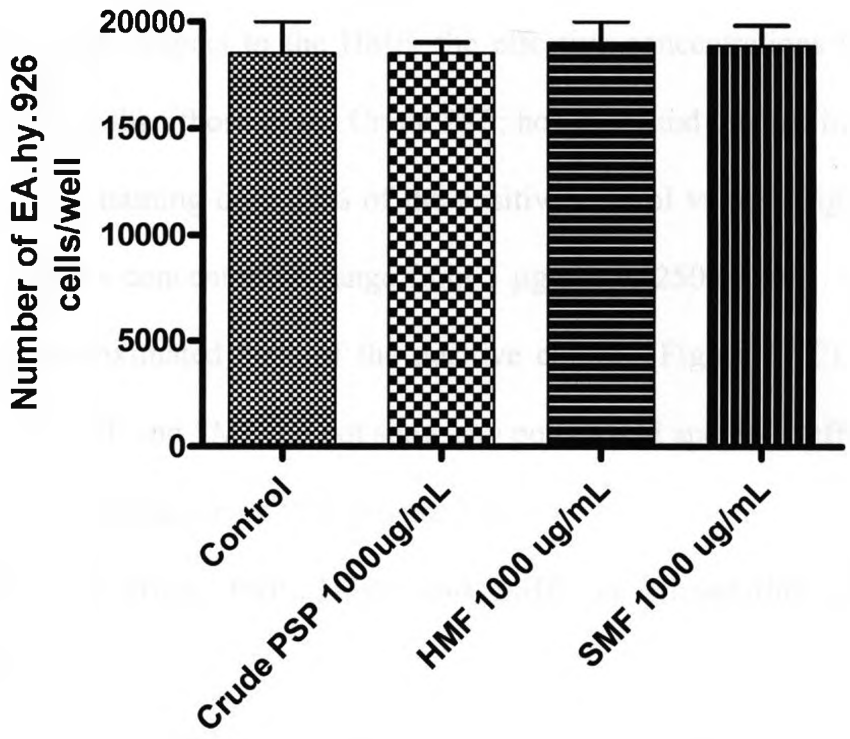

Density of branching pionts(pionts/mm2)
7.5
5.0
2.5
0.0
control
0.1
1
10
100
1000
SMF concentration(ug/mL)

Figure 3.4 Effect of crude PSP, HMF and SMF on 24 hr viability of EA. hy 926 cells. EA. hy 926 cells (1×10^4 cells per well) were treated with crude PSP (1000 μg/mL), HMF (1000 μg/mL), SMF (1000 μg/mL), control (culture medium) for 24 hours. The changes in color were determined by a spectrophotometer at 570 nm. Data are expressed as mean ± S.E.M of six independent experiments (n=6), and each experiment was performed in duplicate. Differences between control and treatment groups were insignificant by one-way ANOVA ($p>0.05$).


Number of EA.hy.926 cells/well
20000
15000
10000
5000
0
Control
Crude PSP 1000ug/mL
HMF 1000 ug/mL
SMF 1000 ug/mL

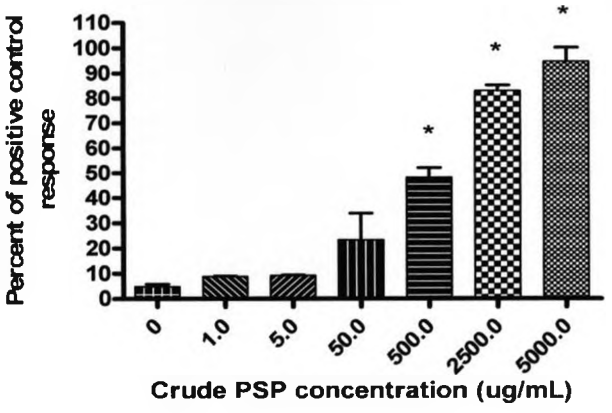
The apoptotic effect of crude PSP and its sub-fractions were studied with melanoma cells. Hypertonic buffer was used as the positive control according to manufacturer's recommendation. The response of positive control was adjusted to 100% response to normalize the response of all treatment groups; and the normalized data were used to generate the dose-response curve. Crude PSP induced dose-related apoptosis in melanoma cells within the dose range from 500 μg/mL to 5000 μg/mL. Moreover, the maximum response of crude PSP group was identical to that of the positive control (Figure 3.5A). With respect to the HMF, the effective concentrations (1 μg/mL to 50 μg/mL) were lower than those of the Crude PSP; however, and the maximum response to HMF was lower, attaining only 32% of the positive control values (Figure 3.5B). SMF showed an effective concentration range from 5 μg/mL to 2500 μg/mL and a maximum response that approximated 50% of the positive control (Figure 3.5C). Moreover, the combination of HMF and SMF did not show any potentiated apoptotic effect and the total effect was still lower than crude PSP (Figure 3.6).

3.3.2.3 Effects of crude PSP, HMF and SMF on stimulation of RAW 264.7 macrophages

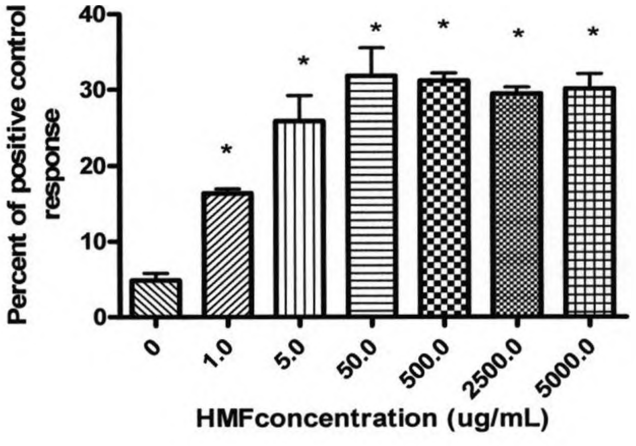
The production of NO by macrophage cells was quantified indirectly by the Griess reaction, which determines nitrite and nitrate accumulation in the culture medium. LPS, which was used as positive control for the experiment, induced a dose-dependent (0.01 to 1.0 μg/mL) increase in the 24 hour NO production in cultured RAW 246.7 macrophage cells, reaching maximal response at 1 μg/mL (data not shown). Results shown in Figure 3.7 (A, B, C) illustrate that crude PSP, HMF and SMF dose-dependently up-regulated the production of NO. The estimated EC_{50} values for the crude PSP, HMF and SMF were

Figure 3.5 Effects of crude PSP and its fractions on the B16 (melanoma) cell apoptosis. B16 cells were exposed for 24 hours at 37 °C to different concentrations of crude PSP (A), HMF (B) and SMF (C). After cell lyses and centrifugation, the supernatant fractions were tested for nucleosomes by ELISA. Hypotonic buffer was used as positive control for this assay. The results were normalized by expressing the treatment response as a percentage of the maximum response of the positive control value. Data are expressed as mean ± S.E.M of four independent experiments (n=4). From 500 μg/mL to 5000 μg/mL, crude PSP induced the apoptosis of melanoma cells; the maximal response was the same as positive control. The effective concentrations of HMF were from 1 μg/mL to 50 μg/mL and the maximal response was 32% of positive control. SMF showed an effective concentration range from 5 μg/mL to 2500 μg/mL and the maximum response was 50% of positive control.

A


Percent of positive control response
110
100
90
80
70
60
50
40
30
20
10
0
0
1.0
5.0
50.0
500.0
2500.0
5000.0
Crude PSP concentration (ug/mL)

B


Percent of positive control response
40
30
20
10
0
0
1.0
5.0
50.0
500.0
2500.0
5000.0
HMFconcentration (ug/mL)

C

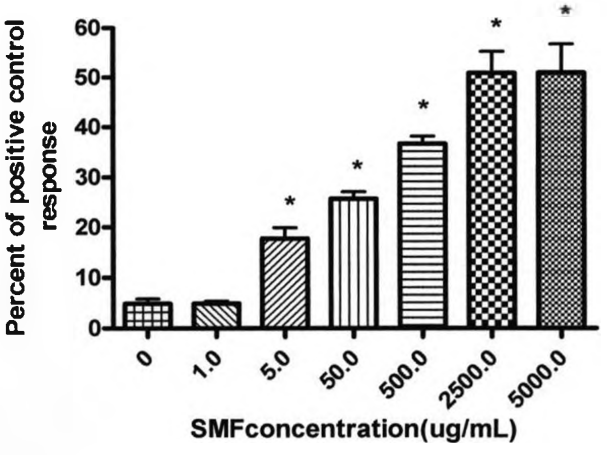
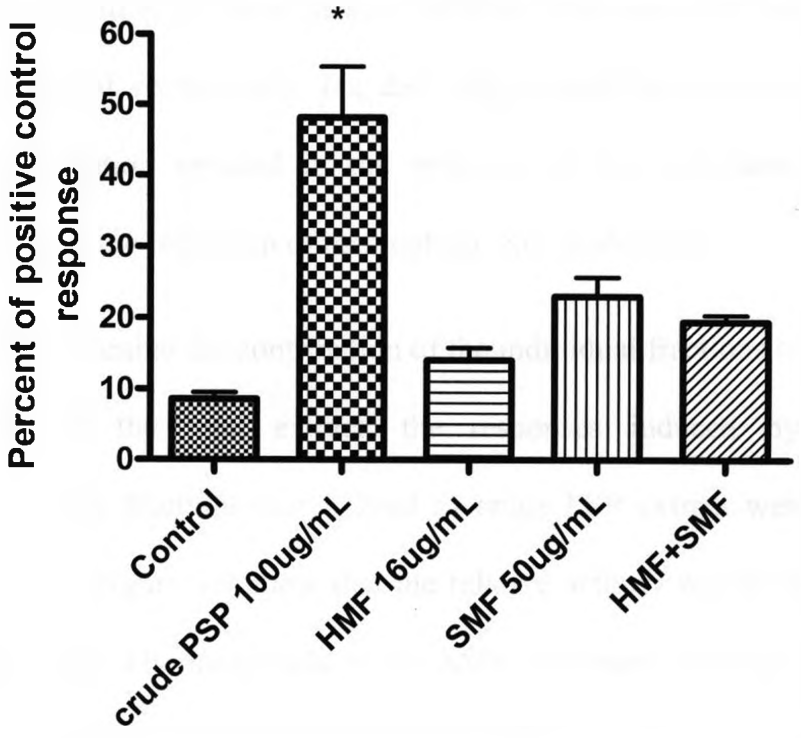

Percent of positive control response
60
50
40
30
20
10
0
0
1.0
5.0
50.0
500.0
2500.0
5000.0
SMFconcentration(ug/mL)

Figure 3.6 Combination effects of HMF and SMF on B16 cells apoptosis. Cells were treated as described in Figure 3.5. Hypotonic buffer was used as positive control which induced melanoma cells apoptosis. The results were normalized by expressing the treatment response as a percentage of the maximum response of the positive control value. The data are expressed as mean ± S.E.M of 4 independent experiments (n=4), and each experiment was performed in duplicate. The Data was analyzed using one-way ANOVA followed by Tukey's test. Crude PSP significantly increased the apoptosis (* $p<0.05$). The combination of HMF and SMF did not show significant difference with HMF and SMF alone ($p>0.05$).

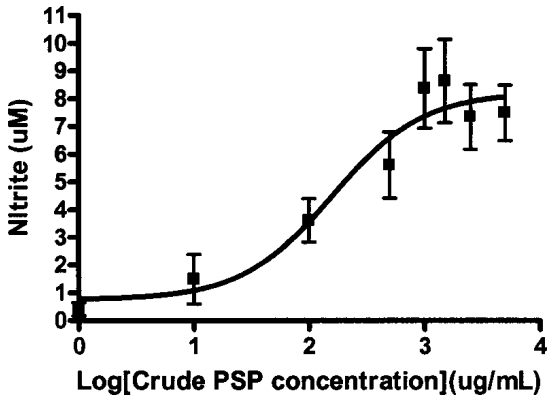

Percent of positive control response
60
50
40
30
20
10
0
*
Control
crude PSP 100ug/mL
HMF 16ug/mL
SMF 50ug/mL
HMF+SMF

158.8, 35.35, 9.71 μg/mL, respectively (Table 3.2). According to the EC_{50} values, SMF was 16 times more active than crude PSP. In addition, the maximum response induced by the three treatments was different and the hillslopes were not identical either (crude PSP 1.109, HMF 1.523, SMF 1.995) which suggested that some inhibitory substance existed in the crude PSP (Figure 3.8). Crude PSP was the least efficacious, producing a maximum response that was about 20% of that produced by the positive control. In contrast, the maximum response of its sub-fractions, SMF and HMF was 100% and 70% of the positive control, respectively. The data suggest that fractionation of the crude PSP extract by gel filtration resulted in the isolation of two sub-fractions that showed enhanced potency in the induction of macrophage NO production.

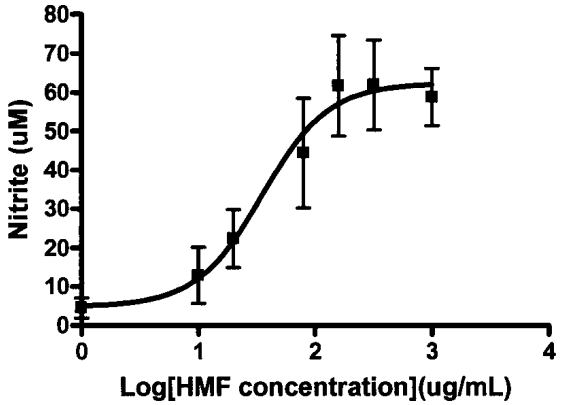
To further examine the contribution of the individual fractions of PSP towards the overall activity of the total extract, the responses induced by the equivalent concentrations of the fractions that existed in crude PSP extract were compared. The results presented in Figure 3.9 show that the relative activity was in the order of: SMF >HMF >>crude PSP. The magnitude of the SMF maximum response was the same as LPS, while HMF and crude PSP produced maximum responses that were 24% and 10% of the maximum LPS response, respectively. Based on the maximal response data, the effect of SMF was 10 times higher than that of the crude PSP on the induction of NO. To determine whether gel filtration of the crude PSP removed an immuno-inhibitory substance(s), thereby unmasking the immunostimulatory of the SMF, materials collected from X (Figure 3.1) were tested for their effect on the induction of NO production. The results (not shown) indicated that fraction X had no such effect. Additionally, adding

Figure 3.7 Dose-response relationships of nitrite accumulation RAW 264.7 cells induced by PSP extract and its sub-fractions. RAW 264.7 cells (5×10^5 cells/mL, 100 μL) were plated into 96-well plate in the absence and presence of different concentrations of crude PSP (A), HMF (B) and SMF (C). After 24 hours incubation, the supernatant was collected and measured for nitrite concentration using Griess reagent. Each concentration was done in duplicate. Dose-response curves of various treatments were generated by nonlinear regression analysis provided by the prism program for each treatment. The EC_{50} values and hillslopes were then estimated. Data were expressed as mean ± S.E.M of 6 independent experiments (n=6).

A


Nitrite (uM)
Log[Crude PSP concentration](ug/mL)

B


Nitrite (uM)
Log[HMF concentration](ug/mL)

C

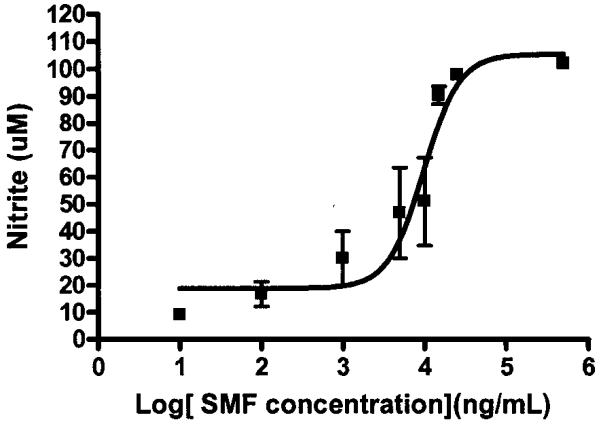

Nitrite (uM)
Log[SMF concentration](ng/mL)

Table 3.2 EC_{50} values of crude PSP, HMF and SMF on up-regulation of RAW 264.7 cells NO production.

	EC_{50} (μg/mL)
Crude PSP	158.80 ± 11.50#
HMF	35.35 ± 5.41*
SMF	9.71 ± 2.23

EC_{50} was estimated by nonlinear regression of prism program. The data were expressed as mean ± S.E.M of six independent experiments (n=6) and analyzed using one-way ANOVA followed by Tukey's test. # $p<0.01$, crude PSP compared with SMF; * $p<0.05$, HMF compared with SMF.

Figure 3.8 Composite dose-response relationships of nitrite accumulation in RAW 264.7 cells induced by crude PSP extract and its sub-fractions. All responses from the treatment groups were normalized and expressed as a % of the maximum response of 1 μg/mL of LPS (positive control). All experimental procedures were identical to what were described in Figure 3.7. Data were expressed as mean ± S.E.M of six independent experiments (n=6). The curves were obtained by nonlinear regression analysis provided by prism program.

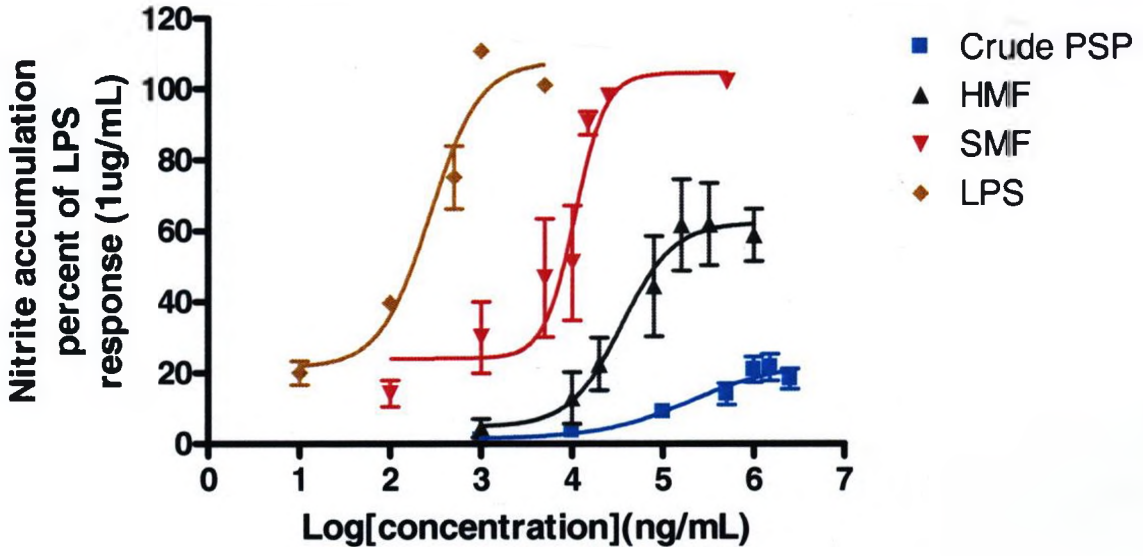
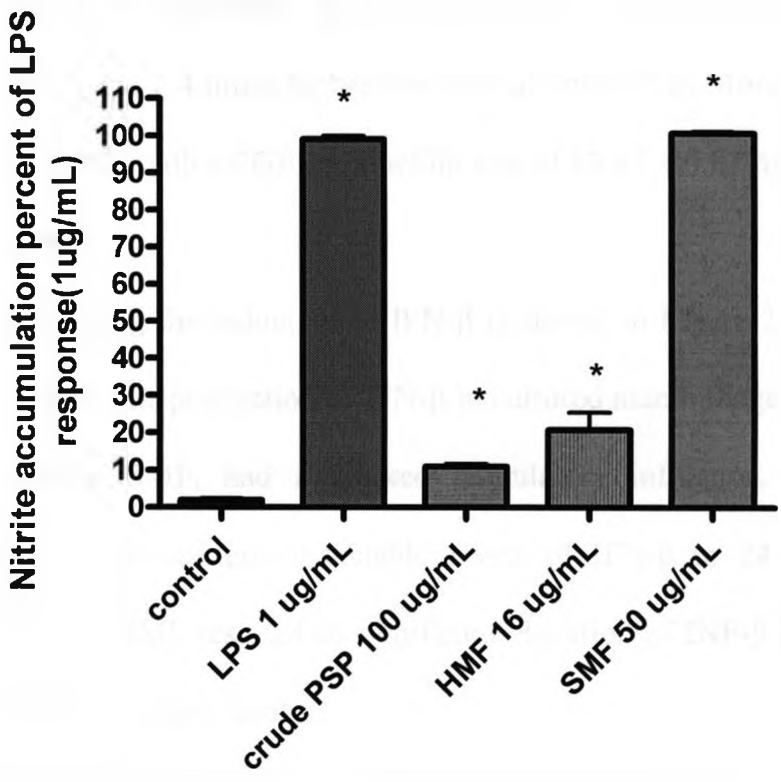

Nitrite accumulation
percent of LPS
response (1ug/mL)
120
100
80
60
40
20
0
0
1
2
3
4
5
6
7
Log[concentration](ng/mL)
Crude PSP
HMF
SMF
LPS

Figure 3.9 Induction of nitrite accumulation in RAW 264.7 cells by crude PSP and equivalent concentration of its sub-fractions. Based on the data shown in Table 3.1, 100 g of crude PSP extract was found to contain 16 g HMF and 50 g SMF and accordingly the equivalent concentration of HMF and SMF to 100 μg/mL crude PSP was 16 μg/mL and 50 μg/mL, respectively. Cells were cultured with LPS (1 μg/mL, positive control), crude PSP (100 μg/mL), HMF (16 μg/mL), SMF (50 μg/mL) or culture medium (control) for 24 hours; and each group was carried out in duplicate. The supernatants were collected for nitrite determination with the Griess reagent. Data are expressed as mean ± S.E.M of six independent experiments (n=6) and analyzed using one-way ANOVA followed by Tukey's test. * $p<0.05$ compared with control.


Nitrite accumulation percent of LPS response(1ug/mL)
110
100
90
80
70
60
50
40
30
20
10
0
*
*
*
*
control
LPS 1 ug/mL
crude PSP 100 ug/mL
HMF 16 ug/mL
SMF 50 ug/mL

together HMF and SMF still did not produce the same effect as crude PSP (data not shown).

Upregulation of PGE_2 production by macrophages was examined to define the spectrum of immunostimulatory activity of the PSP fractions. To determine the relative potency of the sub-fractions, responses to the concentrations that were originally found in the crude PSP extract were compared. As shown in Figure 3.10, the induction of PGE_2 by both SMF and HMF was 2-4 times higher than that of crude PSP. Moreover, SMF was more active than the HMF, with a PGE_2 production rate of 13.47 ± 0.07 ng/mL vs. 6.02 ± 0.15 ng/mL, respectively.

The data concerning the induction of IFN-β is shown in Figure 3.11. The Crude PSP extract failed to alter the production of IFN-β in cultured macrophages; however, the sub-fractions, especially SMF, had a marked stimulatory influence. While control macrophages failed to produce any detectable levels of IFN-β in 24 hr incubation, treatment with SMF and HMF resulted in significant elevation of INF-β levels (77.57 ± 1.93 and 2.85±1.51 pg/mL, respectively).

3.3.3 IFN-β activity and the combination effect of SMF and IFN-β

PSP had been reported to induce IFN in human white blood cells (Yang *et al.*, 1999b). Also, IFN plays an important role in the immune response of the host against cancer. IFN-β is the most potent one amongst the other IFNs with respect to anticancer activity, especially for the treatment of melanoma (Damdinsuren *et al.*, 2003). In view of the similarity in pharmacological activity between PSP and IFN-β, it was decided to examine the interaction between these two agents when given concurrently. Firstly, IFN-

Figure 3.10 Twenty four hours Prostaglandin E_2 production in RAW 264.7 cells induced by crude PSP and its sub-fractions (HMF and SMF). LPS was used as a positive control. Cells were prepared and treated as described previously in Figure 3.7. The values represented the accumulation of PGE_2 in culture medium. Data are expressed as mean ± S.E.M of four independent experiments (n=4) and analyzed using one-way ANOVA followed by Tukey's test. The SMF group was significantly different from control ($p<0.001$) and crude PSP ($p<0.01$).

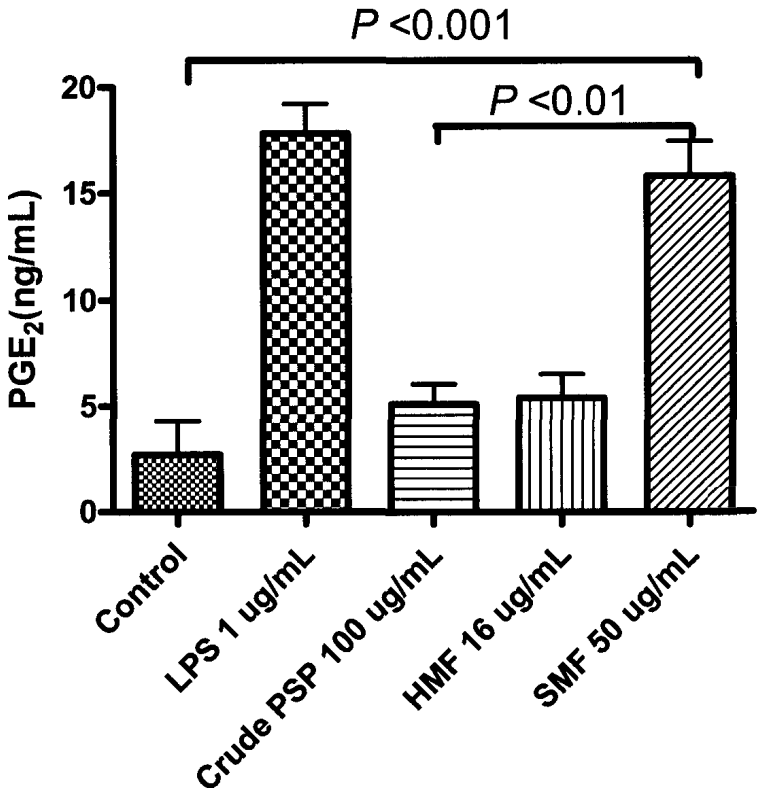
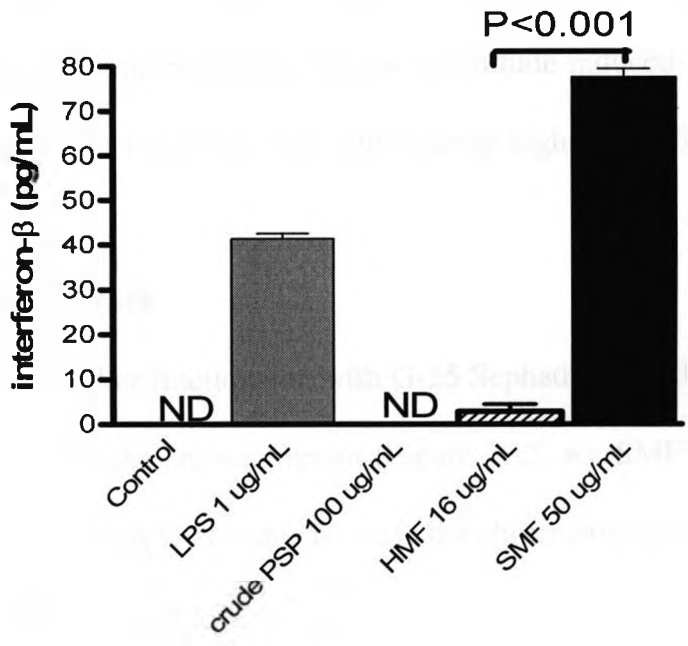

P <0.001
P <0.01
20
15
10
5
0
PGE2(ng/mL)
Control
LPS 1 ug/mL
Crude PSP 100 ug/mL
HMF 16 ug/mL
SMF 50 ug/mL

Figure 3.11 Modulation of IFN-β accumulation in RAW 264.7 cells by LPS, crude PSP, HMF and SMF. Cells were prepared and treated as described on Figure 3.7. IFN-β was quantified by measuring IFN-β level in the cultured supernatant with ELISA kit, the assay range was from 1 to 1000 pg/mL. The data are expressed as mean ± S.E.M of four independent experiments (n=4) and analyzed using one-way ANOVA followed by Tukey's test. ND represents not detectable levels in the group. The SMF group was significantly higher than the HMF group ($p<0.001$).


P<0.001
interferon-β (pg/mL)
80
70
60
50
40
30
20
10
0
ND
ND
Control
LPS 1 ug/mL
crude PSP 100 ug/mL
HMF 16 ug/mL
SMF 50 ug/mL

β dose-dependently inhibited the matrigel-based tubulization of endothelial cells (Figure 3.12A). Sub-effective concentration of IFN-β (0.1 U/mL) and SMF (0.1 μg/mL) were used to test the combination effect, as shown in Figure 3.12B, the combination significantly inhibited the density of branching points ($p<0.05$). Secondly, from 10 to 2500 U/mL IFN-β led to a dose-related induction of B16 cell apoptosis (Figure 3.13A). SMF 10 μg/mL and IFN-β 1 U/mL were combined to test their combination effect together. The results showed that the combination was significantly different from either of them on induction of B16 cells apoptosis (Figure 3.13B). Finally, IFN-β did not induce the NO production of RAW macrophages, but the magnitude induced by combination of IFN-β (50 U/mL) and SMF (1 μg/mL) was significantly higher than SMF alone (Figure 3.14).

3.3.4 Characterization of SMF

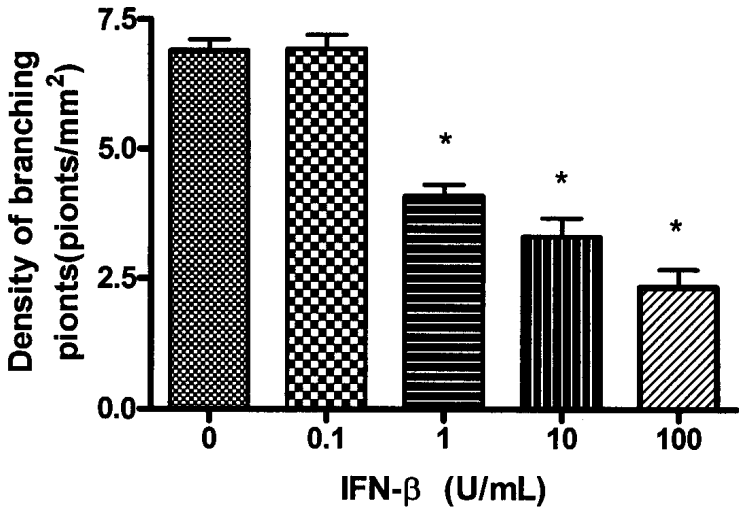
When SMF was further fractionated with G-25 Sephadex gel filtration, there were multiple peaks presented in the chromatogram (Figure 3.15, ■). SMF was in a powdery form and brown in color. It was heat stable because the chromatographic profile remained unchanged after the SMF was heated at 100 °C for 30 min (Figure 3.15, ▲) and the ability of upregulation of macrophages NO production was not changed either (Figure 3.16).

3.4 Discussion

Water extracts of PSP isolated from *Coriolus versicolor* (crude PSP) have been used as a tonic and as adjunct therapy for the treatment of cancer in Asia (Yang, 1999b). Although the mechanism underlying its anticancer is far from clear, it has been recognized as a "biological response modifier" on the basis of its ability to modify the

Figure 3.12 Inhibition of matrigel-based tubulization of EA. hy 926 cells by IFN- β and its interaction with SMF. A: IFN-β; B: Combination effect of IFN-β and SMF. Cells were prepared as mentioned in Figure 3.4 and treated with different concentrations of treatments. Each treatment group was performed in duplicate in each experiment, and the experiment was repeated six times (n=6). The values were number of points/mm^2. The data are expressed as mean ± S.E.M and analyzed using one-way ANOVA followed by Tukey's test. A: IFN-β significantly decreased the density of branching points at concentrations from 1 to 100 U/mL (* p<0.05). B: The combination group significantly decreased the density of branching points (* p<0.05).

A


Density of branching pionts(pionts/mm2)
7.5
5.0
2.5
0.0
*
*
*
0
0.1
1
10
100
IFN-β (U/mL)

B

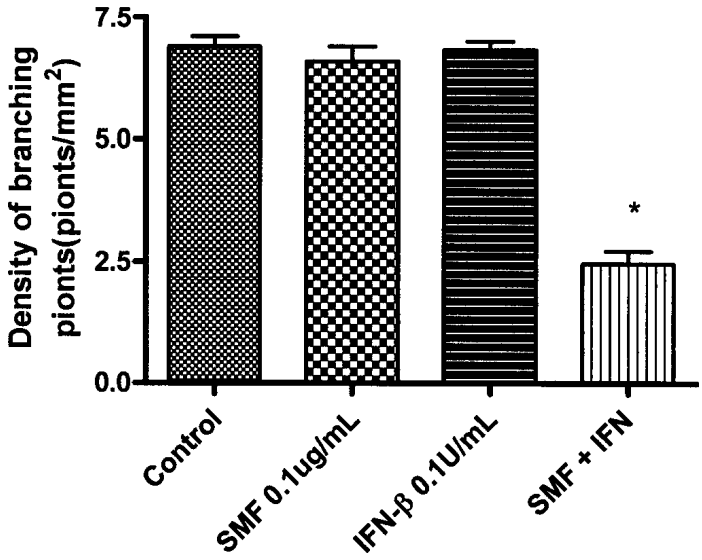
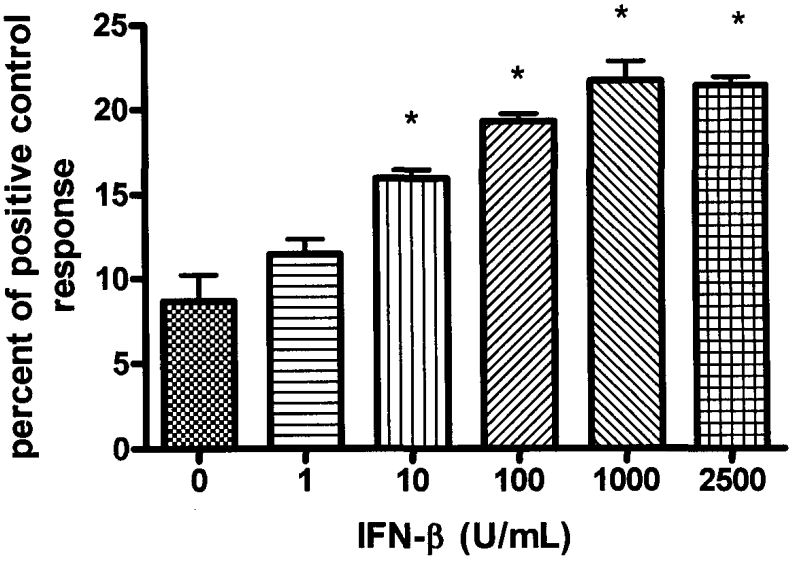

Density of branching pionts(pionts/mm2)
7.5
5.0
2.5
0.0
*
Control
SMF 0.1ug/mL
IFN-β 0.1U/mL
SMF + IFN

Figure 3.13 Apoptotic effect of IFN-β on the B16 (melanoma) cells and modulation by concurrent SMF treatment. A: IFN-β; B: Concurrent treatment. B16 cells were exposed for 24 hours at 37 °C to control and different concentration of treatments. After cell lysis and centrifugation, the pellets were tested for nucleosomes by ELISA. Hypotonic buffer was used as a positive control. All data were expressed as a % of positive control. Each group was performed in duplicate in each experiment and the experiment was repeated four times (n=4). Data are expressed as mean ± S.E.M and analyzed using one-way ANOVA. IFN-β (10 to 2500 U/mL) significantly increased the apoptosis of B16 cells ($p<0.05$). The combinational effect was significantly higher than either IFN-β ($p<0.01$) or SMF alone ($p<0.05$).

A


percent of positive control response
25
20
15
10
5
0
*
*
*
*
0
1
10
100
1000
2500
IFN-β (U/mL)

B

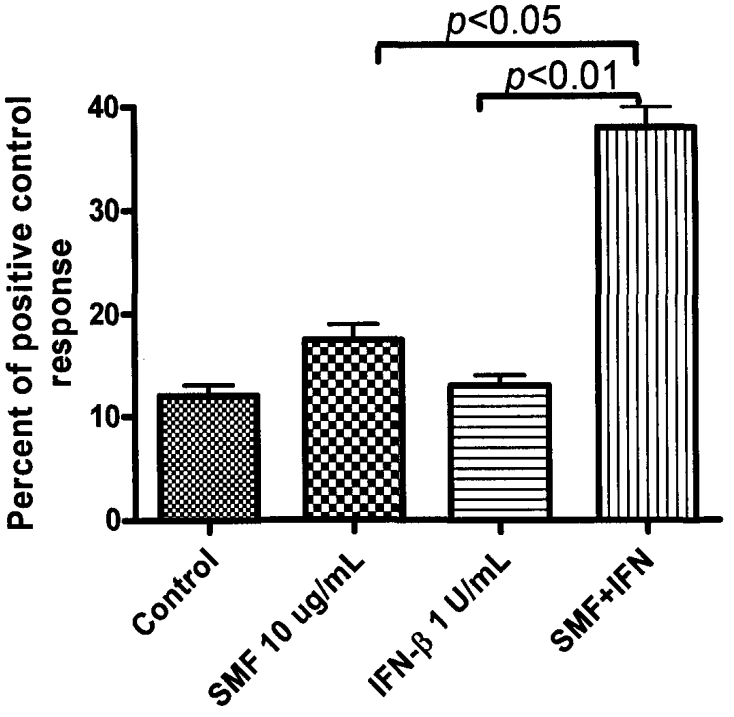

p<0.05
p<0.01
Percent of positive control response
40
30
20
10
0
Control
SMF 10 ug/mL
IFN-β 1 U/mL
SMF+IFN

Figure 3.14 Combination effect of SMF and IFN-β on induction of RAW264.7 cells NO production. RAW264.7 cells (5×10^5 cells/mL) were exposed for 24 hours to SMF (1 μg/mL), mouse IFN-β (50 U/mL), or the combination of SMF and IFN-β. Nitrite concentration accumulated in each culture supernatant during the incubation period was determined as described before. Each group was performed in duplicate in each experiment and the experiment was repeated four times (n=4). Data are expressed as mean ± S.E.M and was analyzed with T-test. ND represents no detectable levels in the group.* $p<0.05$ compared with SMF.

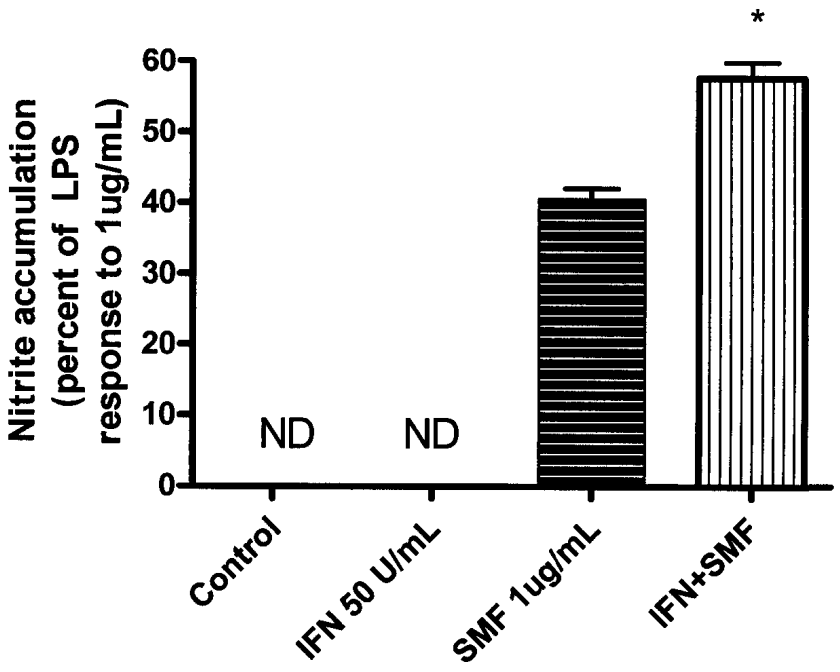

Nitrite accumulation
(percent of LPS
response to 1ug/mL)
60
50
40
30
20
10
0
ND
ND
*
Control
IFN 50 U/mL
SMF 1ug/mL
IFN+SMF

Figure 3.15 Sephadex G-25 gel filtration chromatography of small molecular weight fraction (SMF). 1.5 mL of normal (■) or heated (▲) SMF (100 mg/mL) was loaded on the G-25 column (80×1.8mm) and eluted with distilled water at a flow rate of 0.5 mL/min at 4 °C. The fractions were pooled based on the absorbance at 230 nm.

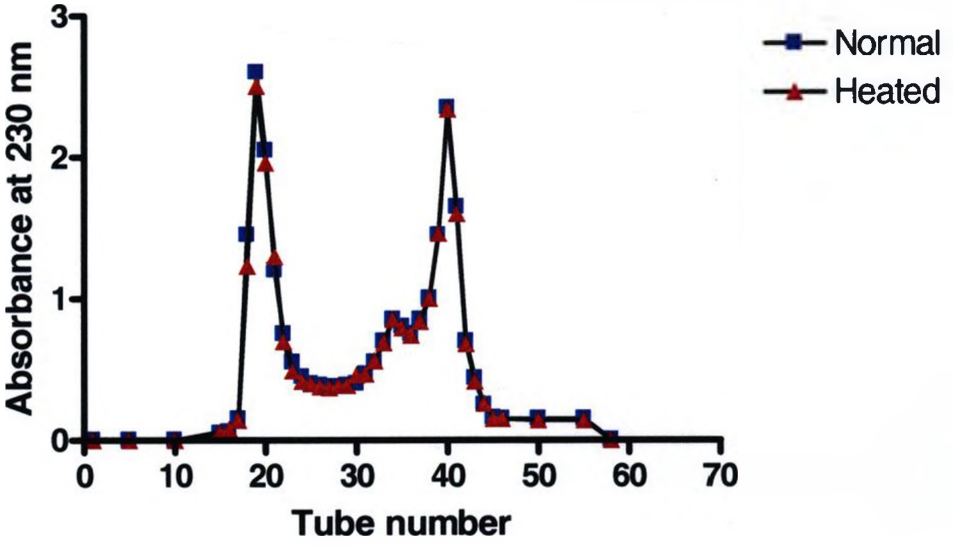
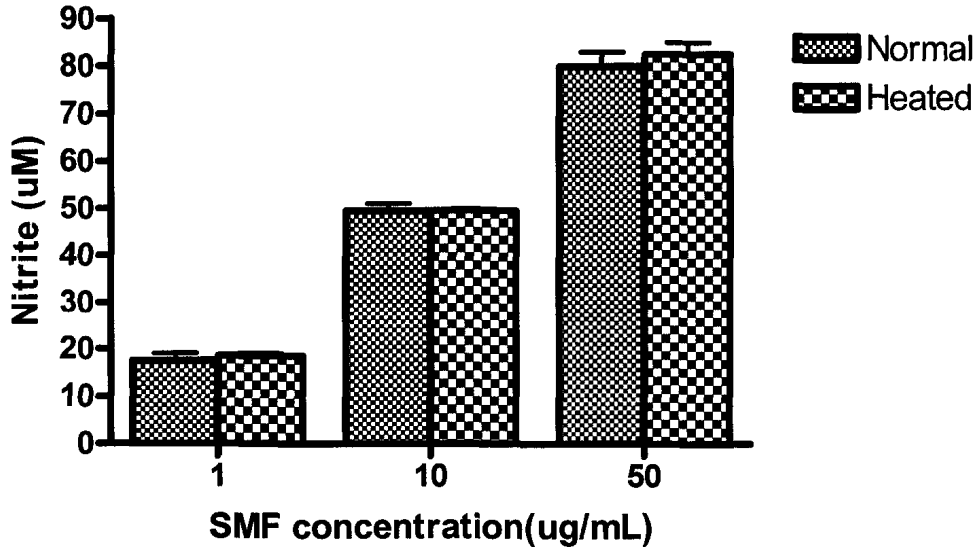

Absorbance at 230 nm
3
2
1
0
0
10
20
30
40
50
60
70
Tube number
Normal
Heated

Figure 3.16 Induction of nitrite accumulation in RAW264.7 cells by normal and heated SMF. SMF was dissolved in distilled water and heated at 100 °C for 30 min, and different concentrations of heated as well as normal SMF were used to treat the macrophages for 24 hours to determine up-regulation of NO production. The supernatant of the cell incubations was collected to test the NO_2^- with the Griess reagent. Each group was performed in duplicate in each experiment and the experiment was repeated six times (n=6). Data are expressed as mean ± S.E.M and analyzed using one-way ANOVA. There was not significant difference between normal and heated SMF groups.


90
80
70
60
50
40
30
20
10
0
Nitrite (uM)
1
10
50
SMF concentration(ug/mL)
Normal
Heated

host's biological response by stimulating the immune system and thereby eliciting various therapeutic effects (Li, 1999). More recent data suggested that they also possessed anti-apoptotic and antiangiogenic effects (Yang *et al.*, 2006; Ho *et al.*, 2004; Hsieh *et al.*, 2006; Zeng *et al.*, 2005; Ye, 2004; Ho *et al.*, 2004). It is generally believed that the primary bioactive components of PSP are associated with the high molecular weight or the polysaccharide-peptide fraction PSP (Chu *et al.*, 2002; Jong and Yang, 1999; Yang, 1999a; Tsukagoshi, 1984). In the present study, we have fractionated crude PSP extract into the polysaccharide-peptide fraction and a small molecular weight fraction to evaluate their bioactivity. The results revealed that crude PSP water extract contained multiple bioactive components that exert antiangiogenic, apoptotic, and a range of immunostimulatory activities. It appears that the antiangiogenic effect was primarily ascribed to HMF, while the immunostimulatory and apoptotic effects were associated with both fractions. Importantly, SMF showed marked and unique immunostimulatory activity with reference to crude PSP and HMF, and a significant interaction with IFN-β.

Medicinal herbal products are known to contain multiple components and it has been speculated that there may be synergistic or antagonistic interactions among different constituents leading to the expression of varying degrees of activity depending on the make-up of the total extract (Ernest, 2000). The results of the present study were supportive of this speculation. The observed antiangiogenic, apoptotic, and immunostimulatory effects associated with the crude PSP and HMF were consistent with the established pharmacological activities for PSP (Yang *et al.*, 1999; Ho *et al.*, 2004; Ye, 2004; Ooi *et al.*, 1999). On the contrary, SMF showed distinct differences from crude PSP extract with respect to immunostimulatory property. In this regard, the maximum

stimulation of macrophages by SMF was 10 times higher (Figure 3.8) and the EC_{50} (Table 3.2) was 16 times lower than crude PSP with respect to the up-regulation of NO production. This was reflected by the difference in the slope of their dose-response curves. Data presented in Figure 3.10 and 3.11 showed that the heightened immunostimulatory potency of SMF could be extended to include the induction of macrophage PGE_2 and IFN-β. The induction of IFN-β was of particular importance in that SMF was almost twice as active as LPS, while crude PSP extract was ineffective in this regard.

IFN-β plays a crucial role in cancer and treatment strategy. Data presented in Figure 3.12a and 3.13a showed its ability *in vitro* to inhibit tubulization of vascular endothelial cells and induce apoptosis in melanoma cells. These findings are consistent with the documentation of its cytotoxicity to tumor cells and inhibition of angiogenesis (Lindner 2002; Chawla-Sarkar, 2001; Leaman, 2003; Murata, 2006; Ikeda, 2002). In clinical practice, IFN-β has been combined with other therapeutic agents to overcome its severe side effect (Uyama *et al.*, 2007). In light of the observed upregulation of macrophage IFN-β by SMF, studies were carried out to examine the combinational effect of SMF and IFN-β on immunostimulation, anti-angiogenesis, as well as apoptosis. As shown in Figure 3.14, IFN-β did not activate macrophages to produce NO, which was consistent with the finding of a previous report (Aaron, 2001); however, it potentiated the stimulation of macrophages by SMF. In this context, it may be speculated that SMF acted like LPS in that the initial induction of macrophage IFN-β by LPS/SMF would act to prime the macrophages to further stimulation for NO production (Aaron, 2001). This may be reflected in the similarity in the dose-response of these two agents in the upregulation of macrophage NO production. Data shown in Figure 3.12B and 3.13B also showed that

the positive combinational effect of SMF and IFN-β could be extended from immunostimulation to antiangiogenesis and apoptosis. This successful combination of results provides plausibility for consideration of future clinical study, although additional studies are required to elucidate the underlying mechanism(s).

The mechanism underlying the increase in immunostimulatory potency of SMF associated with the fractionation of crude PSP extract is not clear. Removal of inactive materials through gel filtration could lead to a corresponding increase in specific activity. This is apparently not the case with SMF in light of the data presented in Figure 3.9, which illustrated the marked differences in the response induced by equivalent concentration of SMF and crude PSP. To explain the remarkable increase in immunostimulatory activity of SMF following its isolation, it is proposed that gel filtration of the crude PSP extract removed an immuno-inhibitory substance thus unmasking the immunostimulatory activity of SMF.

Studies on the chemical characteristics of crude PSP have been focused on the PSP (HMF) component alone because it was thought to be the only pharmacologically active component. The remaining components were discarded during purification procedure. In the current study, SMF was isolated from crude PSP water extract by Sephadex G-75 gel filtration; and it was heterogeneous in nature because the Sephadex G-25 chromatogram of SMF showed the existence of at least 3 sub-fractions. It was heat stable since the Sephadex G-25 chromatogram did not change after the sample was heated at 100 °C for 30 minutes (Figure 3.15), and the stimulatory effect on macrophage NO production remained unchanged with heat treatment (Figure 3.16). There is only one report in the literature describing the isolation of a small peptide from crude PSP extract

of *Coriolus versicolor* by heat treatment of the crude extract (Yang *et al.*, 1993a). Their analysis showed that it had molecular weight of 10 kDa and showed a significant inhibition of proliferation of cancer cell lines of HL-60, LS174-T, SMMU-7721 and SCG-7901 as well as immunopotentiating effect in up-regulating WBC and IgG levels *in vivo*. This peptide is probably different from the SMF identified in the present study on the basis of heat treatment requirement. There are reports on the recovery of small moleculer components from another medicinal mushroom extracts. For example, triterpenoids and steroids isolated from *Ganoderma lucidum* have been shown to possess potent immuno-modulating action (Gao and Zhou, 2002). This could serve as a good lead for future studies on SMF.

3.5 References

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Chapter 4

Anti-metastatic and immunostimulatory effect *in vivo* of a small molecular weight fraction isolated from *Coriolus Versicolor*

4.1 Introduction

Polysaccharide peptide (PSP), isolated from medicinal mushroom *Coriolus versicolor*, is believed to possess significant medicinal value (Yang *et al.*, 1999; Ng, 1998; Hsieh *et al.*, 2001). It has been used clinically as an adjunct for cancer patients undergoing chemo- or radio-therapy in Asia (Tsang *et al.*, 2003). Extensive clinical studies have been undertaken in China (Liu *et al.*, 1999; Ng, 1998); and a clinical study sponsored by NIH is in progress in the US to validate its anticancer ability (http://nccam.nih.gov/research/extramural/awards/2004/). In animal studies, PSP has been found to be effective at inhibiting melanoma, sarcoma S180 and human lung adenocarcinoma (Ho *et al.*, 2004, Zeng *et al.*, 1999). A wide range of pharmacological activities *in vitro* and *in vivo* of PSP including anti-angiogenesis, apoptosis, anti-inflammatory, analgesic, antiviral and activation of immune function have been reported (Shigefumi *et al.*, 2005; Ho, 2004; Chu *et al.*, 2002; Ooi *et al.*, 2000; Kidd, 2000; Ho *et al.*, 2004; Qian *et al.*, 1997; Wu *et al.*, 2006; Gong *et al.*, 1998), but the exact mechanism(s) of action underlying the anticancer effect is/are not clear.

Immunotherapy represents a powerful weapon in the arsenal of anticancer treatments (Ben-Efraim, 1996). For example, immunotherapy agents, such as IL-2, are able to harness and enhance the body's natural tendency to defend itself against malignant tumors. Significant numbers of herbal medicines are known to have immunostimulatory effects, and some of them have been shown to be effective against some forms of cancer (Stevens *et al.*, 1990). Some of these were regarded as biological response modifiers (BRM), which are defined as agents that can modify the host's biological response by stimulating the immune system and thereby eliciting various therapeutic effects (Li,

1999). The polysaccharide peptide (PSP) was designated as a BRM on the basis of its immunostimulatory property (Yang *et al.*, 1993); and it has been shown to activate lymphocytes, monocytes/macrophages, bone marrow cells, natural killer cells, and lymphocyte-activated killer cells *in vitro* (Liu *et al.*, 2002; Pang *et al.*, 2000; Asai *et al.*, 2000; Wang *et al.*, 1996) as well as to promote the proliferation and/or production of chemokines and cytokines such as interleukin (IL)-2 and IL-6, interferons, and tumor necrotic factor (Song, 2002; Fisher and Yang, 2002; Tsukagoshi *et al.*, 1984; Gu *et al.*, 1999; Ng *et al.*, 1999). In clinical studies, PSP was found to upregulate the immune response in patients undergoing cancer radiotherapy (Qian *et al.*, 1997), and restore the immunosupression caused by cancer or anticancer drugs (Mao *et al.*, 2001; Wang *et al.*, 2003).

The chemistry of PSP has not been investigated extensively because of the limitations of the methodologies for studying polysaccharides. Available data suggested that the major chemical constituent(s) of PSP are a group of polysaccharide-peptides with an average molecular weight of approximately 100 kDa, and the polysaccharides are thought to chemically link to a core peptide chain. The polypeptide moieties are rich in aspartic acid and glutamic acid. Monosaccharides with α-1, 4 and β-1, 3 glucosidic linkages constitute the polysaccharide moieties (Chu *et al.*, 2002; Jong and Yang, 1999; Yang, 1999; Tsukagoshi, 1984). Most published studies have focused on the high molecular weight materials, believing them to be the bioactive constituents, and have ignored the compounds with smaller molecular weights. Our laboratory has recently reported the isolation from a crude PSP aqueous extract by G-75 chromatography, a high molecular weight fraction (HMF) and a small molecular weight fraction (SMF) (Lui,

2006; Ye, 2004). The former is believed to correspond to the PSP fraction, which is generally considered to contain the principal pharmacologically active component (Borchers *et al.*, 1999); however, our preliminary study has shown that the latter, small molecular weight fraction, was more active than the HMF and crude PSP extract in upregulating NO production from macrophages (Lui, 2006). Moreover, our more recent *in vitro* studies have shown that although the SMF had limited anti-apoptotic and antiangiogenic activities as compared to the HMF and the crude PSP extract, it possessed remarkable immunostimulatory activity (Section 3). We hypothesized that SMF is more effective than crude PSP as an anticancer agent *in vivo* because of its significant immunostimulatory activity. We have previously shown that crude PSP was effective in the mouse liver metastasis model; the objective of this study was to evaluate the anti-metastatic effect of SMF as well as its immunomodulatory effects with crude PSP as a standard reference with the mouse liver metastases model.

4.2 Methods

4.2.1 Materials

The polysaccharide peptide of *Coriolus versicolor* was purchased from the Shanghai Xinkang Pharmaceutical Company (Shanghai, China, Lot: Z10980124). Crude PSP was the aqueous extract of the commercial PSP and the SMF was isolated from crude PSP aqueous extract by G-75 chromatography. Sephadex G-75 was purchased from GE healthcare bio-sciences AB (Uppsala, Sweden). Cell culture medium and reagents were purchased from Gibco Laboratories (Grand Island, NY, USA). Lipopolysaccharide (LPS), Concanavalin A (Con-A), colorimetric 3-(4, 5-dimethylthiazol-2-yl)-2,5-

diphenltetrazolum bromide (MTT) and Griess reagent were purchased from Sigma Aldrich (St. Louis, MO, USA). IFN-β ELISA kit was purchased from PBL Biomedical Laboratories (Piscataway, NJ, USA).

4.2.2 Cell culture

B16F10 is a spontaneously derived malignant melanoma cell line originating from C57BL/6 mice. This cell line was kindly provided by Dr. Anne Chambers (London Health Sciences Centre, LHSC). The cells were cultured in DMEM supplemented with 10% fetal bovine serum and 1% antibiotics (100 units/mL of penicillin-G and 100 mg/mL streptomycin) in a 75 cm^2 tissue culture flask (Becton Dickinson Labware, Franklin lakes, NJ). The flask was maintained in a cell culture incubator (Krendro Laboratory Products, Asheville NC) with 5% CO_2 at 37 °C. The medium was replaced every two days. When cells grew to 90% confluence, they were detached by trypsin EDTA and sub-cultured.

4.2.3 Animals

Four to six week-old C57 BL mice (Charles River Laboratories, Wilmington, MA) weighing approximately 20 grams, were housed and cared for in accordance with standards of the Canadian Council on Animal Care; and the experimental procedures used in the present study (protocol# 2003-101-11) were approved by The University of Western Ontario Council on Animal Care.

4.2.4 Liver metastases assay

Male C57BL mice (syngeneic to B16F10 cells) were injected with B16 cells (a suspension of 1×10^5 cells in 100 μL PBS) through the portal vein while anesthetized (1.6

mg of ketamine and 0.08 mg of xylazine per 15 g of body weight), and they were sacrificed 2 weeks later to examine the liver metastasis and to evaluate immune function. To determine the treatment effect of crude PSP water extract and SMF, the mice were pretreated with crude PSP aqueous extract (500 mg/kg) and SMF (25 mg/kg and 250 mg/kg) by gastric gavage for two days (n=10 for each group) prior to the treatment with B16F10 cells. The same daily treatment was given one day thereafter and continued for 12 more days. The control group (n=10) was treated with saline instead. The animals were sacrificed 24 hours after the last dose. The blood was collected for the measurement of IFN-β;, the abdominal macrophages were collected. The spleen was removed for the further isolation of lymphocytes, and the liver was removed for tumor evaluation.

4.2.5 Evaluation of tumor burden on the mouse liver and density of micro foci in the liver

The extent of liver metastases was estimated by measuring the surface area and fractional area of tumor in liver histology sections as well as the density of micro foci in the histology section. The mouse liver was excised and fixed in 10% neutral buffered formalin (pH 7.6) for 48 hours. The liver was separated to 5 lobules; the areas of surface tumors in each lobule were measured with a calliper. The liver lobules were then cut into 6 sections, which were processed for histological evaluation with Haematoxylin and Eosin (H&E) staining. The stained histological sections were photographed with a Motican 2000 digital camera (Independent Products Co., UK). The total liver areas and tumor areas in the sections were measured with Image J program. The micro foci were counted under microscopy.

4.2.6 Isolation and culturing of mouse abdominal macrophages

The macrophages were isolated from the peritoneal cavity of tumor-bearing mice (control or treated) before the removal of the liver. Five milliliters of sterile Hank's balanced salt solution were injected into the peritoneal cavity of the mice and the peritoneal fluid containing the macrophages was withdrawn; the procedure was repeated once. The collected fluid was added to sterile Petri dishes and incubated at 37 °C for 2 hours to allow the adherence of the macrophages. The non-adherent cells were aspirated out gently and the adhered cells were washed repeatedly with PBS. The viability was assessed to be >95% by a Trypan Blue dye exclusion test. The viable macrophages were processed for further experiments.

4.2.7 Production of NO by cultured peritoneal macrophages

Mouse abdominal macrophages were seeded into the 96-well tissue culture plate at a density of 10^5 cells per well. Cells were treated with LPS (1 μg/mL) to upregulate iNOS and NO production. Synthesis of NO was determined by assay of culture supernatants for NO_2^-, a stable reaction product of NO with molecular oxygen. NO_2^- production was measured after 24 hours (Stuehr and Marletta, 1987). Briefly, 50 μL of culture supernatant was incubated with an equal volume of Griess reagent (Sigma-Aldrich, Inc) in 96-well tissue culture plate for 10 minutes at room temperature. The absorbance at 550 nm was measured in a spectrophotometer (Multiscan, USA) along with $NaNO_2$ standards.

4.2.8 Isolation of mouse splenic lymphocytes

Spleens were removed aseptically and put in a Petri dish with RPMI 1640 medium containing 10% FBS. The spleen was chopped and suspended in the medium. The suspension was passed through sterile 200-mesh stainless steel screen, and then the filtered medium was centrifuged with 350 g for 5 minutes to remove the supernatant. The pellet was re-suspended in 5 mL of erythrocyte lysis buffer (20.728 g NH_4Cl, 1.978 g NH_4HCO_3 and 0.0925 g EDTA in 2.5 liters distilled water) for 1 minute, then 5 mL of medium was added to stop the reaction, and the resulting mixture was centrifuged to collect the cells. Viable splenic lymphocytes were counted with a hemocytometer by a Trypan blue exclusion test and used for further analysis

4.2.9 Con-A-induced proliferation of splenic lymphocytes

The proliferation of spleen lymphocytes was based on the increase in the number of viable cells in 24 hours; and the cell density was roughly determined by the MTT assay (Wang *et al.*, 2006). Briefly, freshly isolated mouse splenic lymphocytes were plated into 96-well cell culture plates in triplicate at a concentration of 1×10^5 cells per 100 μL; and Con-A was added to the wells (final concentration 5 μg/mL) and incubated at 37 °C for 20 hours. MTT was added into each well to give a final concentration of 0.5 mg/mL and incubated for 4 hours for the formation of formazan crystals. The medium was removed and dried in an incubator for 30 minutes, and stop solution (100 μL) was added to each well and incubated at 37 °C for 1 hour. The absorbance of the resulting solution was read at 570 nm with a spectrophotometer.

4.2.10 Measurement of serum IFN-β

The levels of IFN-β in the serum were quantified by ELISA, as described previously (Odaka *et al.*, 2001). Flat-bottomed 96-well microlitre plates were coated with 50 μL of the serum samples obtained from treated control and tumor-bearing mice or purified standard IFN-β and incubated for 1 hour. Plates were washed four times with diluted washing buffer, and then a labeled secondary antibody was added to each well. Plates were incubated for 2 hours at room temperature and then washed with washing buffer. One hundred microlitres of goat anti-rabbit conjugate was added to each well and incubated for 1 hour at room temperature. Plates were washed and 100 μL of substrate solution was added into each well. Then stop solution was added to stop the reaction and the optical density of the resulting solution was determined on a microplate reader at 405 nm. The results are expressed as picograms of IFN-β per milliliter.

4.2.11 Data analysis

Data are expressed as Means ± the standard error of the mean (S.E.M). For comparing multiple means, the overall difference among groups was determined by one-way ANOVA followed by Tukey's test to determine the statistical significance between individual groups. A p value <0.05 was considered to be statistically significant. All statistical analysis was calculated with the Graph-pad Prism computer program (Graphpad Software, version 4.1, San Diego, CA).

4.3 Results

4.3.1 *In vivo* anti-metastasis effect of crude PSP and SMF

In the control group, tumors were successfully induced in the liver 12 days following the injection of melanoma cells via the portal vein (shown in figure 4.1A). Surface melanoma could be seen in the photograph as irregularly shaped darkened regions (due to the presence of pigment). The mean surface tumor area measured with a caliper was 4.12 ± 0.91 mm^2 per liver of the melanoma control group, which was in accordance with the report of Luzzi *et al.* (1998). Crude PSP treatment significantly reduced the liver surface tumor area (0.14 ±0.04 mm^2). Treatment with the high dose of SMF, which was equivalent to its concentration in the crude PSP, completely obliterated the appearance of surface tumors; however, the low dose failed to produce a statistically significant reduction from control levels (Figure 4.1B).

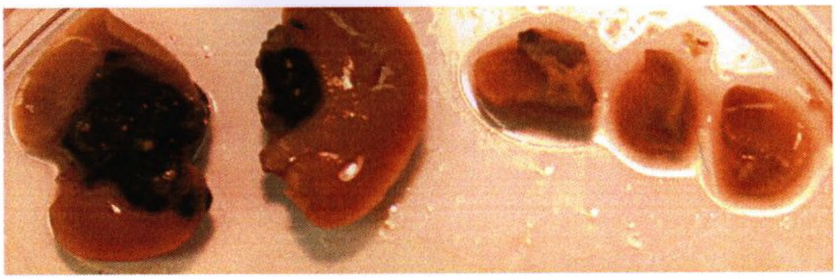
Figure 4.2A shows the representative histology of a liver section from the melanoma-treated group. The quantitative analysis results of of the histological findings (Figure 4.2B) show that the tumor burden in the liver of control group was 1.372±0.03 per unit histology area, and this parameter was significantly reduced in all treatment groups ($p<0.01$).

Histological evaluation of liver sections also shows the presence of micro foci, which were defined as small metastases with 4-16 cells (Figure 4.3A). Quantitative analysis data show that the density of micro foci in the liver of melanoma cell-treated animals was $1.13\pm0.05\times10^{-6}$ per unit area (Figure 4.3B). This parameter was significantly reduced in the high and low SMF-treatment groups ($p<0.01$), but the crude PSP extract had no significant effects.

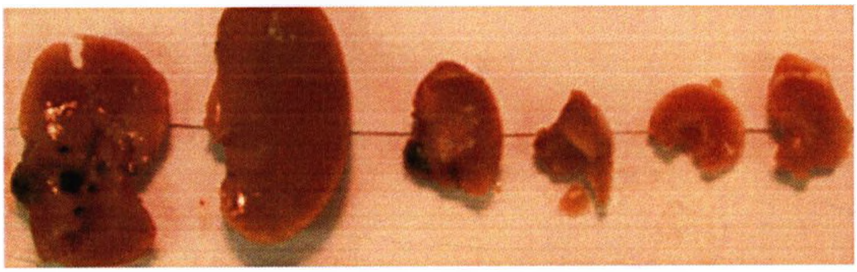
To determine whether crude PSP and SMF produce any undesirable effects on

Figure 4.1 Mouse liver surface tumor areas. A: representative control and SMF treated liver surface tumor; B: quantitative data of all treatment groups. Each group contained 10 mice (n=10). Data are expressed as mean ± S.E.M and analyzed using one-way ANOVA followed by Tukey's test. M represents melanoma. The data show that crude PSP significantly inhibited the surface tumor area (*p<0.05), the same results was observed in SMF 250 mg/kg group. SMF 25 mg/kg group did not show a significant difference with control group.

A



Melanoma control



Melanoma and SMF treated

B

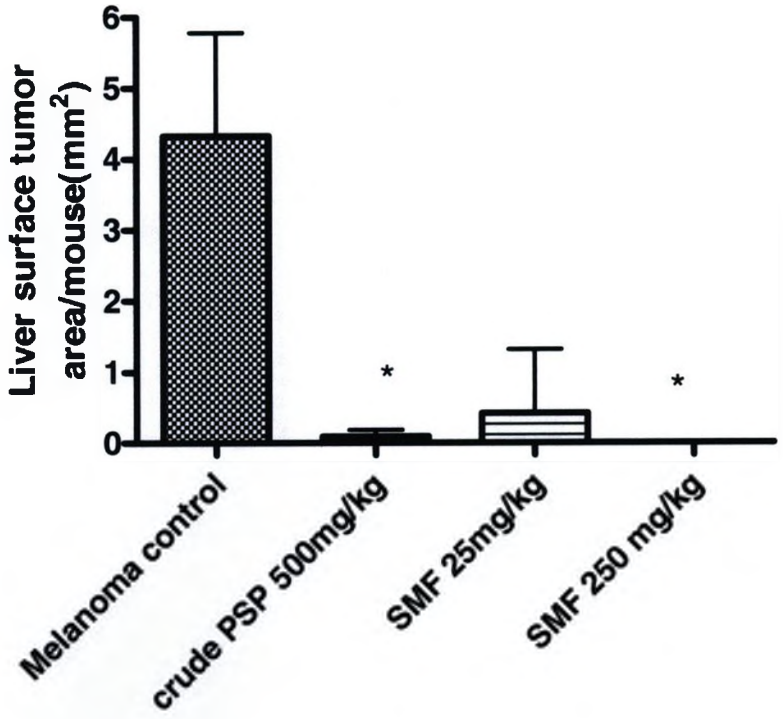
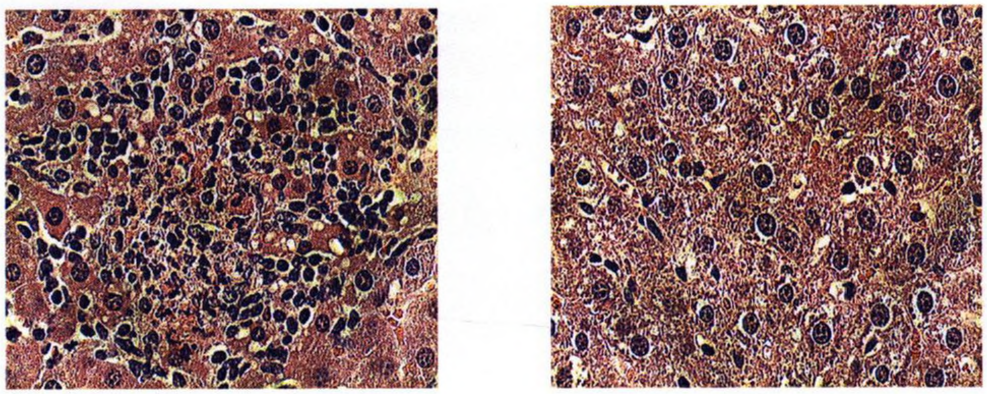


Figure 4.2 Histological evaluation of mouse liver tumor burden. A: representative photomicrographs of H&E stained liver histological sections showing tumor areas from control and SMF treated group (400X); B: Relative tumor burdens of various treatment groups. M represents melanoma. Each group contained 10 mice (n=10). The data are expressed as Mean ± S.E.M and were analyzed using one-way ANOVA followed by Tukey's test. Crude PSP significantly inhibited the tumor burden (* $p<0.05$).The tumor burdens of both high dose and low dose of SMF treated groups were also significantly lower than control (** $p<0.01$).

A



Melanoma control (400X)

Melanoma and SMF treated (400X)

B

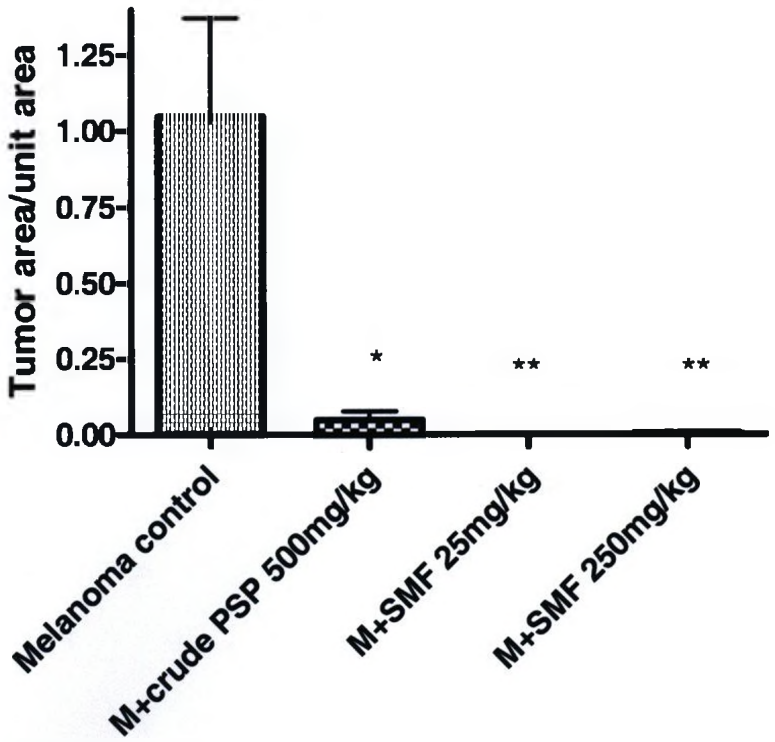
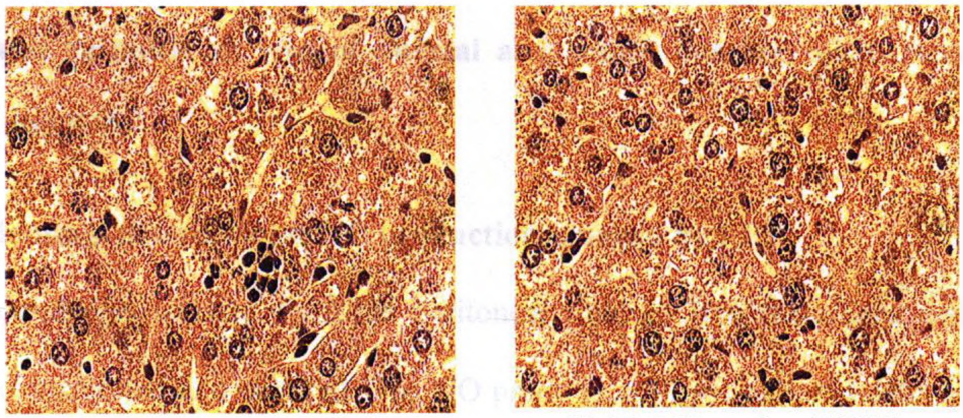


Figure 4.3 Effects of PSP and SMF treatments on micro foci of melanoma cells in mouse liver. A: Representative control and SMF treated histology liver micro foci (400X); B: Quantifies data of micro foci of various treatment groups. M represents melanoma. Each group contained 10 mice (n=10). Data are expressed as mean ± S.E.M and were analyzed using one-way ANOVA followed by Tukey's test. Crude PSP did not significantly reduce the density of micro foci. Both low and high concentrations of SMF significantly inhibited the density of micro foci (*p<0.05).

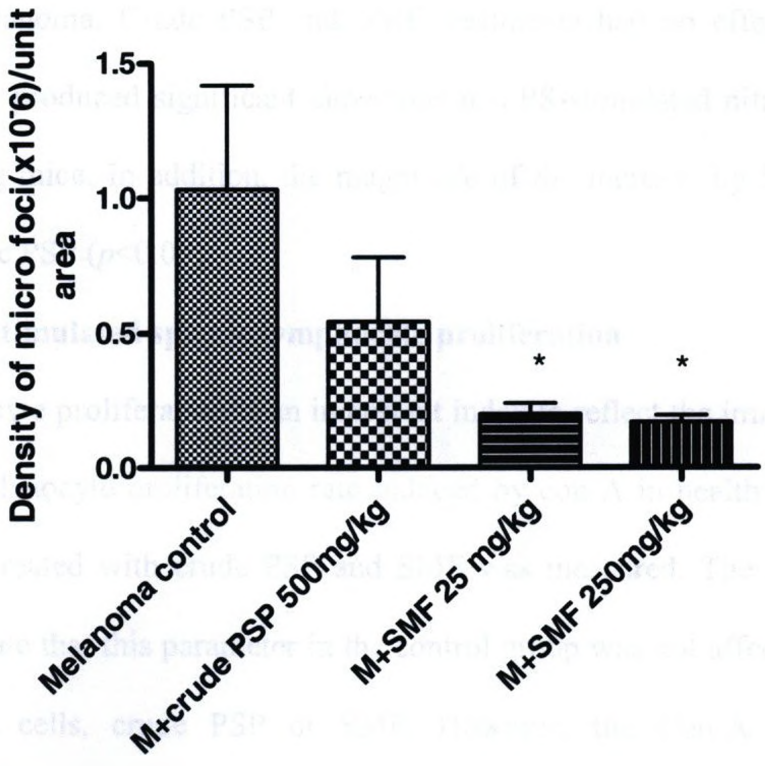
A



Melanoma control (400X)

SMF 250 mg/kg (400X)

B



animals, body weights and major organ weights were recorded. As shown in Figure 4.4 and Table 4.1, crude PSP and SMF treatments did not have any significant effect on these parameters.

4.3.2 Immune response *ex vivo* of normal and tumor bearing mice treated with crude PSP and SMF

4.3.2.1 Abdominal macrophages NO production induced by LPS

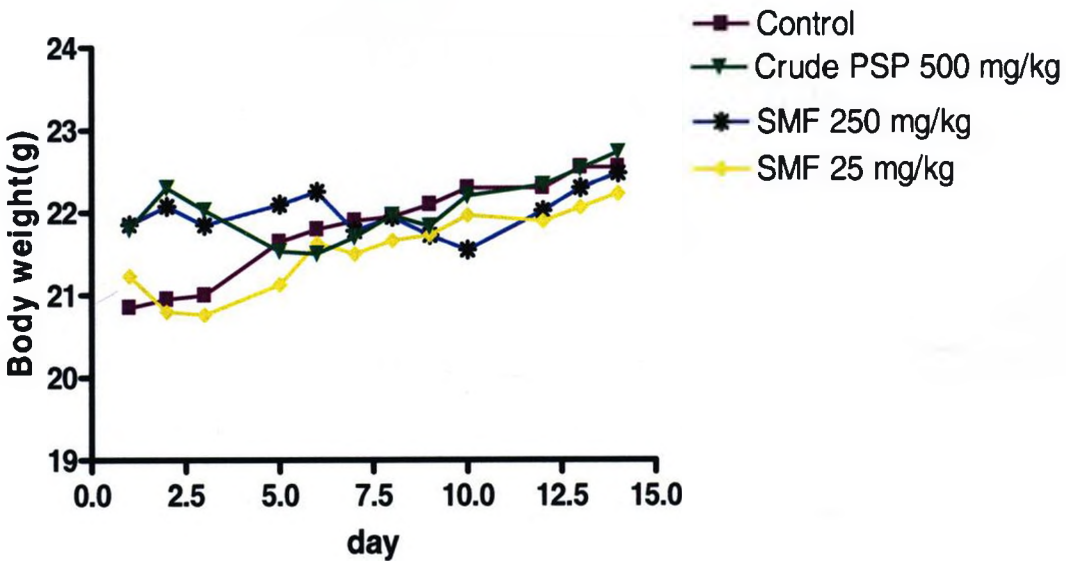
Macrophages isolated from the peritoneal fluid of B16 melanoma cell-treated animals were harvested and cultured. The NO production of the cultured cells following LPS-stimulation was evaluated to determine whether crude PSP and SMF enhanced their responsiveness to stimulation. Figure 4.5 showed LPS-stimulated nitrite accumulation in peritoneal macrophages isolated from control animals and this was not affected by the presence of melanoma. Crude PSP and SMF treatments had no effect in the control groups, but they produced significant elevations in LPS-stimulated nitrite accumulation in tumor-bearing mice. In addition, the magnitude of the increase by SMF was greater than that of crude PSP ($p<0.05$).

4. 3.2.2 Con-A stimulated splenic lymphocyte proliferation

Lymphocyte proliferation is an important index to reflect the immune response of the host. The splenocyte proliferation rate induced by con-A in healthy and B16-tumor bearing mouse treated with crude PSP and SMF was measured. The results shown in Figure 4.6 indicate that this parameter in the control group was not affected by treatment with melanoma cells, crude PSP or SMF. However, the Con-A induced splenic lymphocyte proliferation rate in crude PSP and SMF treated tumor-bearing mouse was

Figure 4.4 Effect of PSP and SMF treatment on body weight of normal and tumor-bearing C57BL mouse. A: Control group. Mice were administered with crude PSP (500 mg/kg), SMF (250 mg/kg and 25 mg/kg) or saline (control) daily for 14 days by gastric gavage (n=10). B: Treated group. Mice were pretreated for two days with PSP, SMF, or saline (control) prior to the injection of B16F10 cells through portal vein under anesthesia; and the same daily treatment was given one day thereafter and continued for 12 more days. The body weight was recorded daily. Data are expressed as mean (n=10) and analyzed using one-way ANOVA followed by Tukey's test. There were no significant difference among all experimental groups ($p>0.05$).

A


Control
Crude PSP 500 mg/kg
SMF 250 mg/kg
SMF 25 mg/kg
Body weight(g)
24
23
22
21
20
19
0.0
2.5
5.0
7.5
10.0
12.5
15.0
day

B

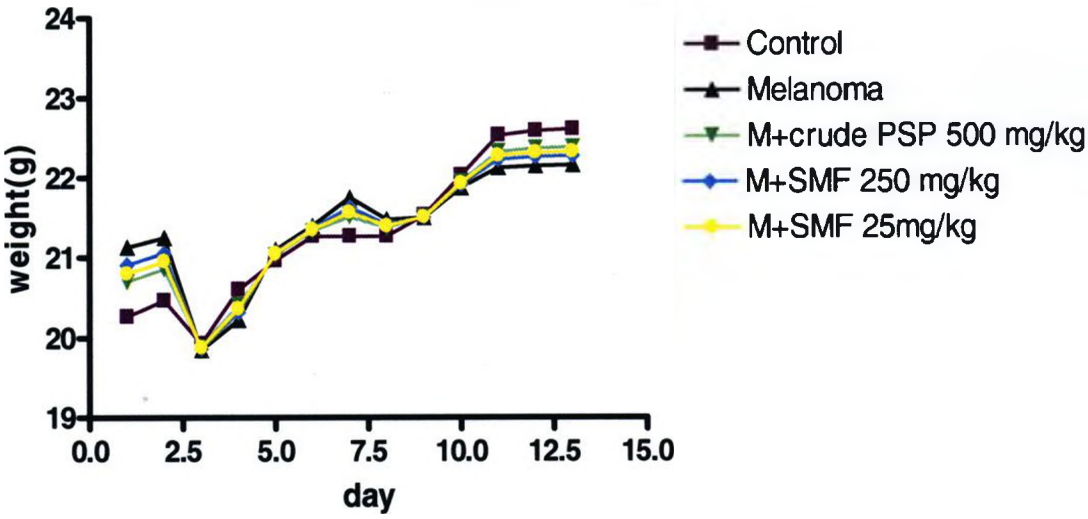

Control
Melanoma
M+crude PSP 500 mg/kg
M+SMF 250 mg/kg
M+SMF 25mg/kg
weight(g)
24
23
22
21
20
19
0.0
2.5
5.0
7.5
10.0
12.5
15.0
day

Table 4.1 Effects of PSP and SMF treatment on major organ weight of mice.

Group	N	Liver (g)	Kidney (g)	Spleen (g)	Lung (g)
Control	10	1.29±0.20	0.32±0.02	0.09±0.02	0.17±0.03
M control	10	1.30± 0.19	0.33± 0.03	0.10 ± 0.04	0.17± 0.06
M+Crude PSP	10	1.31± 0.20	0.33± 0.02	0.09± 0.01	0.17± 0.03
M+SMF 0.5	10	1.26± 0.19	0.30± 0.02	0.09± 0.01	0.17±0.05
M+SMF 5.0	10	1.35± 0.27	0.31± 0.02	0.10± 0.02	0.19±0.04

Mice were treated as previously described in material and method 4.2.4. Data are expressed as Mean ± S.E.M (n=10) and analyzed using one-way ANOVA followed by Tukey's test. There were no significant differences in control and treatment groups on organ weights (p>0.05).

Figure 4.5 Twenty four hours LPS-induced nitrite accumulation in abdominal macrophages isolated from mice. Mice abdominal macropgages were collected and treated with LPS (1 μg/mL) as described in material and method 4.2.6 and 4.2.7. Data are expressed as Mean ± S.E.M and analyzed using one-way ANOVA followed by Tukey's test. Each analysis was performed in duplicate in each experiment and the experiment was repeated six times (n=6). M represents melanoma. Treatment with melanoma cells, crude PSP or SMF did not affect the response in normal mice. This parameter was elevated by crude PSP and SMF treatment in melanoma-treated tumor-bearing animals. * $p<0.05$ compared with control; the level of nitrite in M+SMF was significantly higher than that of M+Crude PSP.

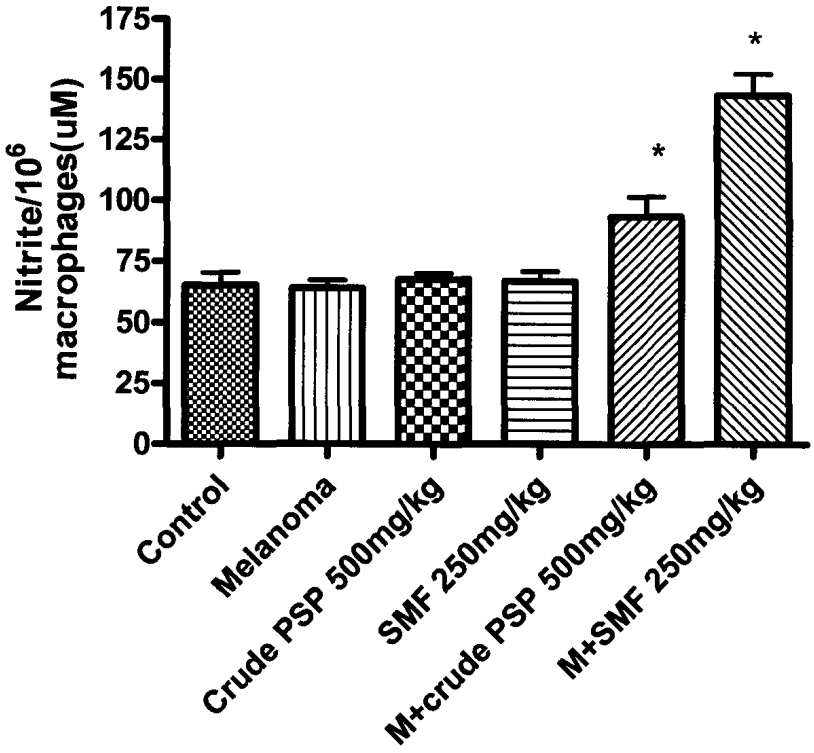
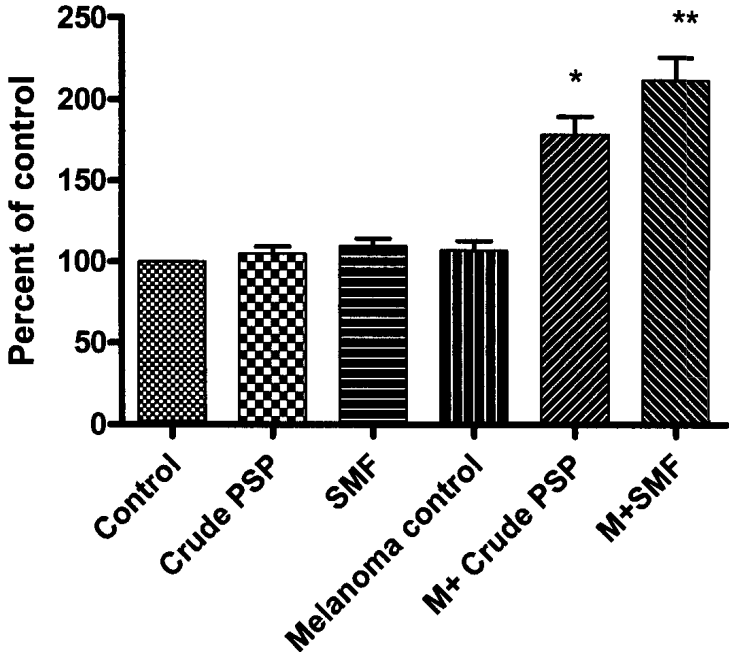

175
150
125
100
75
50
25
0
Nitrite/10^6 macrophages(uM)
*
*
Control
Melanoma
Crude PSP 500mg/kg
SMF 250mg/kg
M+crude PSP 500mg/kg
M+SMF 250mg/kg

Figure 4.6 Effect of PSP and SMF treatment on Con-A induced-splenic lymphocytes proliferation *ex-vivo*. Mice spleenocytes were collected and treated with Con-A (5 μg/mL) as described in material and method 4.2.8 and 4.2.9. Data are expressed as mean ± S.E.M and were analyzed using one-way ANOVA followed by Tukey's test. Each analysis was performed in triplicate in each experiment and the experiment was repeated six times (n=6). M represents melanoma. The data show that treatment with melanoma cells, crude PSP or SMF did not affect the normal mice lymphocyte proliferation. Both crude PSP and SMF stimulated the proliferation of lymphocytes in tumor-bearing mouse. * $p<0.05$ compared with control; ** $p<0.01$ compared with control.


Percent of control
250
200
150
100
50
0
*
**
Control
Crude PSP
SMF
Melanoma control
M+ Crude PSP
M+SMF

significantly elevated.

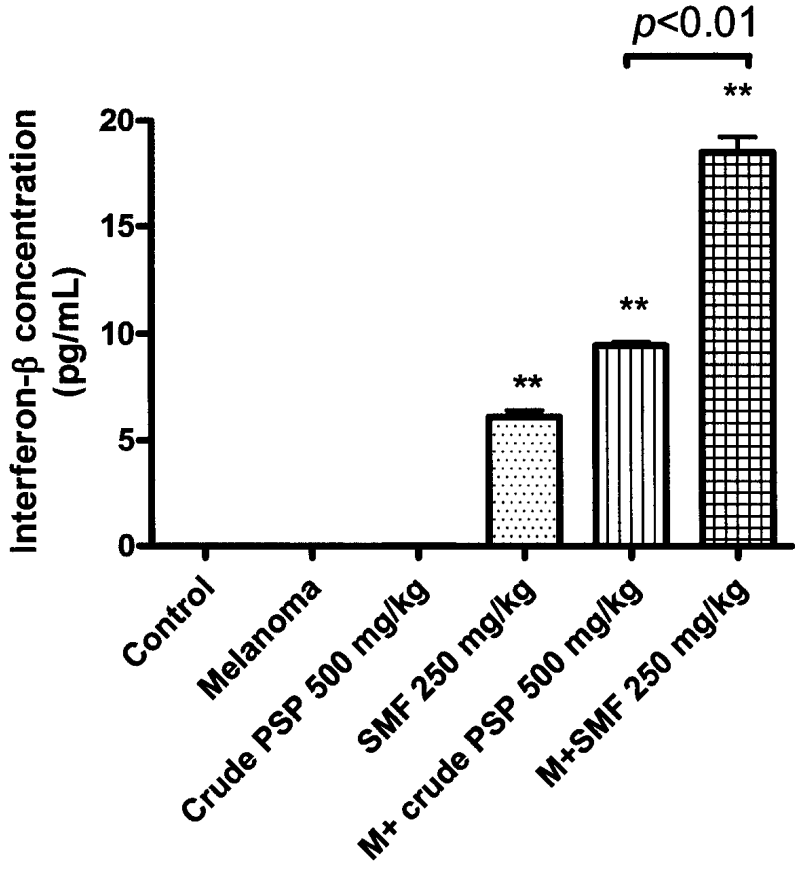
4.3.2.3 Serum IFN-β level

Data presented in Figure 4.7 showed the serum IFN-β concentrations of mice from different treatment groups. There were no detectable levels of serum IFN-β in control or tumor-bearing mice. Although PSP failed to modify this parameter in controls, the values were markedly elevated in tumor-bearing animals. In contrast, SMF treatment significantly increased serum IFN-β levels in both control and melanoma cell-treated groups. Data shown in Figure 4.7 also show that the serum level of IFN-β in SMF treated tumor-bearing mice was significantly higher than that obtained with crude PSP.

4.4 Discussion

Commercially available crude polysaccharide-peptide (PSP) extracts isolated from *Coriolus versicolor* have been on the market since 1987 and have become one of the most popular medicinal mushrooms among consumers. It has been used clinically as an adjunct for cancer patients undergoing chemo-or radio-therapy (Kidd, 2000; Ho *et al.*, 2004; Yang *et al.*, 1999). Our present study has demonstrated the ability of crude PSP to inhibit liver metastases in a mouse liver metastasis model. This could be added to the list of pharmacological activities that is known to be associated with PSP, such as anti-apoptosis, anti-proliferation, anti-angiogenesis, analgesic and antiviral effects (Chan *et al.*, 2006; Ho *et al.*, 2006; Hsieh *et al.*, 2006; Zeng *et al.*, 2005; Kidd, 2000; Chu *et al.*, 2002; Ho *et al.*, 2004; Lui, 2006). The upregulation of cytokine production *in vivo* observed in the present study extends our understanding of the reported immunomodulatory activity of PSP (Chan *et al.*, 2006; Ooi *et al.*, 2000) to include IFN-β as a new pharmacological

Figure 4.7 Serum IFN-β levels of crude PSP and SMF treated healthy and tumor-bearing mice. Mice serum was collected and mouse serum IFN-β concentration was measured by ELISA as described in material and method 4.2.9. Assay range is from 1.0-1000 pg/mL. Data are expressed as mean ± S.E.M and were analyzed using one-way ANOVA followed by Tukey's test. Each analysis was performed in duplicates in each experiment and the experiment was repeated four times (n=4). M represents melanoma. ** $p<0.01$ compared with control. The IFN- β level of M+SMF group was significantly higher than that of M+crude PSP group ($p<0.01$).


p<0.01
**
**
**
Interferon-β concentration (pg/mL)
20
15
10
5
0
Control
Melanoma
Crude PSP 500 mg/kg
SMF 250 mg/kg
M+ crude PSP 500 mg/kg
M+SMF 250 mg/kg

target for PSP. Our research has thus provided additional insight into the mechanism(s) of PSP that supports its current application as an adjunct therapeutic agent for solid tumors. Our study also revealed for the first time the isolation of a SMF from crude PSP extract, with molecular mass much smaller than that of the purported pharmacologically active PSP molecule and potent immunostimulatory activity *in vivo*, which is consistent with the heightened immunostimulatory potency of this fraction *in vitro* reported by us (Section 3). Moreover, our present study has shown that it was more effective than crude PSP extract at inhibiting tumor metastases in the mouse liver metastases model.

Different experimental models have been used to study the anticancer effect of PSP and related products (Ooi *et al.*, 2000; Yang *et al.*, 1999; Ye, 2004). Our previous study showed that crude PSP inhibited the matrigel-based tubulization of vascular endothelial cells *in vitro* and suppressed angiogenesis in the Chick Chorioallantoic Membrane (CAM) model (Ye, 2004). In addition, the anti-angiogenic effect of crude PSP was confirmed with the mouse liver metastases model (Lui, 2006). In this model, the injection of 2×10^5 melanoma cells into the portal vein induced about a 40% increase in tumor burden by histological evaluation and a significant presence of microvasculature in the tumor tissues. It is known that the growth of larger metastases depends on angiogenesis as well as immune regulation (Luzzi *et al.*, 1998; Folkman, 1992; Searle and Young, 1996). This model has enabled estimation of the inhibitory effect of crude PSP on angiogenesis and metastasis (Lui, 2006). The primary objective of the current study was to examine the anti-metastatic effect of SMF, which has been shown to possess a marked immunostimulatory effect *in vitro*, but to be devoid of anti-angiogenic property *in vitro* (Section 3). Accordingly, this liver metastasis model protocol was modified by

reducing the melanoma cells injection by 50%, which gave rise to lower than 1% incidence of solid tumor burden in the liver and the occurrence of melanoma micro foci (≤1-2mm) within the liver. Since suppression of micro-foci development and the initial progression into solid tumors does not involve angiogenesis, the observed anti-metastatic effect of SMF in the current model suggests that its anti-metastatic effect could perhaps be mediated, at least in part, by a mechanism that is independent of the suppression of angiogenesis.

Several other studies have reported the anticancer effect of PSP with different animal models. Zhang *et al* injected B16 cells (5×10^5) into adult mice through the tail vein to induce significant lung metastases in 14 days, which could be reduced by 72.5% with daily administration of 400 mg/kg of crude PSP extract (Zhang *et al.*, 2004). Ho and his colleagues (2004) have reported the induction of tumor growth in mice at the site of subcutaneous implantation with murine sarcoma S180 cells (1×10^6 cells) in the back and a 48% reduction in tumor growth following the feeding with a PSP solution in drinking water (1.75 mg/kg) for 29 days. In a study by Wang *et al.* (1993), PSP administered intraperitoneally at 50 mg/kg per day for 3 weeks was found to produce 45% inhibition of the growth of Lewis lung cancer. These published data as well as those from the present study indicate that PSP possesses both anticancer and anti-metastatic effects and is effective in different tissues. However, the relative potency of PSP is difficult to ascertain because of differences in the type of PSP used, routes and duration of administration as well as the use of different experimental models. The effective PSP dosage used in the present study (500 mg/kg) was five time higher than those recommended by the manufacturer (100 mg/kg). Small rodents have higher metabolic

rate and larger body surface area than human and they are known to tolerate drugs better. On this basis, it has been recommended that a factor of 5 be used in across species extrapolation of dosages per unit body weight for chemo-tharapeutic agents (www. fda.gov/cder/cancer/animalframe.html). Therefore, the effective PSP dosage used in the present study is considered equivalent to the recommended human dose after correction with this extrapolation factor.

The immunostimulatory effect *in vitro* of PSP has been reported in many studies, but limited data is available *in vivo* (Chu *et al.*, 2002; Qian *et al.*, 1997; Yang *et al.*, 1993; Chu *et al.*, 2002; Yang *et al.*, 1999; Zhang *et al.*, 2004). Induction of cytokines and activation of immune cells are both mechanisms of the immunostimulatory action of PSP. Cytokines play an important role in PSP action. The observed upregulation of serum IFN-β level (Figure 4.7) complements the reported elevation of cytokines induced by PSP, such as IFN-α and γ , IL-2, TNF-α, and IL-1β (Mao *et al*, 1996; Yang *et al.*, 1999b; Liu *et al.*, 1993; Chan and Yeung, 2006b). The observed selective immunostimulatory effect of crude PSP seen in tumor-bearing mice rather than in normal mice is consistent with the recognition of PSP as a BRM, which is characterized by its action in stressed or immune-suppressed hosts only (Li, 1999; Liu, 1999; Gu, 1999; Yang, 1998; Chu *et al.*, 2002). The immunosuppression caused by tumor cells might increase the responsiveness of immune cells to PSP activation for IFN-β production. It is also possible that PSP induces melanoma cells to produce IFN-β (Satomi *et al.*, 2002), but further investigation is required to validate this. Unlike crude PSP, SMF induced IFN-β production in both normal and tumor-bearing mice, suggesting that the action of SMF was not confined to an immunosuppressed host. This observation is consistent with the result of our *in vitro*

study that SMF, but not crude PSP, induced RAW macrophage IFN-β production (Section 3). While identification of the source of PSP-induced elevation of serum IFN-β requires additional studies, our present study did reveal that some of the immune cells in tumor-bearing mice were targets of PSP and SMF.

Data presented in Figure 4.6 and 4.7 showed that such treatments enhanced the responsiveness of i) NO production in peritoneal macrophages in response to LPS stimulation and ii) splenic lymphocytes to undergo proliferation in response to Con-A. Furthermore, several immune cell types, such as Kupffer cells, lymphokine-activated-killer (LAK) cells, nature killer (NK) cells, tumor infiltrating lymphocytes (TIL) and cytotoxicity T lymphocytes (CTL) have been reported to be activated by crude PSP in several studies (Jong and Yang, 1999; Li, 1999; Yang *et al.*, 1993b).

Medicinal mushroom polysaccharides including PSP are known to produce a range of pharmacological activities; however, conclusive evidence linking specific mechanism(s) to their anticancer effects is lacking (Wasser, 2002). It is likely that multiple mechanisms may be involved and that certain pathways are more relevant to specific types of cancer. Although immunostimulation has long been thought to be involved in the anti-cancer effect of PSP, there have been limited direct experimental data to support this speculation (Yang *et al.*, 1999; Ooi *et al.*, 2000; Ho *et al.*, 2004). Data presented in the present study concerning SMF, a constituent of crude PSP, suggested that its anti-metastatic effect may be mediated primarily by its immunostimulatory effect. The significant upregulation of serum IFN-β by SMF is expected to have dual roles. Firstly, IFNs and in particular IFN-β are known to have an important anticancer role, especially for melanoma (Ernest, 2005; Chawla-Sarkar, 2001). It can activate immune

cells to be cytotoxic as well as to directly inhibit angiogenesis. In addition, the potentiation effect involving SMF and IFN-β on immunostimulation, anti-angiogenesis and apoptosis as reported in our *in vitro* studies (Section 3) may also contribute to the anti-metastasis effect. Secondly, Kupffer cells (differentiated macrophages) in the liver are known to play a crucial role in arresting and killing blood-disseminated tumor cells, thereby greatly contributing to prevention of metastases formation (van der Bij *et al.*, 2005). Moreover, it has been reported that stimulation of Kupffer cells with stimuli such as IFN-γ and GM-CSF resulted in powerful antitumor effects in animal models (Klimp *et al.*, 2002; Schuurman *et al.*, 1997; van der Bij *et al.*, 2005). Although the influence of SMF and the associated elevation in serum IFN-β level on Kupffer cell function has not been examined in the present study, the observed up-regulation of peritoneal macrophage function by SMF *in vivo* and the immunostimulatory function *in vitro* reported earlier (Section 3) would strongly support this possibilities and could explain the observed effectiveness of SMF to reduce the melanoma micro foci in the liver of treated animals.

Herbal medicines are known to contain multiple unidentified compounds. There are challenges to identify those bioactivities since synergistic and antagonistic interactions exist among different constituents. As reported previously (Section 3), the SMF isolated from the crude PSP extract was water-soluble and consisted of at least 4 components as revealed by Sephadex G-25 chromatography. It was heat stable with respect to its pharmacological activities *in vitro*. Since the polysaccharide-peptide fraction is generally believed to be the primary pharmacological active component, there has been no previous report on other constituents (Yang, 1999a; Cui and Chisti, 2003). Yang *et al.* (1993) reported the recovery of a small peptide from a *Coriolus versicolor*

extract showing an immuno-potentiating effect in increasing WBC and IgG levels as well as and an antitumor effect in nude mice. It was believed to be a small peptide released from PSP upon heat treatment. It appears to be different from the SMF based on the heat stability characteristic.

In conclusion, our data shows that SMF isolated from crude PSP extract by gel filtration possesses a marked immunostimulatory effect *in vitro* but is devoid of antiangiogenic activity. It was shown to be effective in the mouse liver metastasis model in reducing melanoma cell-induced tumor burden and micro foci in the liver. In addition, SMF was more effective as an anti-metastatic agent than crude PSP extract, which was in accordance with the observations *in vitro* in terms of the immunostimulatory effect. The present study also validated the immunostimulatory effect *in vitro* of SMF in the mouse liver metastasis model and our data suggest that the immunostimulatory effect of SMF may mediate its anti-metastatic effect observed *in vivo*. Additional studies are in progress to elucidate the structural characteristics of SMF and identify the bioactive component(s) that mediate the immunostimulatory effect and the underlying mechanisms.

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Chapter 5 General discussion

5.1 Important findings of this research

Crude PSP has been used as an adjunct for cancer therapy for a long time. The purported activities include anti-cancer and immunomodulation (Yang, 1999a). In this study, the crude PSP water extract was fractionated and investigated the activities of crude PSP and its fractions on angiogenesis, apoptosis and immunostimulation. The results indicate that crude PSP and SMF are promising immunostimulatory agents for cancer therapy. This study also confirms that the immunostimulation effect does contribute to the anti-cancer effect. Previously, SMF was considered as a substance without bioactivity activity (Yang, 1999a; Cui and Christ, 2003). This is the first report that demonstrates immunostimulatory effects of SMF. Immunomodulation has become one of the most exciting approaches in the development of anti-cancer drugs without the disadvantages of traditional anticancer drugs (Melero *et al.*, 2007). Based on the findings of this research, it is hoped that this immunostimulatory agent, developed from crude PSP, will benefit patients with immune function inhibition and also provide a possible application of SMF on preventing immune deficiency disease such as AIDS (acquired immune deficiency syndrome) (See http://www.patentstorm.us/patents/6087335-description.html).

5.2 The bioactive components of crude PSP: SMF and HMF

Fractionation of crude PSP with Sephadex G-75 chromatography resulted in two major fractions (HMF and SMF). Traditionally, the high molecular weight fraction (HMF) was considered to be the primary pharmacologically active component(s) of crude PSP (Chu *et al.*, 2002; Jong and Yang, 1999; Yang, 1999a). On the other hand, the small molecular weight fraction (SMF) of crude PSP has been considered to be

pharmacologically inactive and thus was usually discarded during the HMF purification procedure. Unlike previous research, this study explored the potential anti-cancer and immunostimulatory effects of SMF isolated from crude PSP.

This is the first study to demonstrate that the small molecular weight fraction (SMF) from the aqueous extract of commercial PSP (crude PSP) contains bioactive components with promising pharmacological activity. The results shown in Figure 3.9 and 3.10 indicate that SMF is more potent than crude PSP on upregulation of NO and PGE_2. Moreover, SMF, but not crude PSP, could up-regulate the IFN-β production in macrophages.

Since IFN-β plays a crucial role in anti-cancer treatment and is used clinically in combination with the other therapeutic agents, it is noteworthy that SMF up-regulated the endogenous IFN-β. For future clinical anti-cancer studies, it will be interesting to observe the combined potentiated effects of SMF and IFN-β on immunostimulation, anti-angiogenesis and apoptosis.

In the current study, HMF was associated with anti-angiogenic, apoptotic, and immunostimulatory effects, which is consistent with the literature (Yang *et al.*, 1999b; Ho *et al.*, 2004; Lui, 2006; Ooi *et al.*, 2000). HMF eluted in the void volume of the column in accordance with the reported active components of crude PSP (Yang *et al.*, 1989; Lui, 2006; Ye, 2004). The pharmacological activity of HMF was compared with crude PSP. Anti-angiogenic and apoptotic effects of HMF were not as effective as crude PSP, but the immunostimulatory effect of HMF was more effective than crude PSP.

In summary, crude PSP contains multiple components and a range of pharmacological activities that may contribute collectively to the treatment of some

diseases. In this study, apoptosis, anti-angiogenesis and immunostimulation may be beneficial to cancer treatment. Our data suggest that crude PSP contains at least two bioactive fractions: HMF and SMF. Their relative potencies are listed in Table 5.1.

5.3 Possible anti-metastasis mechanism of SMF

The anti-metastatic effect of crude PSP was consistent with the report of Zhang and colleagues, who injected B16 cells (5×10^5) into the mice through the tail vein and administered 400 mg/kg PSP per day. After 14 days, a 72.5% inhibition of lung metastasis rate was observed (Zhang *et al.*, 2004). The difference in inhibitory effect may be a result of the PSP dosage, method of administration and organ sensitivity. The in vivo anti-metastic effect of SMF has not been reported elsewhere. In the current study, SMF was found to be more effective than crude PSP on inhibition of small metastases (micro foci). Although SMF also inhibited large metastases (tumor burden), there was no significant difference between crude PSP and SMF. This might be due to the lower incidence rate of tumors (<1%) and high inhibitory rate of crude PSP and SMF (95% and 99%). SMF did not only reduce the large metastases, but also functioned to reduce the micro foci at various dosages.

The inhibition of metastases by SMF might be due to its immunostimulatory activity. Small metastases ($\leqslant$1-2mm, such as micro foci) lack angiogenesis and mainly reflect immune regulation (Wasser, 2002; Luzzi *et al.*, 1998). The observed results of activation of immune cells and up-regulation of cytokines by SMF (Figure 4.5, 4.6, and 4.7) may provide a biological basis for its anti-metastatic effects. These can be summarized as follows (Figure 5.1). Firstly, upon SMF stimulation, immune cells such as

Table 5.1 Summary of the pharmacological activity of crude PSP and its fractions (The ranking based on per unit dry weight).

Activity	Crude PSP	HMF	SMF
Anti-angiogenesis	+++	++	-
Apoptosis	+++	+	++
Immunostimulatory	+	++	+++

macrophages were first activated, and exerted direct cytotoxicity such as macrophage-mediated tumor cytotoxicity (MTC) and antibody-dependent cellular cytotoxicity (ADCC) to kill tumor cells. SMF can therefore potentially facilitate the up-regulation of the innate immune response to foreign pathogens. Secondly, upon oral administration, SMF is delivered to the liver via portal circulation and activates Kupffer cells, differentiated macrophages, in the liver to arrest and kill tumor cells that have been distributed to the liver. This mechanism was proposed by van der Bij *et al.* (2005) to explain the significance of Kupffer cell function in the development of liver metastases. Additional study on the stimulation of Kupffer cells by SMF is required for validation. Thirdly, SMF may also induce immune cells or tumor cells to produce cytokines to further combat tumors. In this regard, it should be noted that SMF up-regulated INF-β production *in vitro* and *in vivo* (Figure 3.14, 4.3). It has been reported that stimulation of Kupffer cells with stimuli such as IFN-γ and GM-CSF resulted in powerful additional anti-tumor effects in animal models (Klimp *et al.*, 2002; Schuurman *et al.*, 1997; van der Bij *et al.*, 2005). IFN has an important anti-cancer role and IFN-β is the most potent IFN, especially for melanoma (Ernest, 2005; Chawla-Sarkar, 2001). It can activate immune cells to become cytotoxic, as well as directly inhibit angiogenesis. Moreover, the potentiated effect of a combination of SMF and IFN-β on immunostimulation, anti-angiogenesis and apoptosis observed *in vitro* (Figure 3.12, 3.13, 3.14) may also contribute to the *in vivo* anti-metastasis effect (Figure 4.1, 4.2, 4.3).

In anticipation of the role of immunostimulatory effect of SMF on anti-metastasis, we have adopted a 3 day pre-treatment with SMF before the initiation of melanoma cell injection in the present study. This would ensure adequate up-regulation of immune

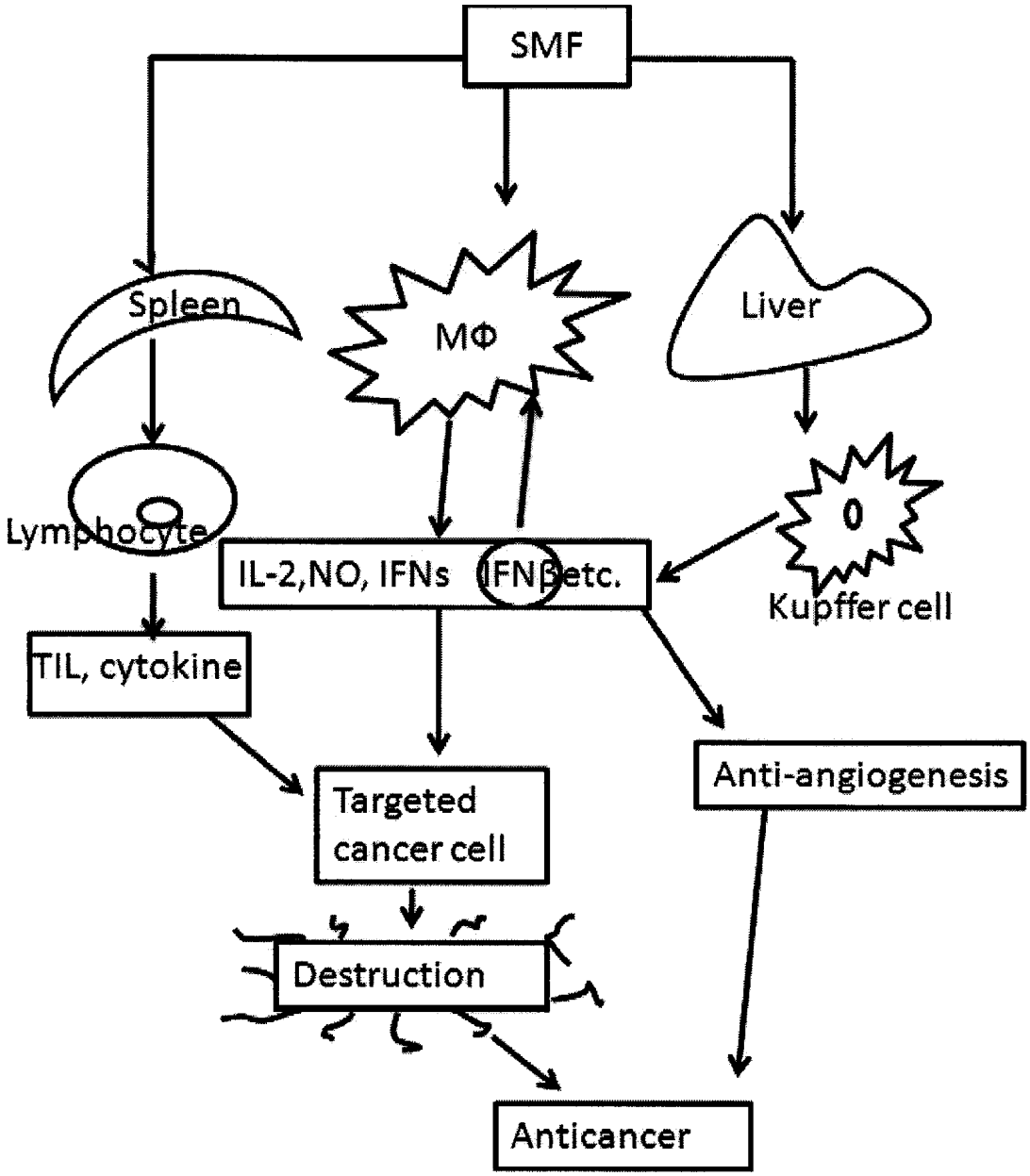

Figure 5.1 The immune-stimulatory mechanisms of SMF on anti-metastasis

function prior to tumor cells treatment. This treatment protocol is consistent with the report by MacDonald *et al.* (2002) which stated that approximately 72 hours is required for cell deposit and micro-metastases formation. It remains to be determined whether the effectiveness of SMF is dependent on a pretreatment protocol.

Overall, SMF was found to be more effective than crude PSP on inhibition of small metastases as well as immunostimulation *in vivo*. The effectiveness of SMF on small metastases may be due to, at least in part, its potent immunostimulatory activity. Since the ideal anti-cancer drug is one that directly inhibits cancer and indirectly stimulates the body's immune system activity while having less toxic side effects on the body, it is apparent that the crude PSP and its fraction SMF possess all of the desired characteristics for an anti-cancer drug. Since SMF is especially potent, it deserves further clinical study.

5.4 Experimental Models and their limitations

Because of the broad pharmacological activity of crude PSP, several experimental models and methodologies were used in this study. In addition to advantages, they also have limitations.

The *in vitro* apoptotic activity of crude PSP had been demonstrated in some tumor cells (Mao *et al.*, 2001; Yang *et al.*, 1999b). Induction of apoptosis is a process that many anticancer drugs possess; and this therapeutic effect has the benefit of not influencing the homeostasis of the whole system (Ryungsa *et al.*, 2002). There are many methods to study apoptosis. The apoptotic program is characterized by membrane blebbing, condensation of cytoplasm and activation of endogenous endonucleoses. This leads to internucleosomal cleavage of DNA and generation of mono- and oligonucleosomes,

which are tightly complexed with histones. Many methods can be used to detect apoptosis, such as cell death ELISA kit to measure the apoptotic DNA fragment, electrocytometry to test the morphology apoptotic body.

In this study, a cell death detection ELISA kit was used to test the melanoma cell apoptosis induced by crude PSP and its fractions. This kit can quantify histone-complexed DNA fragments (mono- and oligonucleosomes, a marker for apoptosis) in the cytoplasm of cells after the induction of apoptosis. Since the assay does not require pre-labeling of cells, it can detect inter-nucleosomal degradation of genomic DNA during apoptosis and differentiate apoptosis from necrosis. The biggest advantage of the kit is that it is designed for a 96-well plate format; hence multiple samples can be run in a single assay. The Cell Death Detection ELISA kit is highly sensitive and detects apoptotic nucleosomes in cell lysates, which requires fewer cells than flow cytometry-based apoptosis assays. This assay is simple to perform and one can obtain results within 5-6 hours. However, the assay background can be significant if re-stimulating quiescent cells is required. Therefore, it is essential to optimize experimental conditions so that the background absorbance is low (Dasgupta, 2005).

The *in vitro* anti-angiogenesis model used in this study is matrigel based endothelial tube formation assay. It is a quick assessment of angiogenesis and the tube formation can be achieved within 18 hours. Endothelial tube formation on Matrigel (ECM gel from engelbreth-holm-Swarm murine sarcoma) is reasonably predictive of the *in vivo* situation. It can be used to test the angiogenesis inhibitor before *in vivo* testing. However, different batches of matrigel from the supplier may vary in activity from time

to time; and this may influence the experimental results. Therefore, several lots from the same batch were purchased at one time (Nehls and drenckhahn, 1995).

There are many *in vivo* metastases models including transplantable cancer models, mice genetically engineered to develop metastatic cancers, and outbred large animals (primarily pet dogs) that develop cancers naturally. No single metastasis model is sufficient to answer all questions. As such, it is crucial to use the optimal model to resolve a specific experimental question. In this study, the aim is to study the anti-metastatic effect of SMF, especially the relationship between immunostimulation and metastases. We modified the syngeneic mouse liver metastases model by reducing the number of cells injected and obtained the small micro foci rather than large metastases. Experimental metastasis models provide several advantages for investigation. The time course for model maturity is generally rapid, the biology of metastasis is reproducible and consistent, and the user has control of the number and type of cells that are introduced to the circulation (Khanna and Hunter, 2005). However, since the cells are from the same species, these model systems lack many of the important features of human tumors. For example, they are usually derived from homozygously inbred mice and therefore lack the genetic complexity of human tumors. In addition, due to species-specific differences in oncogenesis, for example, differences in carcinogenic xenobiotic metabolism, they may not bear the same constellation of mutations observed in human patients (Gonzalez and Kimura, 2001). Thus, care must be taken to validate observations and conclusions drawn from these models and to confirm their relevance to human cancers.

5.5 Variability of different brands of crude PSP and SMF

Many factors are known to influence quality of herbal products. The same herb from different suppliers might be different due to their growing environment and processing procedures. The present study was carried out initially with a product purchased from Hong Kong (manufactured by Xinkang Company of China, Lot Z10980124). It is important to determine whether the findings of the present study on one product could be extrapolated to other commercially available PSP products. Two additional brands were examined to determine the variability (Appendix 2). Results suggested that although the profile of the chromatograms in different brands of PSP product was similar qualitatively, both the yields of the two subfractions and immunostimulatory activity of different brands of crude PSP and SMF were different (Appendix 2). Since we did not examine different batches from each brand, it is not possible to conclude whether the observed differences were due to brand- or batch-related variability in product quality. Nevertheless, our data raised the question of whether data obtained from one brand/batch of PSP could be extrapolated onto other brands/batches.

5.7 Future directions

Firstly, identification and isolation of active components of SMF by G-25 chromatography and characterization of the chemical components should be conducted.

Secondly, IFN-β is widely used clinically and SMF showed a potentiated effect with IFN-β in vitro. It is important to validate the combination effect on in vivo models, as this will serve as a direction for future clinical combination study.

Thirdly, the detailed mechanism of SMF on anti-metastases is still not clearly understood. The immunostimulation effect of SMF might place an important role on anti-metastasis. It is known that Kupffer cells are an effective inhibitor of anti-metastasis

(Klimp *et al.*, 2002; van der Bij *et al.*, 2005). In this study, we only tested the activity of abdominal macrophages, but the differentiated macrophage (Kupffer cell) which directly relates to metastases was not studied. Thus, the function of Kupffer cells deserves further investigation.

Furthermore, Furthermore, SMF was isolated from crude PSP which is known as a safe herbal medicine. This study focused on the pharmacological activity of SMF which has never been reported previously. Therefore, it is suggested that studies concerning the safety of SMF still need to be carried out.

Last of all, further *in vitro* and *in vivo* experiments for different brands of SMF are required to determine their bioequivalence on the same pharmacological activity.

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Zhang WY, Wang Y and Hou YY. Effects of Chinese medicinal fungus water extract on tumor metastasis and some parameters of immune function. *International immunopharmacology.* 4: 461-468, 2004.

Appendix 1: General methodology

1. Nitrite quantification

Due to the short half-life of NO, its direct measurement requires specialized and expensive equipment that uses chemiluminescence detection. Thus, NO was not measured directly in the present study. Since the NO produced in the culture medium is converted primarily to nitrite, measurement of the accumulation of nitrite in the culture medium can be used as an indirect method for quantifying NO production. Nitrite concentrations of standard solutions (0, 5, 10, 20, 40, 60, 80, and 100 μM) and samples were determined by the reaction with the Griess reagent. Fifty μL standards and samples were added into the 96-well plate in triplicate; and 50 μL of Griess reagent (Sigma-Aldrich, Inc.) was added to each well and incubated for 10 minutes at room temperature. Thereafter, the absorbance at 550 nm was measured in a microplate reader. A standard curve relating the concentrations of nitrite to their corresponding absorbance values was obtained and the equation of $Y=0.0037X+0.0047$ ($R^2 = 0.9978$) was used to estimate the nitrite concentrations of the samples.

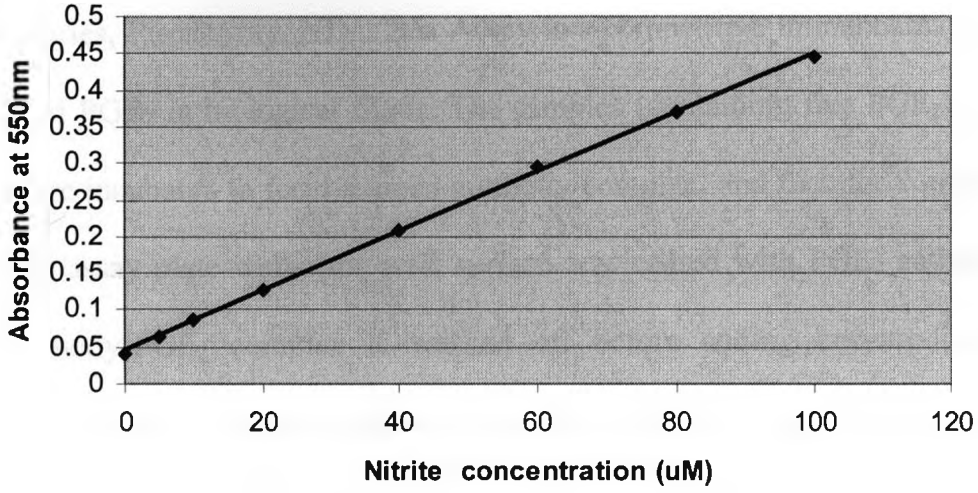


Figure A1.1 Standard curve of Nitrite as determined by the Griess reagent method.

2. Measurement of PGE_2

An enzyme-linked immunosorbent assay (ELISA) was used to estimate concentration (ng/mL to pg/mL) of PGE_2 in culture medium (PGE_2 ELISA kit, PBL biomedical laboratories, Piscataway, NJ). This Assay is a competitive immunoassay for the determination of PGE_2 in biological fluids. The samples (containing free PGE_2) and primary antibody are incubated to form antigen-antibody complex, and then the complex was added into the assay plate wells (the well surface was coated with PGE_2 antigen). The unbound antibody-PGE_2 complex is washed off before adding enzyme-linked secondary antibody, which is directed against the primary antibody. The p-nitrophenyl phosphate (pNpp) substrate is then added. After a short incubation time the enzyme reaction was stopped and the observed yellow color generated was read with a spectrophotometer at 405 nm. The intensity of the yellow color is inversely proportional to the primary antibody, which was directly related to the concentration of PGE_2 in either standards or samples. The PGE_2 concentration of the samples was calculated from the standard curve prepared from PGE_2 standards (39.1, 78.1, 156, 313, 625, 1250, and 2500 pg/mL). The standards were calculated by the following method: B was the net OD value of the standard PGE_2, B0 was the OD value of 0 pg/mL standard: B/B0% was obtained with the following formula: Net OD/Net B0 OD ×100. A standard curve was obtained by plotting percent of bound versus concentrations of the standard PGE_2. The sensitivity of the assay was 13.6 pg/mL and the antibody provided by this kit had cross reactivity with PGE_1 (70%) and PGE_3 (17%).

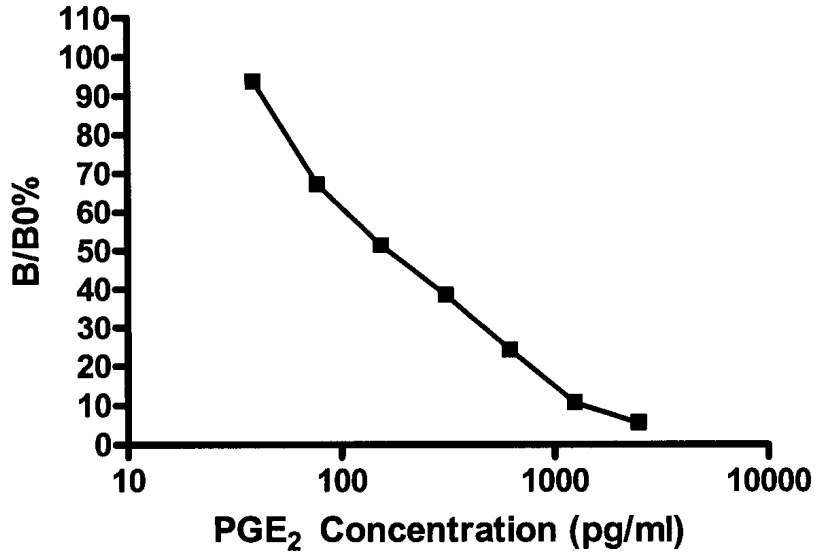


Figure A1.2 Standard curve of PGE_2

3. Quantification of IFN-β with mouse interferon-beta ELISA kit

Concentration of IFN-β in serum or culture medium was measured with a commercial ELISA kit (PBL biomedical laboratories, Piscataway, NJ) that is based on a "sandwich" enzyme immunoassay. This enzyme immunoassay can specifically detect and quantify the concentration of interferon-β in the samples. The basic principle is described as following: the highly-purified anti-interferon-β antibodies are non-covalently bound onto the surface of plastic microwell plates. After plate washing, the immobilized antibodies serve to specifically capture soluble interferon-β present in samples. The captured interferons are detected by biotin-conjugated anti-interferon antibodies and then an enzyme-labeled avidin is added into the biotin-conjugated anti-interferon antibodies and incubated. Following the addition of a chromogenic substrate, the level of colored product generated by the bound, enzyme-linked detection reagents can be measured spectrophotometrically using an ELISA-plate reader at 450 nm. This IFN-β kit does not have cross reactivity with IFN-α and IFN-γ. A standard curve which relates the concentrations of IFN-β (0, 15.6, 31.3, 62.5, 125, 500, and 1000 pg/mL) to their corresponding absorbance was obtained. This relationship could be described by $Y=0.0037X+0.0686$ ($R^2=0.9972$). This curve was used to estimate the IFN-β concentration of the sample.

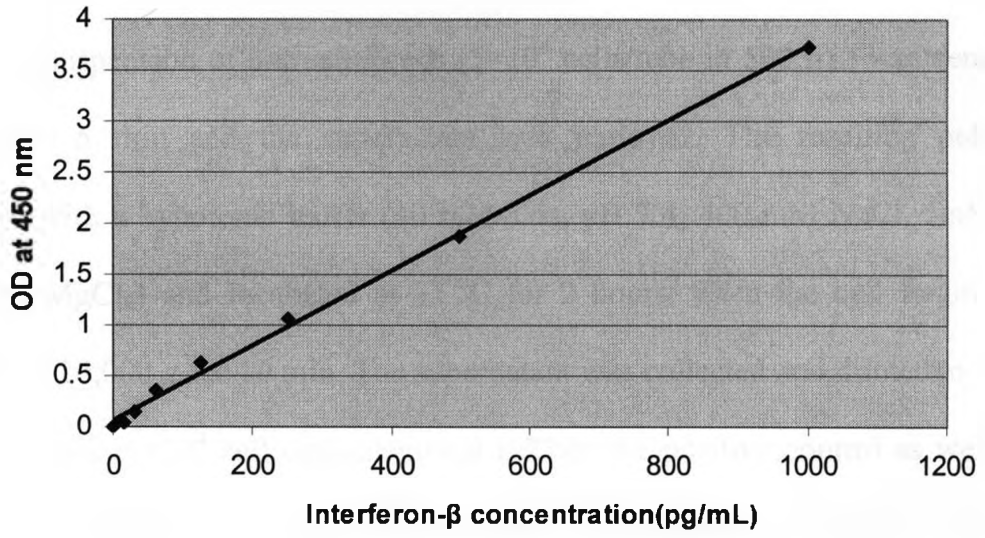


Figure A1.3 Standard curve of IFN-β determined by mouse interferon-β ELISA kit.

5. Apoptosis of melanoma cells

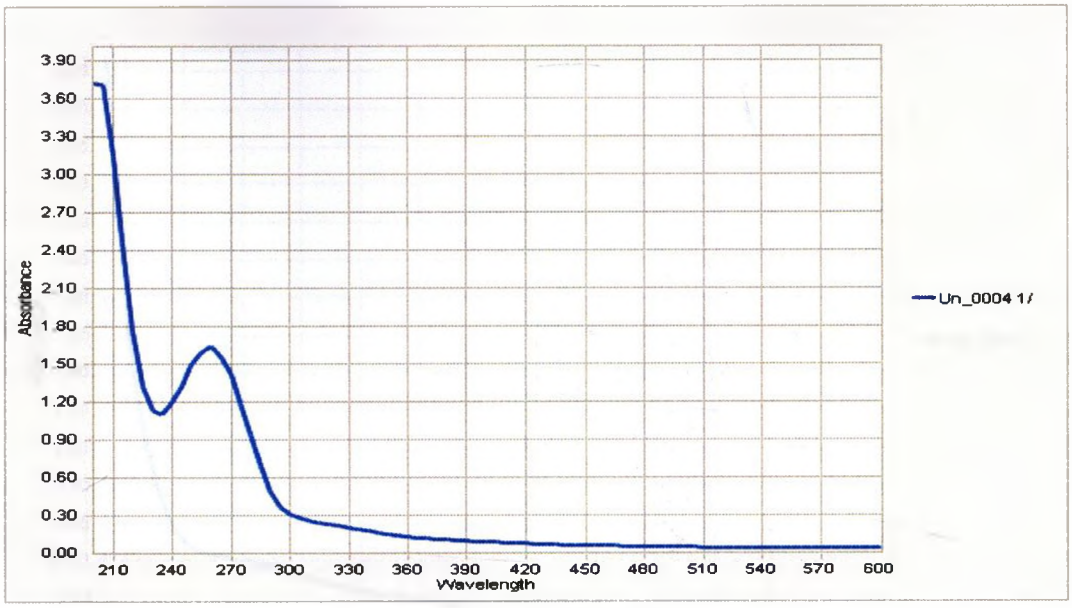
Apoptosis of melanoma cells in culture were quantified with a cell death detection ELISA based assay (Roche Diagnostics GmbH, Mannheim, Germany). This assay is for the qualitative and quantitative *in vitro* determination of cytoplasmic histone-associated DNA fragments (mono - and oligonucleosomes) associated with apoptosis. The positive control was prepared by treating the cells with hypotonic buffers and is described in the following. A suspension of untreated cells (5×10^4 cells/tube in 500 µL) was centrifuged at 300 g for 5 min and the supernatant was removed. The resulting pellet was resuspended with a hypotonic buffer (10 mM Tris, pH 7.4, 400 mM NaCl, 5mM $CaCl_2$ and 10 mM $MgCl_2$) and incubated at 37 °C for 2 hours. Then the cell fragment was centrifuged at 20,000 g for 10 min. The supernatant was collected and diluted to 1:5 with incubation buffer (1×10^4 cell equivalents/mL). Then the positive control as well as the samples was prepared following the procedures described in Chapter 3.2.6. The absorbance of positive control was used as a reference standard for expressing the relative magnitude of apoptosis of the treated samples.

Appendix 2: Physiochemical Characterization of PSP

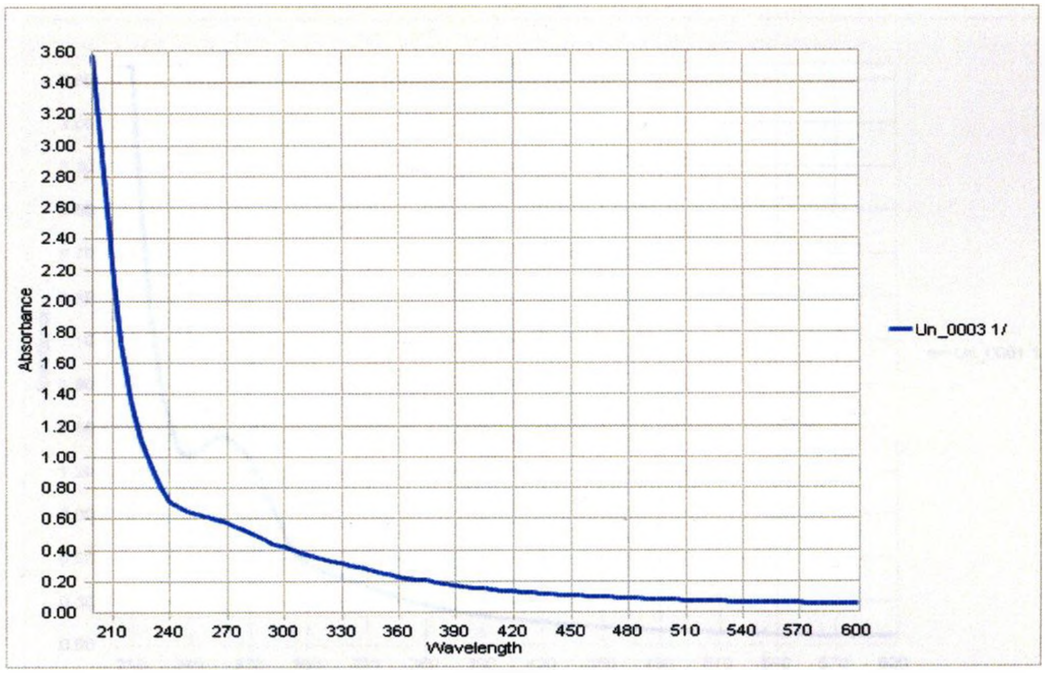
1. Spectral analysis of crude PSP and its fractions obtained from the Xinkang Company

Crude PSP water extract was fractionated by Sephadex G-75 chromatography into four fractions (HMF, PSP-X, SMF, and PSP-Y) with respect to their molecular size. Because peptide bonding generally shows absorption in the range of 190-230 nm, the chromatographic eluates were monitored at 230 nm. The spectral scans (200-600 nm) of crude PSP water extract and solution of HMF, PSP-X, and SMF were shown in Figure 1. The spectrum of Crude PSP and SMF was qualitatively similar with a max absorption at 260 nm. The magnitude of the SMF peak (2.5 mg/mL which was equivalent to crude PSP 5 mg/mL) was similar to that of the crude PSP. In contrast, HMF and PSP-X showed a little or no absorbance at 260 nm. This suggested that SMF contributed to the absorbance of the crude PSP peak at 260 nm.

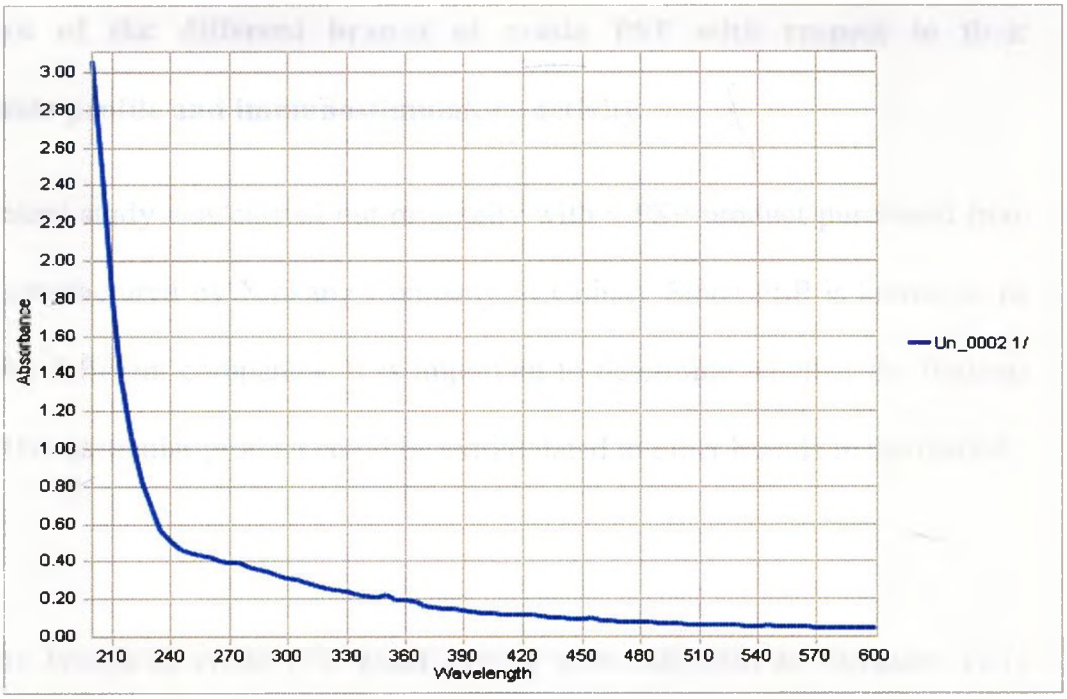
Figure A2.1 Spectral scanning results of crude PSP and its fractions. A: Crude PSP 5 mg/mL; B: HMF 5 mg/mL; C: PSP-X 5 mg/mL; D: SMF 2.5 mg/mL. Crude PSP and its fractions (HMF, X and SMF) were dissolved in nanopure water separately, the solutions were then scanned with spectrophotometer with the wavelength range from 200 to 600 nm.



A: Crude PSP



B: HMF


Absorbance
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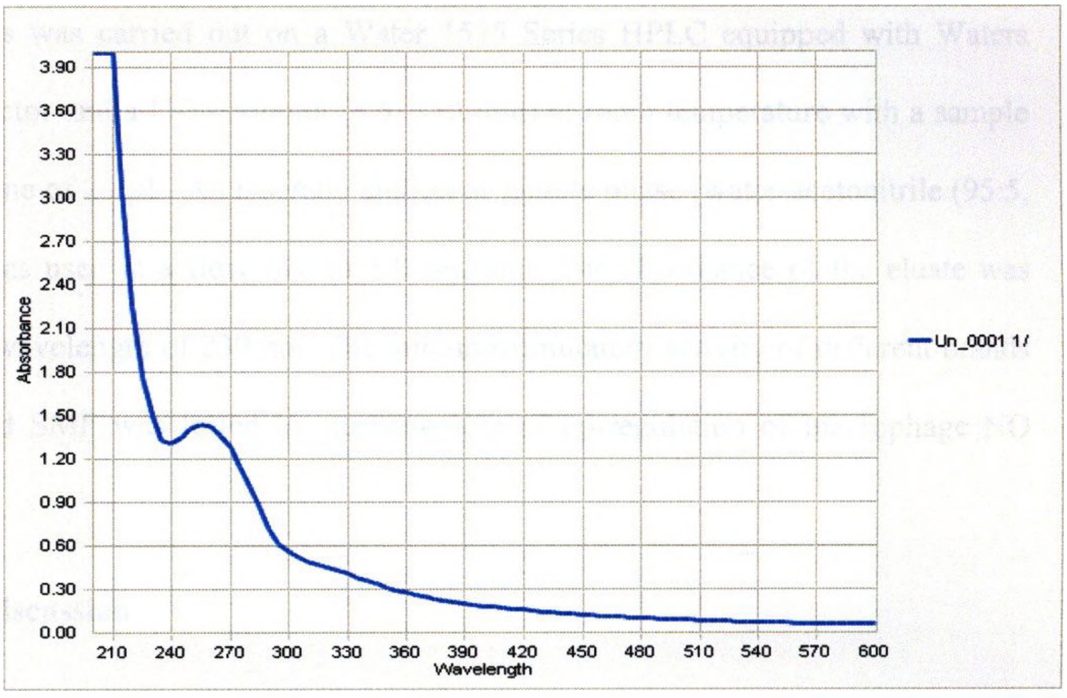
2.80
2.60
2.40
2.20
2.00
1.80
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
210 240 270 300 330 360 390 420 450 480 510 540 570 600
Wavelength
Un_0002 1/

C: PSP-X


Absorbance
3.90
3.60
3.30
3.00
2.70
2.40
2.10
1.80
1.50
1.20
0.90
0.60
0.30
0.00
210 240 270 300 330 360 390 420 450 480 510 540 570 600
Wavelength
Un_0001 1/

D: SMF

2. Comparison of the different brands of crude PSP with respect to their chromatographic profile and immunostimulatory activity

The present study was carried out originally with a PSP product purchased from Hong Kong (manufactured by Xinkang Company of China). Since PSP is known to be manufactured by different companies, it is important to determine whether the findings observed with this particular product could be extrapolated to other brands in the market.

Methods

Different brands of crude PSP water extract were subjected to Sephadex G-75 Chromatography (See Section 3.2.2). Furthermore, high performance liquid chromatography (HPLC) was used to analyze different brands of crude PSP and SMF. HPLC analysis was carried out on a Water 1525 Series HPLC equipped with Waters 2487 UV detector and a C-18 column (4.6×150 mm) at room temperature with a sample injection volume of 20 μL. An isocratic elution of mobile phase [water-acetonitrile (95:5, v/v), pH 7] was used at a flow rate of 1.0 mL/min. The absorbance of the eluate was recorded at a wavelength of 230 nm. The immunostimulatory activity of different brands crude PSP and SMF was tested by measurement of up-regulation of macrophage NO production.

Results and discussion

Data presented in Figure A2.1 A, B, and C shows the G-75 chromatographic profiles of the crude water soluble PSP extract prepared from products obtained by the Xinkang Co., Chongqing Co. and the Signature Inc., respectively. All three profiles show

the two major peaks (HMF and SMF); however, the relative abundance of the fractions was different. Table A2.1 shows the recovery of water-soluble PSP extract as a percentage of commercial crude PSP as well as the yield of SMF as a percentage of crude water-soluble PSP subjected to chromatography from the three different brands. The yield of crude PSP from Xinkang, Chongqing and Signature commercial product was 77.7%, 92.9% and 99.8%, respectively. The yield of SMF from the water extract of crude PSP was 50%, 68.1% and 40.5%, respectively.

High performance liquid chromatography (HPLC) was used to compare the chemical composition of different brands of crude PSP and SMF. They were superimposed to show the identity of the HMF and SMF components (Figure A2.2). Crude PSP contains 4 peaks and SMF had 2 peaks (Peak 2 and 3). It is apparent that Peak 1 and 4 in the chromatogram could be ascribed to the HMF; and peak 2 represented the major SMF component(s). Since the major HMF and SMF fractions (peak 1 and 2) have similar retention time, suggesting that they have similar water-solubility. Crude PSP from Chongqing Co. do not show the presence of peak 1; and this is consistent with the almost absence of the HMF in the G-75 profiles. Peak 2 and 3 are both presented in different brands of SMF, the relative abundances are different.

The immunostimulatory activity of different brands of crude PSP and SMF is shown in Figure A2.3. The activity of crude PSP was not different among brands on stimulation of macrophages NO production. SMF of Xinkang and Chongqing showed similar activity and they were significantly higher than SMF of Signature ($p<0.05$).

The similar immunostimulatory activity of crude PSP suggests that peak 1 or

HMF is not bioactive in this regard. On the other hand, the difference of immunostimulatory activity of SMF might be linked to the difference presented on the HPLC profile (Table A2.2). For example, the relative abundance of peak 2 was increased from SMF of Xinkang, Chongqing and Signature, while the activity was decreased; on the contrary, the relative abundances of peak 3 were the same order with the activity. This points out that our future work should concentrate on the peak 2 and peak 3 to study the single component as well as the activity.

Figure A2.2 Isolation of different brands of crude PSP with Sephadex G-75 chromatography. A: Xinkang Company; B Chongqing Company China; C Signature Inc., Canada. The absorption at 230 nm was measured for each fraction. Five mL of crude PSP water-soluble extract (100 mg/mL) was loaded on the column and eluted with distilled water at flow rate of 0.8 mL/min at 4 °C. The fractions were collected according the absorbance at 230 nm.

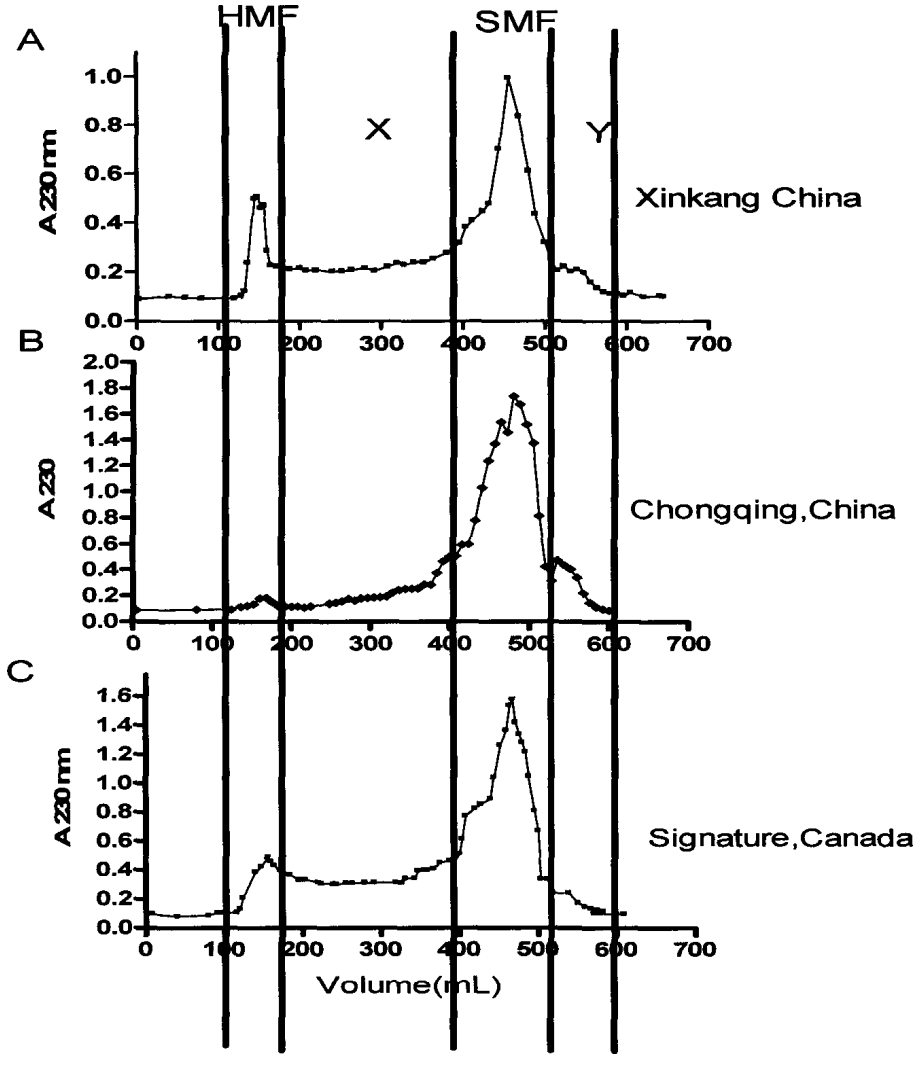

A
HMF
SMF
X
Y
A230nm
1.0
0.8
0.6
0.4
0.2
0.0
0
100
200
300
400
500
600
700
Xinkang China
B
A230
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Chongqing,China
C
A230nm
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Signature,Canada
Volume(mL)

Table A2.1 Yields of Crude water-soluble PSP and SMF from different brands of PSP.

PSP product	%water-soluble extract	% yield of SMF from water-soluble extract
Xinkang Co.	77.7	50
Chongqing Co.	92.9	68.1
Signature Inc.	99.8	70.5

Three brands of PSP powder were weighted and dissolved in nanopure water separately, and then the solutions were centrifuged to recover the supernatant (water-soluble fraction). The water insoluble residues were dried and weighed. The yield was calculated by the following formula: [(the weight of crude PSP powders - the weight of residues) / the weight of crude PSP powder] ×100. The SMF from the three brands of crude PSP were collected and dried and the yield as a % of the water-soluble PSP extract was calculated by the following formula: (Weight of SMF / Weight of water-soluble PSP extract) ×100.

Figure A2.2 C18- HPLC profiles of crude PSP and SMF obtained from three different brands. Crude PSP 2.0 mg/mL (—); SMF 1.0 mg/mL (—). The elution system was water, acetonitrile (95:5) (pH 7) at a flow rate 1.0 mL/min.

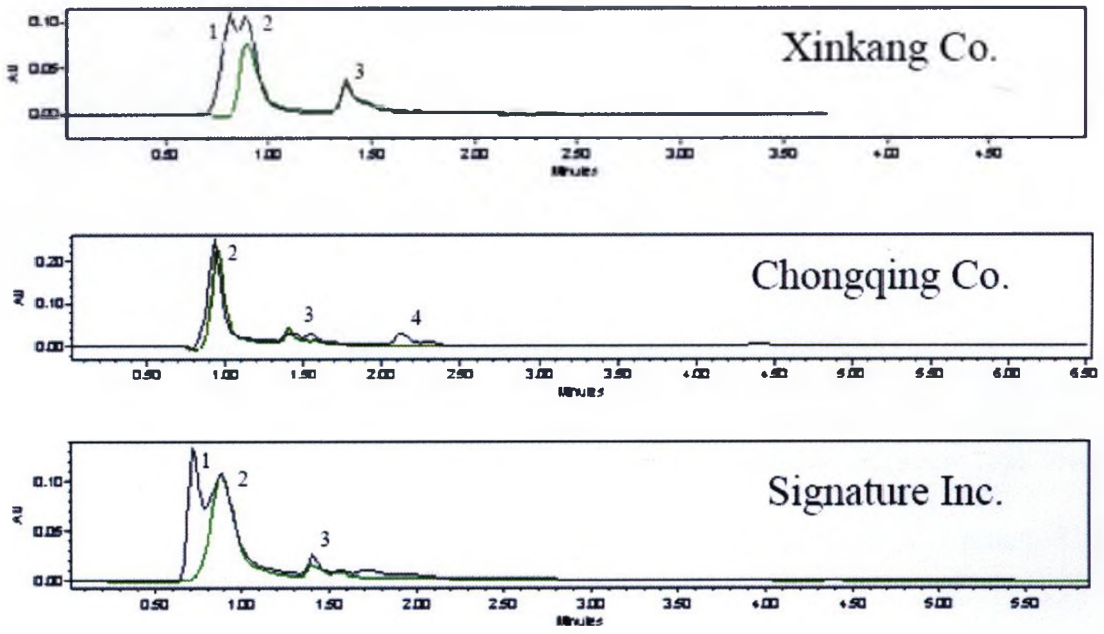

Xinkang Co.
Chongqing Co.
Signature Inc.

Figure A2.3 Different brands of crude PSP and SMF on induction of NO by RAW 264.7 cells. Macrophages were prepared as described in Chapter 3.2.7 and treated with different concentration of crude PSP (10 μg/mL) and SMF (5μg/mL) for 24 hours; the culture medium was collected and the NO accumulation was calculated. Each treatment group and control group was performed in duplicate per experiment. The experiment was, therefore, repeated six times (n=6). Data is expressed as mean ± S.E.M. *p<0.05 for SMF of Signature versus Xinkang and Chongqing.

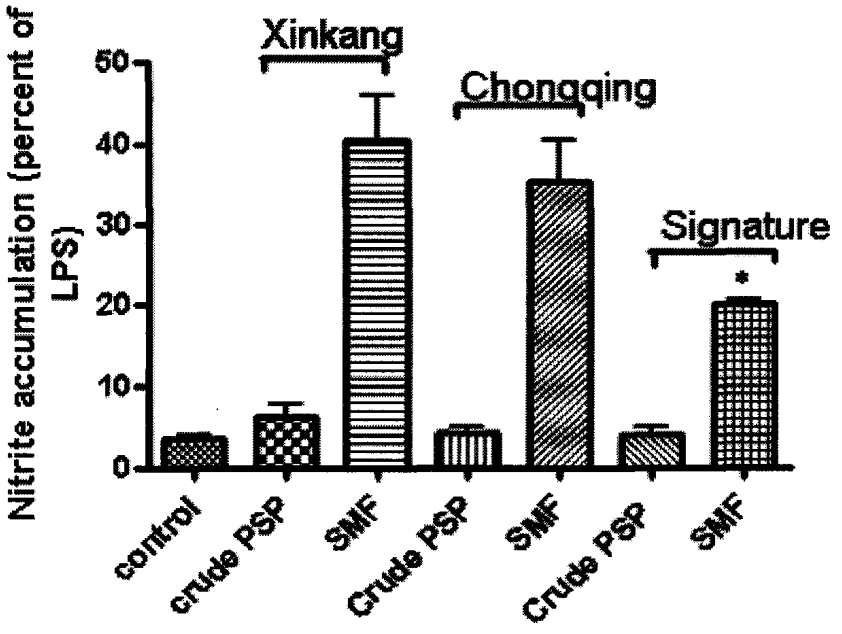

Nitrite accumulation (percent of LPS)
50
40
30
20
10
0
Xinkang
Chongqing
Signature
*
control
crude PSP
SMF
Crude PSP
SMF
Crude PSP
SMF

Table A2.2 Relationship of the immunostimulatory activity with the relative abundances of SMF components on HPLC area (Peak 2 and 3 were peaks shown in the HPLC analysis, see figure A2.2).

SMF	Immunostimulatory activity	% Total HPLC area	
		Peak 2	Peak 3
Xinkang	+++	49.08	19.12
Chongqing	++	61.80	8.34
Signature	+	84.90	6.42