ANTI-TUMOUR AND IMMUNOMODULATORY ACTIVITIES OF A POLYSACCHARIDE FRACTION FROM *Solanum nigrum* L. *nigrum* TOWARDS BREAST CANCER

FAIZAN NAEEM BIN RAZALI

FACULTY OF SCIENCE
UNIVERSITY OF MALAYA
KUALA LUMPUR

2017

ANTI-TUMOUR AND IMMUNOMODULATORY ACTIVITIES OF A POLYSACCHARIDE FRACTION FROM *Solanum nigrum* L. *nigrum* TOWARDS BREAST CANCER

FAIZAN NAEEM BIN RAZALI

THESIS SUBMITTED IN FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

INSTITUTE OF BIOLOGICAL SCIENCES
FACULTY OF SCIENCE
UNIVERSITY OF MALAYA
KUALA LUMPUR

2017

UNIVERSITI MALAYA

ORIGINAL LITERARY WORK DECLARATION

Name of Candidate: FAIZAN NAEEM BIN RAZALI

Registration/Matric No: SHC 130101

Name of Degree: DEGREE OF DOCTOR OF PHILOSOPHY

Title of Project Paper/Research Report/Dissertation/Thesis ("this Work"):

ANTI-TUMOUR AND IMMUNOMODULATORY ACTIVITIES OF A POLYSACCHARIDE FRACTION FROM Solanum nigrum L. nigrum TOWARDS BREAST CANCER.

Field of Study: BIOCHEMISTRY & IMMUNOLOGY

I do solemnly and sincerely declare that:

(1) I am the sole author/writer of this Work;
(2) This Work is original;
(3) Any use of any work in which copyright exists was done by way of fair dealing and for permitted purposes and any excerpt or extract from, or reference to or reproduction of any copyright work has been disclosed expressly and sufficiently and the title of the Work and its authorship have been acknowledged in this Work;
(4) I do not have any actual knowledge nor do I ought reasonably to know that the making of this work constitutes an infringement of any copyright work;
(5) I hereby assign all and every rights in the copyright to this Work to the University of Malaya ("UM"), who henceforth shall be owner of the copyright in this Work and that any reproduction or use in any form or by any means whatsoever is prohibited without the written consent of UM having been first had and obtained;
(6) I am fully aware that if in the course of making this Work I have infringed any copyright whether intentionally or otherwise, I may be subject to legal action or any other action as may be determined by UM.

Candidate's Signature Date

Subscribed and solemnly declared before,

Witness's Signature Date

Name:
Designation:

ABSTRACT

Breast cancer is the most frequently diagnosed cancer worldwide. In the year of 2012, approximately 1.67 million new incidences were reported, with a mortality rate of 15.4%. Approximately 5,410 of Malaysian women were affected, with the mortality rate of 11.9% reported in the same year. Conventional therapies for cancer indeed improve mortality and cure rate in patients. However, the side effects due to toxicity of the therapeutic agents are unavoidable and negatively affecting patient's quality of live. *Solanum nigrum* is an herbal plant that has been extensively studied because of folklore belief that this plant has various health benefits, including curing cancer. Currently, immunotherapy is gaining much interest among pharmacologist as an approach in cancer treatment. The aim of this study was to evaluate the ability of *S. nigrum* extract to inhibit breast cancer progression through immunomodulation. In the *in vitro* study, the immunomodulating activity of polysaccharide extract from *S. nigrum* was analysed by looking at its effects on RAW 264.7 murine macrophage cells. Crude polysaccharide extracted from the stem of *S. nigrum* was partially purified through ion-exchange chromatography, yielding five polysaccharide fractions. The fractions were then evaluated for cytotoxicity and nitric oxide production. The ability of the polysaccharide extracts to activate macrophages and to induce phagocytosis and cytokine production were also measured. Though not cytotoxic (IC_{50} > 100 mg/kg), all polysaccharide fractions were able to induce nitric oxide production in RAW 264.7 cells. Of the five fractions tested, SN-ppF3 had the lowest cytotoxicity and induced the highest amount of nitric oxide, which tally with the increase in inducible nitric oxide synthase expression detected in the cell lysate. This fraction also significantly induced phagocytic activity and stimulated the production of tumour necrosis factor-α and interleukin-6. Based on the cytokines produced, it could be concluded that classically activated macrophages could be induced by SN-ppF3. The *in vitro* results confirmed that SN-ppF3 possessed

the immunomodulatory effect, and *in vivo* evaluation was carried out to further analyse the possible mechanism on how SN-ppF3 could inhibit tumour progression in 4T1 tumour-bearing BALB/c mice. The sample dose of 500 mg/kg/bw was shown to have non-toxic effect to the healthy mice as 100% of the mice were alive after 14 days of treatment (n=5). Tumour volume and weight were significantly (p<0.05) inhibited by 65% and 40% respectively after oral administration of 500 mg/kg of SN-ppF3 for 10 days (n=6). To further analyse the ability of SN-ppF3 in suppressing tumour progression, the production of cytokines in the serum were evaluated. The level of tumour necrosis factor-α, interferon-γ and interleukin-4 were significantly (p<0.05) elevated by 12%, 22% and 12% respectively, while the level of interleukin-6 was decreased by 52%. Histological observations showed that treatment with SN-ppF3 resulted in disruption of cell morphology due to apoptosis and enhanced the infiltration capability of natural killer cells and cytotoxic T cells directly into the solid tumour. These results suggested that the treatment of tumour-bearing mice with SN-ppF3 was able to suppress tumour progression by improving host immune responses thus potentially develop as novel anti-tumour agent.

ABSTRAK

Kanser payudara merupakan kanser yang paling kerap didiagnosis di seluruh dunia. Pada tahun 2012, sebanyak 1.67 juta insiden baharu dilaporkan dengan kadar kematian sebanyak 15.4%. Seramai 5,410 orang wanita Malaysia telah didiagnosis, dengan kadar kematian sebanyak 11.9% telah dilaporkan pada tahun yang sama. Terapi konvensional untuk kanser sememangnya dapat memperbaiki kadar kematian dan kesembuhan pesakit. Walau bagaimanapun, kesan sampingan disebabkan oleh bahan terapeutik tidak dapat dielakkan dan memberi kesan kepada kualiti hidup pasakit. Pokok herba *Solanum nigrum* telah dikaji secara meluas disebabkan oleh kepercayaan masyarakat bahawa pokok ini mengandungi pelbagai manfaat kesihatan termasuk merawat kanser. Ketika ini, terapi imuno telah menarik minat dalam kalangan ahli farmakologi sebagai satu pendekatan yang berkesan kepada rawatan kanser. Tujuan kajian ini dijalankan adalah untuk menilai kemampuan ekstrak daripada *S. nigrum* untuk merencat pertumbuhan kanser payudara melalui proses imunomodulasi. Dalam kajian *in vitro* ini, aktiviti imunomodulasi ekstrak polisakarida daripada *S. nigrum* telah dikaji dengan memerhati kesannya terhadap sel makrofaj tikus RAW 264.7. Polisakarida mentah telah diekstrak daripada bahagian batang *S. nigrum* dan ditulenkan secara separa melalui kromatografi penukaran ion, menghasilkan lima fraksi polisakarida. Semua fraksi polisakarida telah dicerakinkan untuk melihat kesan sitotoksik dan penghasilan nitrik oksida. Kemampuan ekstrak polisakarida untuk mengaktifkan sel makrofaj dan untuk mendorong fagositosis dan menghasilkan sitokina juga telah diukur. Walaupun tidak sitotoksik (IC_{50} > 100 mg/kg), semua fraksi polisakarida mampu mendorong penghasilan nitrik oksida oleh sel makrofaj. Daripada lima fraksi polisakarida yang telah diuji, fraksi SN-ppF3 menunjukkan tahap ketoksikan paling rendah dan mendorong penghasilan nitrik oksida yang tertinggi selari dengan tahap peningkatan ekspresi *inducible nitric oxide synthase* yang dikenalpasti di dalam lisat sel. Fraksi ini dapat meningkatkan aktiviti fagositosis

secara ketara dan meransang penghasilan faktor nekrosis tumor-α dan interleukin-6. Berdasarkan kesan penghasilan sitokina, boleh dirumuskan bahawa pengaktifan makrofaj secara klasik telah didorong oleh SN-ppF3. Hasil kajian *in vitro* ini mengesahkan SN-ppF3 mempunyai kesan imunomodulatori, dan analisis lanjut kajian *in vivo* telah dijalankan untuk mengkaji mekanisme bagaimana SN-ppF3 dapat merencat tumbersaran tumor pada tikus BALB/c pembawa tumor 4T1. Sukatan sampel pada 500 mg/kg telah menunjukkan kesan tidak toksik kepada tikus-tikus yang sihat di mana 100% tikus masih hidup selapas 14 hari tempoh rawatan (n=5). Isipadu dan berat tumor, masing-masing telah direncat secara ketara (p<0.05) sebanyak 65% dan 40% selepas rawatan secara oral dengan sukatan SN-ppF3 pada 500 mg/kg selama 10 hari (n=6). Sebagai analisa lanjutan terhadap kemampuan SN-ppF3 dalam merencat tumbesaran tumor, penghasilan sitokina di dalam serum telah disiasat, dan tahap faktor nekrosis tumor-α, interferon-γ dan interleukin-4, masing-masing telah meningkat secara ketara (p<0.05) sebanyak 12%, 22% dan 12%, dan tahap IL-6 telah berkurangan sebanyak 52%. Pemerhatian histologi menunjukkan rawatan SN-ppF3 telah mengakibatkan perubahan morfologi sel yang disebabkan oleh apoptosis dan peningkatan keupayaan penyusupan sel NK dan sel T sitotoksik ke dalam tumor solid. Keputusan daripada kajian ini mencadangkan bahawa rawatan tikus pembawa tumor menggunakan SN-ppF3 dapat merencat tumbesaran tumor melalui peningkatan keupayaan tindak balas imuniti hos dan berpotensi untuk dijadikan sebagai bahan anti-tumor yang novel.

ACKNOWLEDGEMENT

I would like to express the deepest gratitude towards my supervisors, Assoc. Prof. Dr. Adawiyah Suriza Shuib and Assoc. Prof. Dr. Nurhayati Zainal Abidin for their guidance, valuable advices and encouragement throughout the preparation and completion of the study. Without them, this study will not be accomplished.

Special appreciations towards the laboratory members in B503 Biology Molecular Laboratory, Institute of Graduate Study and E1.1 Proteomic Laboratory, Institute of Biological Sciences for their kindness help and endless support while I am working in the labs. My deepest appreciation to my parent, family members, and friends, and also to everyone who involved in making this project accomplished.

Last but not least, I would like to acknowledge the Minister of Higher Education (MOHE) and University of Malaya for the research funds.

TABLE OF CONTENTS

CHAPTER 2: MATERIALS AND METHODS

CHAPTER 3: RESULTS

CHAPTER 4: DISCUSSION

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ADCC	Antibody-dependent cell-mediated cytotoxicity
APCs	Antigen presenting cells
APS	Ammonium persulfate
ASR	Age-standardised incidence rate
BrCa	Breast cancer
CD	Cluster of differentiation protein
COX-2	Cyclooxygenase-2
CRs	Complement receptors
CTLs	Cytotoxic T lymphocytes
CTX	Cyclophosphamide anti-cancer drug
DCs	Dendritic cells
DEAE	Diethylaminoethyl
DNA	Deoxyribonucleic acid
dsRNA	Double stranded ribonucleic acid
FADD	Fas-associated death domain
FITC	Fluorescein isothiocyanate
FT-IR	Fourier transform infrared
HPLC	High performance liquid chromatography
IFN	Interferon
IFN-R	Interferon receptor
IgG	Immunoglobulin-G
iNOS	Inducible nitric oxide synthase
IL	Interleukin
IRAK	IL-1R-associated kinase

ITAM	Immunoreceptor tyrosine-based activation motif
JAK	Janus kinase
JNK	Jun N-terminal kinase
LPS	Lipopolysaccharide
MAC	Membrane attack complex
MAPk	Mitogen-activated protein kinase
MHC	Major histocompatibility complex
mRNA	Messenger ribonucleic acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide
NED	N-1-napthylethylenediamine dihydrochloride
NF-κB	Nuclear factor kappa B
NK	Natural killer
NO	Nitric oxide
OD	Optical density
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral mononuclear cell
PGE2	Prostaglandin E2
POI	Percentage of inhibition
PrCr	Programmed cell removal
PRRs	Pathogen recognition receptors
RIP	Receptor interacting protein
ROS	Reactive oxygen species
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SN-ppF1–5	*Solanum nigrum* polysaccharide fraction 1–5
SOCS	Suppressor of cytokines signalling

STAT	Signal transducer and activator of transcription
TAA	Tumour-associated antigen
TCR	T-cells receptor
Th	Helper T cells
TDLU	Terminal duct lobular unit
TEMED	N, N, N'tetramethylethylenediamine
TGF-β	Transforming growth factor beta
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TNFR	Tumour necrosis factor receptor
TRADD	TNF receptor-associate death domain
TRAF	TNF receptor-associate factor
TRAIL	TNF-related apoptosis-inducing ligand
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labelling

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

1.1 Breast cancer

1.1.1 Epidemiology

Breast cancer (BrCa) is the leading cause of cancer death among females in both economically developed and developing countries. According to International Agency for Research in Cancer GLOBOCAN 2012, BrCa is the second most frequently diagnosed cancer in the world, after lung cancer with approximately 1.67 million new cancer cases. BrCa constitutes 25% of all cancers diagnosed until 2012. By far, BrCa was listed as the fifth cause of death from all cancer cases and approximately 14.0% and 15.4% of total death for female cancers worldwide in 2011 and 2012, respectively (Jemal *et al.,* 2011). In the year of 2015, BrCa contributes approximately 29% of all female cancers in United State (Siegel *et al*., 2015).

In the year of 2007, BrCa is the top listed cancer in Malaysia, accounting for 32.1% of all common female cancers and affecting approximately 18.1% of the Malaysian women (Kuan *et al*., 2015). Referring to the data provided by National Cancer Registry of Malaysia for 2004, the age-standardised incidence rate (ASR) of BrCa is 46.2 out of 100,000 females. Statistically, approximately 1 in 20 of Malaysian women is affected with BrCa in their lifetime. However, the incidence rate differs between the main races in Malaysia with the highest ASR is among the Malaysian Chinese population (59.7 per 100,000), followed by Malaysian Indians (55.8 per 100,000) and the Malays (33.9 per 100,000). The prevalent age group is between 40–49 year of age group and more than 50% of cases below the age of 50 (Yip *et al*., 2006).

1.1.2 Pathological classification

Most invasive BrCa arises from the epithelial cells of the terminal duct lobular unit of the breast. BrCa is extremely heterogeneous due to the broad variability in morphology, gene expression patterns and individual tumour cell behaviour. Over the past few years, BrCa was characterised into five subtypes, namely luminal A, luminal B, HER2/*neu* over-expressing, basal-like and normal breast-like tumour tissues. These sub-categories were defined based on the disease manifested by differences at the molecular pattern, histopathological and clinical assessment (Campbell and Polyak, 2007).

1.1.3 Aetiology

The major causes of human BrCa still remain unclear. Several factors are linked to human BrCa development. Genetically, women with history or having relatives with BrCa or ovarian cancer are more susceptible to develop BrCa. Moreover, women who carried the BrCa related inherited-genes, *BRCA1* and *BRCA2* (Dutt and Stambolic, 2013) are at higher risk (up to 85%) of getting BrCa and ovarian cancer (Veronesi *et al*., 2005). In addition to BrCa, *BRCA1* and *BRCA2* carriers are now categorised in highly potential risk of getting ovarian cancer, and male who carries *BRCA2* are prone to prostate cancer (Dutt and Stambolic, 2013). Other than that, *TP53* gene is also linked to a greater BrCa risk (Hulka and Stark, 1995).

History reported that most of cancer patients are women at the age of 50 and older (Hulka and Stark, 1995). According to America Cancer Society, women who are exposed longer time to oestrogen and progesterone, beginning from early puberty to late menopause, have higher risk of getting BrCa in older age. Luminal A is one of the BrCa subtypes, which is frequently diagnosed as oestrogen-related BrCa. Moreover,

menopause women who received combination hormones treatment are also having approximately 0.8% chances of BrCa development.

1.1.4 Staging

Before the computerised era, BrCa was staged based on the tumour size and its primary lesion. It starts with the primary BrCa cells T1, which commonly be divided into three subgroups namely T1a, T1b and T1c. Nowadays, more precise staging procedure of BrCa was achieved where the 'T' classification will probably be determined by a continuous numerical description of the tumour size in centimetre (example: T0.9 or T2.4). The same system could be applied to number of affected nodes (N), and as well as metastases (M), indicates the present of BrCa metastases. The staging of BrCa by the American Join Committee of Cancer (AJCC) is simplified as in Table 1.1.

1.1.5 Treatment option for BrCa patient

Currently, there are limited options for the treatment of BrCa. Surgery remains as the most popular option, along with chemotherapy, radiotherapy and hormone therapy as an adjunctive therapy (Yip *et al*., 2014). Although the major modern therapies and techniques have become possible in treating this cancer, the treatments still have some drawbacks such as unavoidable toxicity due to nonspecific action towards cancer cells as well as rapidly-dividing normal cells which would affect the quality of patients' lives (Palumbo *et al.,* 2013). Evidences have demonstrated that the anti-tumour activities of many chemotherapeutic agents have resulted in the toxicity of normal cells and organ damages as well (Antonarakis and Drake, 2010). Thus the identification of novel anti-cancer treatment with better effectiveness and lower toxicity is necessary. Numerous studies on BrCa therapies are still on-going, and one of the possible strategies to encounter this disease is through immunomodulation.

Table 1.1: [1]Staging of BrCa

Stage	T (size)	N/M	[2]Survival rate (%)
0	Tis (no tumour, Lump)	N0/M0	93
IA	T1/T1mi (≤ 0.1cm / ≤ 2.0 cm)	N0/M0	88
IB	T0	N1mi/M0	88
	T1/T1mi (≤ 0.1cm / ≤ 2.0 cm)	N1mi/M0	
IIA	T0	N1+/M0	81
	T1/T1mi (≤ 0.1cm / ≤ 2.0 cm)	N1+/M0	
	T2 (> 2.0 cm, < 5.0 cm)	N0/M0	
IIB	T2	N1/M0	74
	T3	N0/M0	
IIIA	T0	N2/M0	64
	T1/T1mi (≤ 0.1cm / ≤ 2.0 cm)	N2/M0	
	T2 (> 2.0 cm, < 5.0 cm)	N2/M0	
	T3 (> 5.0 cm)	N1/M0	
	T3 (> 5.0 cm)	N2/M0	
IIIB	T4	N0/M0	41
	T4	N1/M0	
	T4	N2/M0	
IIIC	Any T	N3/M0	49
IV	Any T	Any N/M1	15

[1]AJCC staging manual (Edge and Compton, 2010)

[2]Information obtained from National Cancer Centre Database

T4=any size with extension to chest wall or skin and with ulceration or skin nodules or inflammatory cancer

N0=no spread to nearby nodes.

N1= spread to 1–3 moveable, low or mid-axillary nodes.

N1mi = N1 nodes with micro-metastases (> 0.2 mm and/or 200 cells, but < 2.0 mm)

N2 = N1 and spread to internal mammary nodes but not axillary nodes / spread to infraclavicular nodes / spread to supraclavicular nodes.

M0 = no metastases

M1 = metastases present

1.2 Cellular immune responses towards cancer

Immune response is a natural defence mechanism in all organisms, against challenges in the living environment. Organisms such as mammals have very complex phalanx defensive system especially towards bacteria, viruses, parasites and cancers. The immune systems generally will not attack the host self-tissues because the system has self-discrimination, which is the ability to distinguish between self and foreign tissues. The ability to avoid attack on self-tissue is referred as 'self-tolerance'. However, the tolerance mechanism is becoming an impediment that needs to be overcome in anti-tumour immunity (Nguyen *et al*., 2013). Thus, a possible strategy such as activating and enhancing the activities of several immune cells against tumour cells have been developed over the last few decades to be used as potent therapy towards cancer.

Tumour-associated antigens (TAAs) are intra- or extracellular proteins that are expressed exclusively on tumour cells, but are often expressed by normal cells. Commonly, TAAs were able be recognised by immune cells through immune cells receptors. Because of that reason, the identification and recognition of TAAs was prioritised in designing immunotherapeutic agents which specifically activates TAA-specific immune cells against tumour cells. There are several types of TAAs that can be classified into broad categories: (1) mutated antigens, (2) cancer-testes antigens, (3) differentiated antigens, (4) overexpressed antigens, (5) viral antigens and (6) unique posttranslational modified antigens (Nguyen *et al*., 2013). Thus, the immune tolerance against most of cancers is incomplete due to the presented TAAs.

1.2.1 Innate immune responses

A. Natural Killer cells

Natural Killer (NK) cells are one the lymphocytes lineage that is able to identify stressed or infected cells and directly respond to them. NK cells constitutes approximately 5 to 20% of all total mononuclear cells in peripheral blood lymphocytes (Waldhauer and Steinle, 2008) and spleen, but are rarely present in any lymphoid organ. The term killer refers to the natural behaviour of the cells that directly kill various targeted cells following detection of the latter. NK cells majorly involves in eliminating many types of tumour cells. Major Histocompatibility Complexes (MHC) class I expression in tumour cells are either absent or reduced. The losses in MHC class I molecule on cancer cells induces NK cells inhibitory signals, leading to the destruction of tumour cell. Some tumour cells also express ligands such as MICA, MICB and ULB that favour the binding with CD94-NKG2D receptor on NK cells and the binding initiates the inhibitory signals of NK cells cytotoxicity (Zamai *et al.*, 2007; Abbas *et al*., 2012).

NK cells will start the killing action following the initiation of inhibitory signals. Once activated, NK cells release their granules content adjacent to the target cells, perforin, which is one of the granule proteins, will facilitate granzyme entry into the target cells, which in turn activates the apoptosis pathway. Because NK cells were shown to kill tumour cells *in vitro*, it was proposed that NK cells may also kill malignant clone *in vivo*. Alternatively, cytokines such as interferon-gamma (IFN-γ) that is released by activated NK cells may serve as macrophages activator and increase macrophages capability to kill microbes and eliminate apoptotic cancer cells via phagocytosis (Abbas *et al*., 2012).

B. Macrophages

Macrophages are tissue-based phagocytic cells that are derived from blood monocytes which majorly participate in both innate and adaptive immune responses. Macrophages occupied approximately 20% population of peripheral mononuclear cell fraction, much lesser as compared to the other major phagocytes population such as polymorphonuclear cells and neutrophilic granulocytes (Hume, 2012). Macrophages can be activated upon interaction with microbial components such as lipopolysaccharide (LPS) and cytokines such as interleukin (IL)-2 (Abbas *et al*., 2012). The pattern of macrophages polarisation depends on the activators surrounding its microenvironment. Typically, macrophages are classified into two main groups, which are (1) classically activated and (2) alternatively activated (Obeid *et al*., 2013).

Classically activated macrophage is an integral cellular component of the organism immune system. This type of macrophages commonly protect the host from intracellular pathogens and also play important role in eliminating cancer cells by initiation of both innate and adaptive immune responses. The activation of macrophages is triggered by antigens or cytokines that bind to the cells' specific receptors, causing the cells to secrete pro-inflammatory cytokines and radical nitrogen species. Classically activated macrophages secrete high level of IL-12, a necessary potent inducer of IFN-γ production by Type 1 helper T cells (Th1), and NK cells (Croxford *et al*., 2014). Circulation of IFN-γ in the system creates a positive feedback loop to maintain the classical activation mode of macrophages.

The direct mechanism on how macrophages eliminate tumour cells still remains unclear. The possible mechanism is probably similar to the action of eliminating infectious antigens. The release of reactive nitrogen species and pro-inflammatory

cytokines by classically activated macrophage could be the potent proxy in eliminating tumour cells (Duque and Descoteaux, 2014; Abbas *et al.*, 2012). In addition, the killing mechanism towards tumour cells also involves indirect action of activated macrophages by supporting the other immune cells function. Excretion of IL-2 by classically activated macrophages is responsible for modulating NK cells and cytotoxic T lymphocytes (CTLs) anti-tumour activities (Wang *et al.*, 2000). In addition to tumour cells elimination mechanism, macrophages are responsible in eliminating apoptotic tumour cells via phagocytosis (Flannagan *et al.*, 2012).

Phagocytosis

In general term, phagocytosis is a process of digesting foreign particles performed by phagocytes. Precisely, phagocytosis is an active, energy dependent engulfment mechanism of large particles, approximately 0.5 μM in diameter or larger. In an organism immune system, phagocytosis is the most prominent mechanism used to remove pathogens and cellular debris. Several immune cells especially macrophages are able to perform phagocytosis and became the major roles in innate immune response against possible invaders (Flannagan *et al.*, 2012).

The process of phagocytosis begins with the recognition of microbes or other possible threats by macrophages. Receptors expressed by macrophages specifically recognise microbial recognition sites called pathogen-associated molecular patterns (PAMPs), and these receptors are functionally linked to phagocytosis. The other type of receptors presented on macrophages surface are able to recognise certain host proteins coating the antigens known as opsonins (Underhill and Ozinsky, 2002).

Antibody, complement molecules and certain types of lectin are present in the blood circulation bind to antigens to activate the opsonisation process. Once microbes or particles bind to receptors on macrophages, plasma membrane of the latter starts to redistribute, forming extensions known as pseudopodia, performing the cup-shaped projection around the microbes. When the engulfment process completes, both cup-shaped edges are pinched-off, trapping the microbes in intracellular vesicle or phagosome. Phagosome that contains the ingested microbes will break away from the plasma membrane and floating in the macrophage cytoplasm. At the exact time, cell surface receptors immediately deliver signals to activate the killing mechanism (Abbas *et al.*, 2012).

Activated macrophages kill microbes in phagosome via oxygen-independent and/or oxygen-independent reactions. Oxygen-independent mechanism involves the action of microbicidal molecules. The fusion between phagosomes and lysosomes will result most of microbicidal mechanisms. Phagolysosome contains proteolytic enzymes produced by macrophages are strong enough to destroy phagocytosed microbes (Abbas *et al.*, 2012). In oxygen-dependent mechanism, oxygen in cell cytoplasm is inadvertently converts into reactive oxygen species (ROS) such as hydrogen peroxides (H_2O_2) and superoxide (O_2^-), which are highly reactive oxidizing agents that can destroy a variety of biomolecules. In addition to production of ROS, macrophages also release reactive nitrogen intermediate such as nitric oxide to kill antigens (Slauch, 2011).

In tumour microenvironment, macrophages are not able to perform a direct programmed cell removal (PrCR) on the living tumour cells. PrCR is a process of recognising and performing phagocytosis on target cells by macrophages-mediated

immunesurveillance (Chao *et al*., 2012). During cancer development, the antiphagocytic molecule, CD47 was highly expressed and protects cancer cells from being phagocytosed. However, the PrCR is commonly active in eliminating apoptotic cells, including apoptotic tumour cells due to the absence of CD47 molecule (Jaiswal *et al*., 2009). Thus, the action of phagocytosis by macrophages is mainly to eliminate apoptotic tumour cells, with the same elimination process to microbes.

Nitric oxide and inducible nitric oxide synthase

Inducible nitric oxide synthase (iNOS) is commonly absent in resting macrophage. However, iNOS can be induced in response to microbial components binding to Toll-like receptors (TLRs). Binding of the receptor leads to the activation of nuclear factor kappa B (NF-κB) signalling pathway, which allows translocation of NF-κB heterodimer into the nucleus. The binding of heterodimer subunits to a specific DNA motif modulates a transcription of several targeted genes, especially pro-inflammatory mediator such iNOS, cyclooxygenase-2 (COX-2) and various of pro-inflammatory cytokines (Chao *et al*., 2010). Nitric oxide (NO) is a by-product of enzymatic conversion reaction of arginine to citrulline, catalysed by iNOS during macrophages activation. In addition to ROS, this reactive nitrogen intermediate possesses a prominent killing activity which functions as a potent microbicidal component to destroy phagocytosed antigens or microbes. During phagocytosis, NO is released within the phagosome and combines with H_2O_2 or O_2^-, which is generated by phagocyte peroxidase, to produce highly reactive peroxynitrite radical ($ONOO^-$) that powerful enough to kill microbes.

Macrophages and some other related immune cells are strongly activated during acute inflammation and generated higher level of NO in the microenvironment. As a

result, this highly toxic and powerful killing compound could possibly injure host normal tissues as well, because of its inability to distinguish between infected and normal tissues. In another cases, it was reported that over production of NO could damage DNA in the tissues and lead to the promotion of cancer (Hofseth *et al*., 2003). Thus, only appropriate level of NO is necessary to be produced in the infected location to kill cancer cells. On the other hand, many types of tumour cells produce its own NO at sub-micromolar level to support the survival mechanism including cell proliferation, migration and metastases. However, the exposure of NO at relatively high level (micromolar range) produced by activated macrophages can be fatal to the tumour cells (Fahey *et al.*, 2015).

1.2.2 Adaptive immune responses

A. T cells

T cell or T lymphocyte is an effector cell that is majorly involve in adaptive immune response. T cells mature in the thymus, circulate in the blood, populate in the secondary lymphoid tissues and recruited to antigen-infected sites. The functional subsets of T cells include $CD4^+$ helper T cells (Th) and $CD8^+$ cytotoxic T cells. Matured T lymphocytes possess the ability to differentiate between self and foreign peptides, due to specificity of T cell receptor (TCR) to a single peptide, giving advantage of recognising only foreign peptides over self-peptides. TCR can only recognise peptide fragment presented by MHC molecules expressed on target cells or infected cells and this complex is stabilised by T cell CD coreceptors (Nguyen *et al*., 2013). CD8 protein that is expressed on CTLs binds to a constant region of MHC class I molecule. The binding of CTLs to the target cells will result in immediate cell death.

There are two main mechanisms of action of CTLs to the target cells. Upon contact with the infected cells, CTLs will immediately release their granules which contain with perforin and facilitate granzyme entry into the target cells, which in turn activates the apoptotic deaths signal, instructing the cells to self-destruct (Elliott and Elliott, 2009). CTL is one of the important immune components in reducing tumour burden by infiltrating into solid tumours. In many studies such as immunohistochemical and genes expression analyses performed on tumour tissues have shown the correlation between immune cells infiltration, especially CTLs with better prognosis (Zhang *et al*., 2003; Pages *et al*., 2010; Galon *et al*., 2014).

On the other hand, the MHC class II molecule is specifically presents an antigen peptide to the TCRs of Th cells and the interaction is stabilised by CD4 coreceptor. Depending on the cytokine produced, Th cells will differentiate into Th1 or Th2 subsets, activated by IL-12 or IL-4, respectively. Those activated Th cells trigger two discrete pathways. Th1 subset expresses transcription factor of T-bet genes that is responsible in high-level expression of cytokine IL-2, TNF-α, IFN-γ and induce production of IL-12 by APCs of innate immune response and activates $CD8^+$ T-lymphocytes (Szabo *et al*., 2000). The Th2 subset mediates humoral immune response by producing several cytokines such as IL-4, IL-5, IL6, IL-10, which are necessary for B cells differentiation, antibody production and involve in chronic inflammation (Nguyen *et al*., 2013). The knowledge of Th direct function towards anti-tumour immunity for solid tumour is still insufficient. However, the role of Th1 subset in secreting anti-tumour cytokines such as TNF-α and IFN-γ explains the indirect anti-tumour capability by Th cells in interacting with the other immune cells, especially CTLs. These cytokines help to up-regulate the expression of MHC class I molecule on tumour cells and increase the sensitivity towards CTLs (Abbas *et al.*, 2012). All in all, the major contribution of $CD4^+$ Th cells

in anti-tumour immunity is to support and improve CTLs efficacy towards tumour elimination process, which includes $CD8^+$ T-lymphocytes activation, proliferation, maintaining and augmenting accumulation of CTLs at the tumour site (Lai *et al.*, 2011).

B. Antibodies

Humoral immunity involves the productions of antibodies, which are soluble proteins circulating in bloodstream, responsible in identifying and binding to foreign antigens (Elliott and Elliott, 2009). B cells differentiate into plasma cells and produce antibody when activated. The binding of antibodies to antigens results effective elimination of extracellular antigens (Karp, 2008). An antibody has two identical binding sites, the Fab regions that can increase the cross-linking between molecules. Antibodies are able to bind to epitopes on antigen surface. Binding of antibodies to those epitopes function as 'tags' for phagocytes and other immune cells in blood circulation for better destruction mechanisms.

Tumour-bearing host produce antibodies in blood serum against various types of tumour antigens. There are several anti-tumour mechanisms that involve antibodies. These include the association of antibody with complement reaction and antibody-dependent cell-mediated cytotoxicity (ADCC) by NK cells or macrophages (Abbas *et al.*, 2012). The Fc domain of immunoglobulin-G (IgG) binds to Fc receptors (FcRs) expressed on macrophages and subsequently activates the cytotoxic activity against antigens and apoptotic tumour cells through phagocytosis. The opsonisation of IgG directly on the surface of tumour cells can initiate the complement cascade and leading to the formation membrane attack complex (MAC). The presence of MAC causes the formation of pores on tumour cells (Nguyen *et al*., 2013), culminating in cell lysis.

C. Cytokines

Cytokines are proteins secreted by wide variety of immune and non-immune cells, and are particularly known by many different names, such as interleukin and interferon. Cytokines production by immune cells occurs in both innate and adaptive immune responses, and play important roles in regulating the immune system function (Ramani *et al.*, 2015). Different cytokines stimulate diverse responses towards cells involved in immunity and inflammation. In the activation phase of adaptive immunity, cytokines stimulate the growth and differentiation of lymphocytes, while in the effector phase, cytokines provide a wide effector spectrums in killing mechanism for microbes or antigens for both adaptive and innate immune responses. Cytokines also stimulate the development of hematopoietic cells and became the important therapeutic agents for numerous immune and inflammatory diseases in clinical medicine (Abbas *et al.*, 2012).

In innate immunity, cytokines are mainly secreted by mononuclear phagocytes, in response to antigens and infectious agents. PAMPs such as LPS and viral double-stranded RNA (dsRNA) bind to TLRs expressed on cells surface or endosomes of macrophages, and the binding stimulates the production of important cytokines involved in killing mechanism afterwards. Some cytokines are also released by activated macrophages can in turn to activate NK cells and T cells. Cytokines such as tumour necrosis factor alpha (TNF-α), IL-1, IL-10, IL-12, IFN-α and IFN-γ are cytokines which mediate the innate immune response (Ramani *et al.*, 2015).

In the adaptive immune response, T lymphocytes mainly secrete cytokines such as transforming growth factor beta (TGF-β), IL-2, IL-4, IL-5, IL-10 and IFN-γ in response to recognition of foreign antigens (Ramani *et al.*, 2015). In the activation phase of T cells-dependent immune response, some cytokines are necessary in regulating

growth, cell development and differentiation for various lymphocytes population. While in the effector phase of this response, the function of cytokines is to activate, regulate and recruit effector cells such as mononuclear phagocytes, neutrophils and eosinophils to eliminate antigens and infectious agents (Abbas *et al.*, 2012).

Tumour necrosis factor

Tumour necrosis factor (TNF) is the principle cytokine involved in acute inflammation in response to microbial infection and is also responsible in any systemic complication of severe infections. TNF is usually undetectable in healthy individual, but the elevation of TNF levels could be observe in inflammatory and infectious condition (Robak *et al*., 1998; Nurnberger *et al*., 1995), depending on severity of infection (Waage *et al*., 1987). There are several isoforms of TNF and one of the isoforms is TNF-α. TNF-α is predominantly secreted by classically activated macrophages and mast cells. This cytokine is largely produced in conjunction to the signal triggered by LPS and TLR engagement. IFN-γ that is produced by T cells and NK cells helps in the augmentation of TNF level by LPS-stimulated macrophages. TNF-α itself can also be a regulator to produce another batch of TNF-α and other inflammatory mediators such as IL-6, COX-2 and NO, by activating TNF-signal transduction pathway through TNF-receptors (TNF-Rs)-1 and TNF-R2. Those receptors are commonly expressed by normal and infected tissues (Bradley, 2008).

Depending on severity of infection, over production of TNF-α could trigger a severe systemic inflammatory response called septic shock. In this condition, body temperature of the host will increase and blood pressure will reduce. If this condition persists, it may lead to death (Campbell and Reece, 2005). Overwhelming inflammatory responses may also lead to asthma, toxic shock syndrome, respiratory distress syndrome

and also rapid heart rate (Karp, 2008; Tortora *et al*., 2007). Moreover, continuous expression of COX-2 by TNF regulatory system could lead to over production of prostaglandin E2 (PGE2), a vasodilator resulting in vasodilation of epithelial tissue. The increase in vascular permeability allows the increase of trans-endothelial passage of fluid and macromolecules causing oedema (Mark *et al*., 2001). In addition, TNF induction could up-regulate the expression of procoagulant protein such as tissue factor and down-regulate anti-coagulant proteins such thrombomudilin, which can cause intravascular thrombosis (Bevilacqua *et al*., 1986).

The term of TNF was originally given at the time when it was discovered that the protein was observed to cause necrosis to tumour cells. It turned out that TNF-α not only induce necrosis, but also apoptosis to tumour cells. Most of cancer cells including BrCa express their TNF-R on the cells surface. Interaction between soluble TNF and its receptors triggers the death signal responses to be activated. TNF-R1 can activate the cells death mechanisms by two distinct pathways. First, TNF-R1 trigger necrosis signal mediated by receptor interacting protein (RIP) and TNF receptor-associate factor (TRAF)-2, leading to the production of ROS and subsequent prolonged activation of cJun N-terminal kinase (JNK) signal (Lin *et al*., 2004). Secondly, TNF-R1 activation can induce caspases-mediated apoptosis, which involves the TNF receptor-associate death domain (TRADD), Fas-associated death domain (FADD) and caspases-8 (Wajant *et al*., 2003) into the killing of cancer cells.

Interferon gamma

The cytokine interferon gamma (IFN-γ) belongs in the family of interferon, which possesses the ability to protect cells from viral infection. Although IFN-γ belongs to type-II interferon, the biological responses are lower in specificity as compared to the

other type-I classical interferon such as IFN-α and -β. However, IFN-γ exerts more immunomodulatory properties than the type-I interferon (Farrar and Schreiber, 1993). The expression of IFN-γ is related to Th1 activity since these cells are the excellent producer of interferon (Teixeira *et al*., 2005). IFN-γ production is regulated by cytokines, produced by APCs, most notably IL-12 and IL-18. Classically activated macrophages produce IL-12 and attract NK cells to the inflammation sites and promote IFN-γ production by NK cells (Schroder *et al*., 2004). In inflammation, IFN-γ does not play many roles in direct killing of infiltrated pathogens. Instead, it helps to activate predominant T cells and macrophages to eliminate intracellular pathogen.

Other than macrophages, IL-12 is also secreted by other APCs such as dendritic cells (DCs) when in contact with matured $CD4^+$ T cells for the first time (Snijders *et al*., 1998). In relation to adaptive immune response, IFN-γ secretion is the principle of Th1 effector function, and it has a crucial role in naïve helper T cells (Th0) differentiation towards Th1 phenotype, which is important in eliminating persistent pathogen. In the opposite direction, IFN-γ is able to exert direct inhibitory effects on Th2 action, which mainly involves in inhibiting inflammation by producing anti-inflammatory cytokines such as IL-4 and IL-5 through activation of T-bet proteins, Th2-supressing transcription factor in IFN-γ signalling pathway (Afkarian *et al*., 2002; Lighvani *et al*., 2001). In fact, the expression of T-bet in ectopic manner could restrain the production of IL-4 and IL-5 (Szabo *et al.,* 2000). However, the balance between Th1/Th2 levels in the system is extremely important to provide appropriate responses towards infection. Massive production of Th1 cytokines can implicate organ-specific autoimmune diseases such as arthritis and multiple sclerosis. Meanwhile, overexpression of Th2 can cause severe allergic disorders (Kidd, 2003).

Decades ago, additional role of IFN-γ became widely recognised in preventing tumour development with consensus that IFN-γ is able to promote host immune responses. As mentioned earlier, IFN-γ involves in tumour inhibition by proxy in both innate and adaptive immune responses, through the production of killing mediators such as TNF-α, NO and PGE2, and the regulation of Th subsets. IFN-γ can also be directly involved in tumour elimination. It has been observed in several tumour cells, including BrCa cells. The activation of signal transduction through IFN-γ receptor (IFNγ-R) expressed mostly on BrCa cells (Garcia-Tunon *et al*., 2007), induces the anti-proliferative effect of the cancer cells by down-regulating the expression of anti-apoptosis Bcl-2 family (Zhang *et al*., 2003).

Interleukin-4

Interleukin-4 (IL-4) is a pleiotropic cytokine, which can exert multiple biological activities on various cells. It is needed by Th0 to differentiate into Th2 cells. Upon activation by IL-4, Th2 starts to produce IL-4 in a positive feedback loop. This cytokine was first identified as inducer for both B cells proliferation and differentiation, and T cells regulation (Howad *et al*., 1982; Yokota *et al*., 1988). In addition, IL-4 was reported to induce resting B cells to enhance the MHC class II expression and low-affinity receptor for the Fc portion of IgE (Fc-ƐR). Although IL-4 actively involves in the activation of adaptive immune response, it cause the opposite action of IFN-γ responses on macrophages where it induces the production of alternatively activated macrophages and inhibit the cell classical activation. Alternatively activated macrophages mainly involve in tissues repair and wound healing (Obeid *et al*., 2013).

Previously, the transfection of IL-4 gene in cancer cell lines and treatment of tumour cells with IL-4 have suggested that IL-4 has potent anti-tumour properties

towards several types of tumour, including BrCa. Studies elucidated the mechanism of tumour inhibition by IL-4 was through apoptosis induction in several BrCa cell lines (Nagai and Toi, 2000). Direct interaction between BrCa and IL-4 was facilitated by the affinity of IL-4 to its receptor, IL-4R that is expressed on most human BrCa cells. It was reported that human BrCa inhibition and apoptosis is mediated via signal transducer and activator of transcription 6 (STAT6) signalling pathway by IL-4 (Gooch *et al*., 2002) through its receptor.

Interleukin-6

Interleukin-6 (IL-6) is a soluble cytokine that pleiotropically involves in both innate and adaptive immune responses. It is mainly synthesised by classically activated macrophages and several other cell types such as vascular endothelial cells, fibroblasts and also other cells that are capable of interacting with microbial components. Some activated T cells also generate IL-6. In inflammation, IL-6 works as warning signals generator and transmits to the entire body system. These signals are mediated when pathogen recognition receptors (PRRs) expressed by macrophages bind to PAMPs that is exclusively presented by the pathogen (Tanaka *et al*., 2014). The interaction stimulates a range of signal transduction involving NF-κB, thus enhances the transcription of mRNA inflammatory cytokines such as TNF-α, IL-1β, and these two cytokines also further activate transcription factor to produce more IL-6.

In order for IL-6 to mediate signal transduction, it has to associate with its specific receptor, IL-6R that is expressed by various types of cells, either on normal or neoplastic cells. Activation of IL-6 signalling pathway triggers several downstream signalling molecules such as JAK-STAT3 and JAK-SHP-2-MAPK pathways. The complete signalling activation regulates various sets of IL-6 responsive genes, including

acute phase protein and transcription factor of signal transducer and activator of transcription 3 (STAT3). Other than that, suppressor of cytokines signalling (SOCS)-1 and -3 are also produced to stop IL-6 signalling in negative feedback loop (Naka *et al*., 1997; Schmitz *et al*., 2000). This cytokine possesses multiple diverse functions. In innate immunity, IL-6 stimulates the production of acute-phase protein, a type of plasma protein involved in acute inflammation at infection site that contributes to the acute phase response. To extend the inflammation process, IL-6 also contributes its function in the intermediate phase changes from acute to chronic inflammation (Gabay, 2006). In adaptive immunity, IL-6 facilitates the development and differentiation of B-lymphocytes into antibody-producer, and as well as T cells differentiation and proliferation. Moreover, IL-6 is also involved in the promotion of cell-mediated immune reactions by stimulating some other pro-inflammatory cytokines. Closely similar to the other inflammatory cytokines, over production of IL-6 in the system may contribute to chronic inflammation and autoimmunity (Hirano *et al*., 1987).

It was currently reported that IL-6 possesses detrimental effects where its action is frequently associated with tumour growth and progression. The elevation of IL-6 level in the serum correlates with poor prognosis in cancer patients (Gao *et al*., 2007; Sansone *et al.*, 2007). The activation of IL-6 signalling pathway further activates JAK tyrosine kinase, leading to the activation of STAT3, which is responsible in cancer growth and differentiation in various cancer types including BrCa (Sansone *et al*., 2007). IL-6 can also induce signalling of Ras, MAPK, Cox-2 and PI3K/Akt pathways, explaining the tumorigenic and anti-apoptotic activities of this cytokine **(**Guo *et al*., 2012**)**.

1.3 Immunomodulation

Immunomodulation refers to any modification towards the immune responses. This modulation can either leads to induction, expression, amplification or inhibition of any part or phase of immune responses (Saroj *et al*., 2012). In the simpler definition, immunomodulation can be defined as an alteration of immune responses by modifying and regulating the immune system, involving both stimulation and suppression of host's immune responses. Currently, immunomodulation or immunotherapy gains major attention especially in immunopharmacology discipline, as an alternative approach in finding promising medications for immune-associated diseases. For example, amplification of immune responses is desirable to avoid infection in a state of immunodeficiency such as in cancer. On the other hand, an immunosuppression method is necessary to counter an over production of immune responses which leads to autoimmune disease and allergy reaction (Gea-Banacloche, 2006).

Biological or non-biological compounds that are used to modify the immune system responses are called immunomodulators (Sarma and Khosa, 1994), which refer to medications or agents that possess immunomodulatory properties. In clinical practice, there are several classifications of immunomodulator, depending on their effect to the immune responses, such as immunosuppression, immunostimulant and immunoadjuvant (El-Enshasy and Hatti-Kaul, 2013). The demand of immunomodulators is rapidly increasing due to worldwide ranging medical application for stimulation and suppression of immune system to treat immune-associated diseases (GBI Research, 2012). The search for powerful and effective, yet safe immunomodulators, especially in clinical perspective are still on-going and become the main objective for many researchers since immune system plays a fundamental role in immune-associated diseases. Immunostimulants are agents responsible for augmenting host immune

responses against foreign pathogens. Immunostimulants usually act non-specifically, and are able to elevate either innate, adaptive immunities or both simultaneously (Billiau and Matthys, 2001; Brunton *et al*., 2011). In healthy individuals, these stimulating agents are expected to serve as immunopotentiators to enhance the basic level of immune responses (Saroj *et al*., 2012). The enhancement of immune responses is important when dealing with certain diseases caused by infection, immunodeficiency and as well as cancer (Agarwal and Singh, 1999). Some examples of drug-based immunostimulants to treat immune-associated diseases are listed in Table 1.2. However, the implication of using those drugs has created dilemmas towards patients as it could exert other health difficulties after the treatment. Thus, the search of natural-based immunostimulants is necessary to discover potential agents that not just only to cure diseases, but implicates minimal side effects to the host.

Table 1.2: Example of immunomodulators for medication of immune-associated diseases

Immunomodulator	Therapeutic use	Adverse effect
Bacillus Calmette-Guerin (BCG)	Attenuated live culture from bacillus and mycobacterium strain for induction granulomas reaction to treat urinary bladder cancer.	Hypersensitivity, shock, chill, fever, malaise and immune-complex diseases (Sharma and Sharma, 2007)
Levamisole	Anti-helminthic agent used as an adjuvant to 5-fluorouracil after surgical resection with Duke's stage of colon cancer. This agent was capable to restore depressed immune function of B and T lymphocytes, monocytes and also macrophages (Sharma and Sharma, 2007).	Flu-like syndrome, allergic manifestation, nausea and muscle pain (Shaha *et al.*, 2011)
Isoprinosine	A complex of pacetamicobenzoate salt and inosine for augmentation of IL-1, IL-2 and IFN-γ cytokines production, increases proliferation of lymphocytes in response to mitogenic and antigenic stimuli and induces T cells surface marker on prothymocytes (Patil *et al.,* 2012).	Minor central nervous system depressant, transient nausea and rise of uric acid in serum and urine (Parnham and Nijkamp, 2005)
Immunocynin	A stable form of haemocynin, a non-heame cooper-associated protein found in anthropod and molluses that is used to treat urinary bladder cancer.	Rare-mild fever (Parnham and Nijkamp, 2005)
Aldesleukin	A recombinant interleukin that is able to activate lymphocyte and initiate production of multiple cytokines (TNF-α, IL-1 and IFN-γ). This agent was used overcome metastatic renal carcinoma and melanoma.	Capillary-leak syndrome, hypotension, reduced organ perfusion and death (Patil *et al*., 2012)
Filgrastin (r-metHuG-Colony stimulating factor)	An agent that can induces the number and differentiation of myeloid progenitors to treat leukopenia and neutropenia.	Mycocardial infraction and anorexia (Patil *et al*., 2012)

1.4 Immunomodulator derived from natural product

1.4.1 Plant-derived immunomodulators

Researchers, especially pharmacologist, express their interest in searching and developing alternative remedies to cure diseases. Without any doubt, the current available drugs, especially for cancer treatment indeed cause side effects and lower patients' quality of life (Palumbo *et al*., 2013). Up until the year of 2013, the World Health Organisation estimated approximately 80% of human populations are still depending on plants medication for primary healthcare and believe it can continuously provide mankind with plenty of new remedies. Approximately 10,000 of plant species were reported for their medicinal values (McChesney *et al*., 2007), and still retain their significant roles as natural resources of the most effective remedies for the new generation of medicines (Itokawa *et al*., 2008).

A number of immunomodulators originated from plants have been discovered (Sarma and Khosa, 1994). Although some plant immunomodulators were well studied (Table 1.3), the in depth researches and evaluations of their immunomodulator property especially on their mechanisms at the molecular level and toxicity are still on-going.

Table 1.3: Example of plant-based immunomodulators

Plant	Phytoconstituent	Part used	Immumomodulatory activity
Boswellia carterii	Boswellic acid	Barks	Alteration of Th1 and Th2 cytokines production by murine splenocytes (Chevrier *et al.*, 2005)
Eclipta alba	Eclalbatin	Whole plant	Increase the phagocytic index and antibody titer (Kumar *et al*., 2011)
Epilobium angustifolium	Oenothain B	Flowers	Activate functional response of neutrophils and macrophages, *in vitro*, and induce keratinocytes and neutrophils recruitment, *in vivo* (Schepetkin *et al.*, 2009)
Heracleum nepalense	Quercetin glycoside	Roots	Increase the *in vitro* phagocytic index and lymphocytes viability, and increase antibody titer in mice (Dash *et al.*, 2006)
Curcuma longa	Curcumin	Rhizomes	Modulate both proliferation and activation of T cells from concanavalin A-treated human splenocytes (Ranjan *et al.*, 2004)
Panax ginseng	Ginsan	Roots	Enhance the production of cytokines and ROS by macrophages, and stimulate phagocytosis activity of macrophages (Song *et al*., 2002)
Luffa cylindrica	Echinocystic acid	Fruits	Enhance phagocytic index of macrophages in humoral and cell-mediated immune responses, *in vivo* (Khajuria *et al*., 2007)

1.4.2 Polysaccharides

Polysaccharide is an important complex macromolecule, which plays critical roles in various biological functions. The functions are closely related to its structure, which is made up of combination between monosaccharide units and their derivatives, and linked together as a polymer. The stability and variety of polysaccharides depend on the glycosidic linkage between the monosaccharides, which structurally can be distinguished between polysaccharide types. Specific polysaccharide structure features can be classified and recognised through (1) the monosaccharide composition, (2) type of glycosidic linkages, (3) length of polymer chain, and (5) the degree of branching (Boyer, 1999). Polysaccharides such as cellulose and chitin are naturally produced polymer which can be found abundantly in nature as plant structural building block (Navard and Navard, 2012). Both cellulose and chitin are the common matrixes of plants (Keegstra, 2010) and fungi (Bowman and Free, 2006) cell walls. These structural polysaccharides are synthesised intracellularly, but extruded out of the cell to provide a protection layer to the intracellular components (Boyer, 1999). The strong and rigid networks of these glycans serve not only as a protection layer, but also create cell to cell communication with the other biological molecules, including cellular interaction to infection (Kiessling and Grim, 2013).

1.4.3 Plant polysaccharides

Plant polysaccharides can be found abundantly in the cell wall of the plant. The structural type of polysaccharide was largely comprised of cellulose, hemicellulose and pectin with approximately less than 10% proteins and around 40% of lignin. These polymers covalently and non-covalently interact to form a functional cell wall (Tan *et al*., 2013). The important features of plant cell walls include providing shape to the cell and act as an intracellular communicator between cells. Moreover, the surface location

of the plant cell walls plays crucial roles for plants-pathogens interaction, including first layer protection from potential pathogens (Keegstra, 2010).

In these recent years, the development in medicinal natural product researches starts to focus on plant polysaccharides. This is due to the diversity of resources, non-toxicity, biodegradability and biocompatibility with capability of undergoing chemical modifications (Li *et al*., 2015). Some bioactive polysaccharides derived from natural sources including herbs, higher plants and fungi are becoming an attraction into biochemistry and pharmacology fields because they have shown diverse biological properties such as wound healing, reticuloendothelial system induction and tumour cure. Moreover, polysaccharides extracted from some higher plants are greatly used as hypoglycemic (Lopez, 2007; Jing *et al.*, 2009) and immunotheraphy agents (Haller, 1990; Atherton, 2002).

1.4.4 Plant polysaccharides as an immunomodulator

Referring to the Scopus Database, up until year of 2014, there are approximately 9004 research findings documenting on immunological potential of polysaccharides with majority originated from microbial resources (Nie *et al*., 2013; Campo *et al*., 2009**).** A good number of researches involving plant polysaccharides interaction with immune system cells including humoral immunity have also been reported. The immunostimulatory polysaccharides could either interact directly or indirectly with immune components, which later trigger several molecular or cellular responses resulting in activation of immune system (Leung *et al*., 2006). Commonly, the innate immune response, which comprises of monocytes, macrophages and neutrophils is the main system responding to the polysaccharides. Among these immune cells, macrophage is the most common model used to study the immunomodulatory properties

of polysaccharides, *in vitro*, where the enhancement of macrophage functions could be measured (Schepetkin and Quinn, 2006). There are plenty of studies reporting on the immunomodulatory properties on dietary polysaccharides, especially on animal model and clinical practice (Table 1.4). The effects of taking polysaccharide on immune responses are diverse, depending on the type and origin of the polysaccharide (Ramberg *et al*., 2010). The responses may reflect the diversity of polysaccharides chemically and structurally.

Table 1.4: Plant and fungal based polysaccharides with immunomodulatory properties

Source	Test subject	Duration	Treatment method	Significant effects	Reference
Grifola fondosa	ICR mice*	36 weeks	Oral administration	Reduce in tumour weight; increase in peritoneal macrophage chemotactic activity	Kurashige *et al*., 1997
Chlorophytum borivilianum (root)	Wistar rats*	14 days	Oral administration	Augment NK cells activity; improve IgG function	Thakur *et al*., 2011
Lepista sordida	Nude mice*	14 days	Oral administration	Suppress tumour weight and tumour volume	Miao *et al*., 2013
Punica granatum (fruit)	BALB/c mice*	14 days	*i.p.* injection	Induce apoptosis in cancer cell lines, reduce tumour burden	Joseph *et al*., 2013
Actinidia eriantha (roots)	ICR mice*	10 days	*i.p.* injection	Inhibit tumour growth by promoting splenocytes, NK cells and IL-2 production	Xu *et al*., 2009
Brassica napus L.	SPF Kumning mice*	10 days	Oral administration	Decrease tumour formation; increase relative spleen and thymus indexes; up-regulate phagocytic function and lymphocyte proliferation	Yang *et al*., 2007

*Tumour induction by using cancerous cell line inoculation method. *i.p.* = intraperitoneal.

1.5 *Solanum nigrum*

1.5.1 Distribution

Solanum nigrum belongs to a *Solanaceae* family under the genus of *Solanum*. The species of *nigrum* L. is the largest and most variable species group among other species in *Solanum* genus. *S. nigrum* or commonly known as black nightshade is recognised as a worldwide weed of arable lands, garden, rubbish tips and nitrogen rich soils (Edmonds and Chweya, 1997). It is widely distributed throughout temperate climate zones to tropical region of Asia (Huang *et al*., 2010) and Southern hemisphere, from sea level to altitudes over 3500 meters. This weed is mostly found in moist habitats and in different kind of soils, including dry, stony, shallow and deep soils. Although this plant is largely distributed in Europe and Asia regions, it is not continently spread, instead it was introduced in North America, Australia, New Zealand, Indonesia and Malaysia (Edmonds and Chweya, 1997). In Malaysia, *S. nigrum* L. subspecies *nigrum* (Figure 1.1) is more popular with the name of *Sayur Ranti,* and is commonly consumed as vegetable especially by Indian and Javanese in their daily diet.

1.5.2 Physical characteristic

The subspecies of *S. nigrum* L. *nigrum* is not very much different with the other subspecies. It was physically recognised with entirely green, simple petiolated and occasionally rhomboidal-shaped leaves. The stems are usually terete and entirely covered with sub-glabrous to pubescent hair. This pedicellate plant is commonly flowers, with broadly triangular to ovate-lancolate white sepal, prior to the fruits production. Matured fruits are usually black or dark purple, while the immature fruits are white-green in colour. The fruits are independently petiolate from stems and contained many seeds in its succulent (Edmonds and Chweya, 1997).

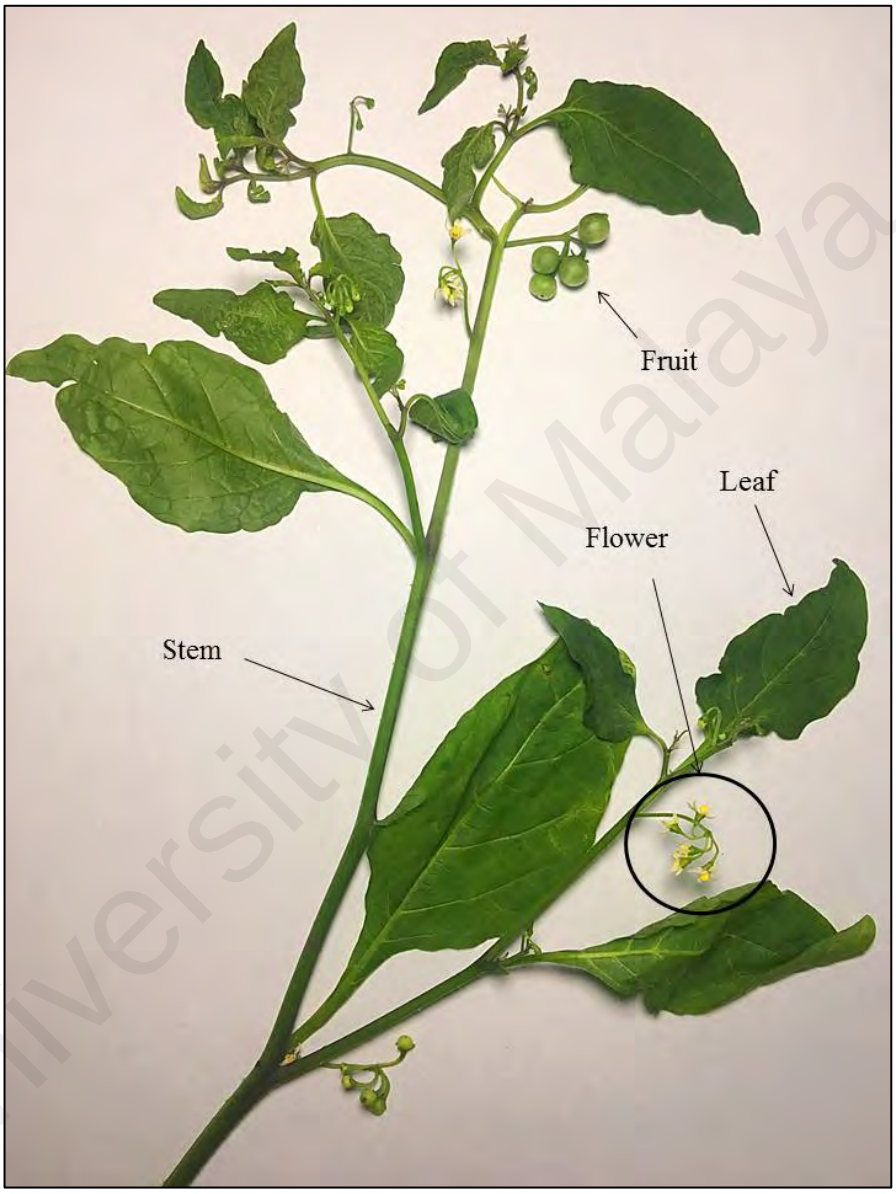

Figure 1.1: *Solanum nigrum* L. *nigrum*

1.5.3 Medicinal values

S. nigrum is usually referred as an herbal plant that has been used traditionally as folk medicine and is believed to have various biological activities (Huang *et al*., 2010). According on folkloric belief, this plant possesses multiple benefits especially to human health (Jain *et al*., 2011). However, the practices and applications of this plant are different, depending on each geographical region. In India, the alcohol extract of *S. nigrum* leaves is used as a traditional remedy for anti-septic and anti-dysentric agent due to its effectiveness against *Staphylococcus aureus* and *Escherichia coli* infection. In China, the dried leaves are used to treat hypertension and urinary system infection. Fresh leaves are also effective for wound healing. Having the same application in China, people in Hawaii traditionally use this plant to treat respiratory tract disorder, wound healing, skin eruption and relieve of abdominal and urinary bladder pain. Aside from leaves, people in East Africa consume the raw fruits of *S. nigrum* for stomach ulcer and continuous general abdominal upset relieve. Various parts of this plant are also believed to be able to cure malaria and black fever (Edmonds and Chweya, 1997). As reported by Sikdar and Dutta (2008), *S. nigrum* contains major classes of bioactive compounds such as glycoalkaloids, glycoproteins, and polysaccharide. It also contains polyphenolic compounds such as gallic acid, catechin, protocat-echuic acid, caffeic acid, rutin and narigenin, which can contribute to diverse biological activities in curing various diseases.

1.5.4 Anti-cancer activity

As the knowledge in science and technology expands, many scientific explanations and reports were documented, thus strengthening the folklore belief on the application of *S. nigrum* in curing multiple diseases including cancer (Jain *et al*., 2011). In the recent years, pharmacologists started to gain interest on this plant extracts in the

search for potent but safe anti-cancer or anti-tumour agents. Several documented studies proved that the crude extract of *S. nigrum* was able to induce necrosis in SC-M1 stomach cancer cells (Akhtar *et al*., 1989), had an anti-neoplastic activity against several human cancer cell lines (Hu *et al*., 1999), suppressed free radical-mediated DNA damaged (Sultana *et al*., 1995) and inhibited the molecules of acetate-induced tumour production in MCF-7 cell line (Heo *et al*., 2004). With reference to glycoalkaloids and polysaccharide, certain phytochemicals such solanine, solasonine, solamargine and chaconine were identified and present in almost all parts of *S. nigrum* plant and believed to be responsible in inhibiting the proliferation of cancer cells (Jain *et al*., 2011). Moreover, polysaccharide fraction extracted from *S. nigrum* L. possessed anti-cancer properties by significantly enhancing $CD4^+/CD8^+$ ratio of T cells subpopulation (Li *et al*., 2010) of cervical tumour-bearing mice. The polysaccharides did not exert a direct killing effect on the cancer cells, but through its ability to improve host's immune responses. Thus, this research suggested that the polysaccharide extracted from *S. nigrum* L. *nigrum* possessed an immunomodulatory activity, which could act as a proxy in fighting against cancer.

1.6 Research objectives and rationale of the study

As addressed earlier, the ability of polysaccharide isolated from *S. nigrum* L. to inhibit cancer progression has been documented. An *in vivo* study on cervical cancer had shown that the tumour growth was inhibited due to the improvement of $CD4^+/CD8^+$ ratio of T lymphocytes subpopulation. However, since the immune response is a very complex system, the cancer inhibition most probably not limited to the improvement of T lymphocytes activity only. It is possible that immune responses synergistically involved in fighting against cancer once activated by the polysaccharide. The anti-cancer activity of *S. nigrum* L. polysaccharide, if identified, provides a better

understanding on the mechanisms of cancer inhibition through the improvement of host's immune responses.

The main aim of this study is to highlight the mechanisms of anti-cancer activity by the polysaccharide extracted from *S. nigrum* L. *nigrum* on breast tumour model. The *in vitro* analyses of this study were carried out to emphasize the immunomodulatory activity of *S. nigrum* L. *nigrum* polysaccharide and its ability to enhance macrophages function. As for the *in vivo* analyses, focus on the ability of the polysaccharide to inhibit breast tumour growth was analysed, as well as elucidating its mechanisms of action.

The specifics objectives for this study were:

a) To identify the polysaccharides fractions of *S. nigrum* L. with immunomodulatory activity.
b) To identify the chemical properties of *S. nigrum* L. polysaccharide fraction
c) To outline the mechanisms on how *S. nigrum* L. polysaccharide fraction could activate RAW 264.7 macrophage cell line.
d) To highlight the possible mechanisms on how *S. nigrum* L. polysaccharide could inhibit breast tumour growth on tumour-bearing mice.

CHAPTER 2: MATERIALS AND METHODS

2.1 Materials

2.1.1 *Solanum nigrum*

Fresh whole plants of *Solanum nigrum* L. *nigrum* were purchased from the local market in Lembah Pantai, Kuala Lumpur, Malaysia in 2012. The plants were identified and authenticated by Dr. Sugumaran Manickam from Institute of Biological Sciences, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia and a voucher specimen was deposited at the Rimba Ilmu Herbarium (Herbarium number: KLU 47872).

2.1.2 Cell lines

Murine macrophage cell line, RAW 264.7 was purchased from American Type Culture Collection (Manassas, VA, USA) and mouse mammary carcinoma cell line, 4T1, was obtained from Jeffery Cheah School of Medicine and Health Sciences, Monash University, Sunway Campus, Malaysia. Both cell lines were maintained in Dulbecco's Modified Eagle Medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum, 2% of 100× penicillin/streptomycin and 1% of 100× amphotericin B (PAA Laboratories, Colbe, Germany). Cells were cultured in a 5% CO_2 and 100% humidified incubator (CelCulture CO_2, Esco Technologies, Hatboro, PA, USA) at 37°C, and cultures were passaged every 2 or 3 days, using accutase solution (PAA Laboratories) as detachment agent.

2.1.3 Animal subjects

Female inbred BALB/c mice (7–8 weeks old) weighing 18–22 g were obtained from Monash University, Sunway Campus, Malaysia and were acclimatised for 14 days before the experiment was carried out. The mice were housed under the controlled

environmental conditions of temperature 25 ± 1°C and 12/12 light/dark cycle and supplied with standard food pellets and tap water, *ad libitum*. Animal care, research and animal sacrificed protocols were in accordance to the principles and guidelines approved by the University of Malaya Institutional Animal Care and Use Committee (ISB/29/06/2014/FNR-R).

2.2 Methods

2.2.1 Extraction of crude polysaccharide from *S. nigrum*

S. nigrum polysaccharides were extracted according to the method as described by Li *et al*., (2009). Briefly, the whole plant, comprising of leaves, stems and fruits, leaves and stems part of *S. nigrum* were washed with tap water, and then dried at 45°C, and ground to a fine powder. To extract the polysaccharides, 300 g of the *S. nigrum* powder was refluxed using a Soxhlet apparatus with 2 L of petroleum ether (60°C–80°C) to remove waxes, fats, and volatile oils from the sample (Houghton and Raman, 1998). Then the sample was refluxed again with 2 L of 80% ethanol to remove monosaccharides and oligosaccharides. The residue was then boiled in 2 L of 95°C water for 5 hours before it was filtered through Whatman no. 3 filter paper. The filtrate was then purified in equal volume of 70% ethanol, allowing the polysaccharide to precipitate overnight at 4°C. The precipitate was collected by centrifugation at 4,000 ×*g* in 4°C for 20 minutes and then washed twice with 95% ethanol. The *S. nigrum* crude polysaccharide pellet was allowed to dry in a desiccator for 7 days before it was stored at 4°C for further use.

2.2.2 Cytotoxic evaluation of crude extract and polysaccharide fractions

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide **(**MTT) assay is one of the common assays used to determine cell viability, proliferation or cytotoxicity.

This assay is based on the theory that active mitochondria of viable cells can reduce MTT into purple formazan crystal (Mosmann, 1983). In this assay, the cytotoxicity of crude extract and the polysaccharide fractions of *S. nigrum* were evaluated using MTT cell proliferation assay against RAW 264.7 cells. Briefly, RAW 264.7 cells (5×10^5 cells/mL) were seeded in a 96-wells culture plate (Orange Scientific, Braine-I'Alleud, Belgium) and incubated for 24 hours. The cells were then treated with polysaccharide samples at sample concentration of 0, 1.56, 3.125, 6.25, 12.5, 25, 50, and 100 µg/mL for 72 hours. To observe cell viability, a 20 µL of MTT solution was added to each well and incubated for another 4 hours. The purple formazan that developed was diluted with 200 µL of dimethyl sulfoxide, and the absorbance values at 570 nm and 690 nm were measured by Multiskan Go microplate spectrophotometer (Thermo Scientific, Tewksbury, MA, USA). The cytotoxicity for each sample was expressed as the IC_{50}, which is the concentration of each extract that reduces cell survival by 50% when compared with the non-treated control. A sample which has IC_{50} value less than 30 µg/mL is deemed actively cytotoxic (Stuffness and Pezzuto, 1990).

2.2.3 Purification of *S. nigrum* polysaccharide

To further purify the crude polysaccharide extract, it was subjected to ion-exchange chromatography column. Diethylaminoethyl (DEAE) cellulose ion-exchanger resin was pre-swollen with 50 mM sodium phosphate buffer, pH 7.4, equilibrated with 5 mM sodium phosphate buffer, pH 7.4 and packed into a glass column (ø20 mm × 250 mm). The crude polysaccharide (300 mg) was dissolved in 5 mL of 5 mM sodium phosphate buffer, pH 7.4 and centrifuged at 4,000 ×*g* in 4°C for 20 minutes before it was loaded into the column. The filtrate was eluted with a linear gradient of 0–1.5 M sodium chloride in 5 mM sodium phosphate buffer. Two-hundred fractions were collected in 5 mL aliquots using a fraction collector. The polysaccharide content of each

fraction was determined by the phenol sulphuric acid method described in Section 2.2.4. Based on the chromatographic profile, the apex fractions of observed peak were pooled and dialysed four times using SnakeSkin Pleated Dialysis Tubing (Thermo Scientific) against 1 L distilled water. Finally, the fractions were freeze dried and stored at -20°C.

2.2.4 Measurement of carbohydrate content

Carbohydrate content in the samples can be detected using phenol sulphuric assay. The concentrated sulphuric acid dehydrates sugar into furfural derivative and forming yellow-orange colour complex after reacts with phenol. Absorbance of the yellow-orange colour complex can be measured using spectrophotometer at 490 nm (Masuko *et al*., 2005). Briefly, 50 µL of 100 mg/mL sample was pipetted into a 96-wells plate (Orange Scientific). Then, 150 µL of absolute sulphuric acid was added into each well, followed immediately by 30 µL of 5% w/v phenol in distilled water. The plate was heated in a 95°C water bath for 10 minutes. The optical density of the samples (490 nm) was measured by Multiskan Go microplate spectrophotometer (Thermo Scientific). The carbohydrate concentration was determined based on standard curve of D-glucose (Appendix C1) (Chen *et al.,* 2010).

2.2.5 Measurement of protein content

The protein content of the samples was quantified using Bradford Protein Quantification Kit. Bradford assay is a protein determination method that involves the binding of Bradford reagent (Coomassie brilliant blue G-250 dye) to protein. When the dye binds to protein, it is converted to a stable unprotonated blue solution that can be measured using spectrophotometer at 595 nm (Bradford, 1976). In this assay, 10 µL of 100 mg/mL sample was pipetted into 96-wells plate (Orange Scientific) and followed by addition of 200 µL Bradford reagent and incubated in a dark condition for 10 minutes.

The optical density of the samples (595 nm) was measured by Multiskan Go microplate spectrophotometer (Thermo Scientific). The protein concentration was determined based on bovine serum albumin standard curve (Appendix C2).

2.2.6 Measurement of nitric oxide production

The presence of nitric oxide (NO) in a culture medium can be detected by measuring nitrite (NO_2^-) by treating the medium with Griess reagent, which consists of sulphanilamide and N-1-napthylethylenediamine dihydrochloride (NED). The reaction of NO_2^- with sulphanilamide under acidic condition results a formation of diazonium cation and produce red violet azo dye after reacted with NED, which can be spectrophotometrically measured at 520 nm (Yucel *et al*., 2012). In this assay, RAW 264.7 cells (5×10^5 cells/mL) were seeded into a 96-wells culture plate and treated with the polysaccharide fractions at the sample concentrations of 12.5, 25, 50, and 100 µg/mL for 24 hours. The positive control used was lipopolysaccharide (LPS) at 100 µg/mL. After the treatment, 100 µL of treated culture media were pipetted into a new 96-wells microplate. Then, 50 µL of sulphanilamide solution was dispensed into all experimental samples and the plate was incubated in the dark for 5–10 minutes at room temperature. After that, 50 µL of NED solution was dispensed to all experimental samples and the plate was incubated in a dark condition for another 5–10 minutes at room temperature. The optical density at 520 nm of the samples was measured using Multiskan Go microplate spectrophotometer (Thermo Scientific). The NO concentration was determined based on the standard curve of 100 µM of nitrite solution (Appendix C3).

2.2.7 Detection of inducible nitric oxide synthase by Western blotting

A. Cell lysate preparation

Based on the highest induction of NO production (Section 2.2.6), only *S. nigrum* polysaccharide fraction number 3, SN-ppF3 was chosen for the inducible nitric oxide synthase (iNOS) detection assay. To assess polysaccharide-induced iNOS expression, RAW 264.7 cells (5×10^5 cells/mL) were seeded in a six-well cell culture plate and treated with polysaccharide fraction SN-ppF3 at 100 µg/mL for 24 hours. LPS was used as a positive control at the same concentration. The cells were harvested using a scraper, washed with 10 mM Tris-sucrose buffer, pH 7.0 and centrifuged at 1,000 ×*g* at room temperature for 5 minutes. The cells were lysed with 2 mL of 2-D protein extraction buffer III (GE Healthcare) for 15 minutes and then pipetted several times on ice. The cell lysate was collected by centrifuging the lysed cells at 42,000 ×*g* at 4°C for 10 minutes.

B. Preparation of stacking and separating gels for SDS-PAGE

Cassette set for gel preparation was assembled and checked for leakage with distilled water. The mixture of 10% separating gel was prepared according to Table 2.1, and it was poured into the glass plate sandwich. The gel was overlaid with a layer of distilled water to achieve an even gel surface. Once the gel has polymerised, the distilled water was discarded and gel sandwich was gently blotted with filter paper to remove excess distilled water. The stacking gel mixture was prepared (Table 2.1) and layered on top of the separating gel along with an insertion of ten-chambered comb before stacking gel started to polymerise.

Table 2.1: Volumes of stock solutions used to prepare the separating and staking gels of SDS-PAGE

Stock solution	Volumes of stock solutions	
	Separating gel (10%)	Stacking gel (4%)
Acrylamide, 30% (mL)	3.34	0.375
Tris-HCl, 1.5 M (mL)	2.0	-
SDS, 10% (µL)	80.0	25
*APS, 10% (µL)	80.0	40
*TEMED (µL)	8.0	4.0
Tris-HCL, 0.5 M (mL)	-	0.625
Distilled water (mL)	2.4	1.45
Total volume (mL)	7.908	2.519

*The solution was prepared prior to use. The total volume of the solution was made for one gel of SDS-PAGE. Stock solutions were prepared as in Appendix B5.2.

C. SDS polyacrylamide gel electrophoresis

The cell lysate was mixed at a ratio of 1:3 with the sample buffer in a micro centrifuge tube and boiled at 95°C for 5 minutes. About 10 µL of samples and broad range (10–250 kDa) molecular weight standard (Bio-Rad) were loaded into the gel wells. Electrophoresis was conducted at a constant voltage of 90 V and stopped once the blue dye front approached approximately 1 cm from the bottom of the gel. The gel was carefully taken out and proceeds with Western blotting.

D. Western blotting

Firstly, the protein bands on the gel were transferred onto a nitrocellulose membrane. Six pieces of blotting filter papers and nitrocellulose were cut into similar size of the gel. The gel, the filter papers and nitrocellulose membrane were immersed into transfer buffer for 15 minutes. Three filter papers were assembled on the anode plate and followed by the membrane, the gel and lastly the remaining three filter papers. The sandwich gel was rolled with a tube roller to expel out any trapped air bubbles. The cathode plate was placed onto the sandwich gel. The protein transfer procedure was carried out for 1 hour at a constant current of 0.8 mA/cm^2.

The membrane was blocked with 3% w/v of gelatin in Tris-buffered saline-Tween 20 buffer, pH 7.4 for 1 hour (Abdullah-Soheimi *et al*., 2010). After removal of nitrocellulose membrane from the paper sandwich, the membrane was cut into two pieces based on the molecular weight markers, in which the piece containing higher molecular weight proteins was incubated with anti-mouse iNOS and the piece containing lower molecular weight proteins was incubated with anti-mouse β-actin overnight at room temperature. After washing three times (5 minutes each wash) with Tris-buffered saline-Tween 20 buffer pH 7.4, the membranes were incubated with

horseradish peroxidase–conjugated anti-rabbit secondary antibody for 1 hour at room temperature. Following the final wash, the membranes were developed by incubating in 3,3'-diaminobenzidine substrate (Thermo Scientific) in the dark for 5 minutes. The membrane was then washed with distilled water and scanned. The intensity of each band was analysed using ImageJ 1.48a (National Institutes of Health, Bethesda, MD, USA).

2.2.8 Pinocytosis analysis

One of the parameters commonly used to determine activation of macrophages is the increase in pinocytosis activity. Activated macrophages are able to uptake massive volume of extracellular fluid by endocytic activity through pinocytosis mechanism (Wang *et al*., 2008). The pinocytosis activity of activated RAW 264.7 cells was analysed by evaluating the uptake of neutral red (NR) solution. To activate the cells, RAW 264.7 cells (5×10^5 cells/mL) were seeded in a 96-wells culture plate (Orange Scientific) and incubated for 24 hours. The cells were then treated with 100 µg/mL of SN-ppF3 for 24 hours. At the end of the incubation period, the medium was replaced with a medium containing NR dye and incubated for another 4 hours. After the incubation period, the medium was discarded and the cells were washed with washing solution. To evaluate the pinocytosis activity, the NR dye was eluted out from the cells by incubating the cells with 200 µL of resorb solution for 30 minutes at room temperature with rapid agitation on a LT BioMax 500 microplate shaker (Thermo scientific). The absorbance at 540 nm was measured by a Multiskan Go microplate spectrophotometer (Thermo Scientific).

2.2.9 Phagocytosis activity

The ability of the activated RAW 264.7 cells to phagocytose foreign particles was tested using fluorescent IgG-coated latex beads. To activate the cells for phagocytosis, RAW 264.7 cells (5×10^5 cells/mL) were seeded in a six-well culture plate (Orange Scientific) and incubated for 24 hours. Then, 100 μg/mL of SN-ppF3 was added to each well and incubated for another 24 hours. LPS was added at 100 μg/mL as a positive control. To evaluate the phagocytosis activity, a Phagocytosis Assay Kit (IgG-FITC conjugated) was used. All reagents and solutions required for this assay were provided in the kit. Briefly, the cells were mixed with 100 μL of latex beads conjugated to rabbit IgG-FITC and incubated for 24 hours. The cells were then centrifuged for 5 minutes at 400 × *g* at room temperature. The supernatant was removed and the cells were washed twice with 1 mL of assay buffer. Finally, the cells were suspended in 500 μL of assay buffer and immediately analysed using a BD FACSCanto II flow cytometer (BD Biosciences, San Jose, CA, USA). The experimental results were compared to both of the non-treated (negative control) and LPS-treated cells (positive control).

2.2.10 *In vitro* assessment of TNF-α and IL-6 production

In classically activated macrophages, TNF-α and IL-6 are the most common cytokines to be produced (Mosser and Edward, 2008). The quantification of these cytokines was carried using ELISA approach. In this assay, the cells were prepared as in the phagocytosis assay (Section 2.2.9). After 24 hours incubation with the SN-ppF3 fraction or LPS, the culture media in each well were collected and the presence of TNF-α and IL-6 were assayed using ELISA kits. All reagents and solutions required for this assay were provided in the ELISA kits. After the treatment, 100 μL of culture medium was pipetted into a 96-wells microplate coated with either anti-mouse TNF-α or anti-

mouse IL-6 and the plate was incubated overnight at 4°C. Then, the medium was discarded, and the wells were washed four times with 300 μL of washing buffer. After that, 100 μL of biotinylated anti-mouse TNF-α or IL-6 antibody was added into each well, and the plate was incubated for 1 hour at room temperature with gentle shaking. The solution was discarded, and the wells were washed again before 100 μL of horseradish peroxidase-streptavidin solution was added into each well. The plate was incubated for 45 minutes at room temperature with gentle shaking. After the final wash, 100 μL TMB One-Step Substrate Reagent was added to each well, and the plate was incubated for another 30 minutes in the dark with gentle shaking. Lastly, 50 μL of stop solution was added into each well to stop the colour development, and the absorbance at 450 nm was immediately measured by a Multiskan Go microplate spectrophotometer (Thermo Scientific). The standard curves for the recombinant TNF-α and IL-6 protein were used to determine the concentration of the cytokines (Appendix C4).

2.2.11 Detection of phosphorylated signalling protein

Protein phosphorylation is one of the common mechanisms in activation of signalling pathways (Elliott and Elliott, 2009). The identification of several phosphorylated proteins involved in inflammation signal transduction is necessary to predict the mechanism on how SN-ppF3 activates the macrophage cells. To predict the pathways taken place in activated macrophages, a PathScan Inflammation Multi-Target Sandwich ELISA kit (Cell Signalling) was used. All reagents and solutions required for this assay were provided in the kit. Cell lysate from non-treated and treated RAW 264.7 cells was prepared as described in Section 2.2.7. One-hundred microliter of cell lysate solution was pipetted into a 96-wells microplate coated with either anti-NF-κB p65, anti-phosphorylated-NF-κB p65, anti-phosphorylated-SAPK/JNK, anti-phosphorylated-p38, anti-phosphorylated-IκB-α or anti-phosphorylated-STAT3 antibodies and plate was

incubated overnight at 4°C. Then, the medium was discarded, and the wells were washed four times with 200 µL of 1× washing buffer. The washing buffer was then discarded and the plate was blotted onto fresh paper towel to remove excess washing buffer. Then, 100 µL of detection antibodies respective to each coated wells was added and the plate was incubated for 1 hour at 37°C. The solution was discarded, and the wells were washed again before 100 µL of horseradish peroxidase-streptavidin-linked secondary antibody solution was added into each well. The plate was incubated for 30 minutes at 37°C. After the final wash, 100 µL TMB One-Step substrate reagent was added to each well, and the plate was incubated at 37°C for another 10 minutes in the dark. Lastly, 50 µL of stop solution was added into each well to stop the colour development, and the absorbance at 450 nm was immediately measured by a Multiskan Go microplate spectrophotometer (Thermo Scientific).

2.2.12 Endotoxin test

The presence of endotoxin in the sample was detected by using qualitative commercial Limulus amebocyte lysate (LAL) E-TOXATE^{TM} kit (Sigma-Aldrich). This test was carried out according to manufacturer's protocol and all reagents and solutions required for this assay were provided in the E-TOXATE^{TM} kit. Briefly, 100 µL of E-TOXATE reagent was added into 100 µL of 100 µg/mL SN-ppF3, 100 µL of endotoxin standard and 100 µL of E-TOXATE water. The mixture were gently mixed, covered with aluminium foil and incubated at 37°C, undisturbed for 1 hour. The incubation period was followed by observing any evidence of gelation by inverting the tubes 180° gently. The presence of endotoxin contamination will result the formation of hard gel in the tubes which permit complete inversion without any disruption.

2.2.13 Chemical characterisation of SN-ppF3

A. Molecular weight estimation

Resin preparation and column packing

To prepare the stationary phase for the size exclusion chromatography column, 80 mL of Sepharose CL-6B resin was filtered through sintered funnel and washed with 1 L of distilled water followed by 1 L of phosphate-buffered saline (PBS). Then, the resin was diluted back in 100 mL of PBS and degassed by using Lab Companion water aspirator pump (Jeio Tech, Seoul, South Korea) to remove possible gases present in the resin mixture. The degassed Sepharose resin was packed into a glass column (ø20 mm × 250 mm). The column was washed with at least 400 mL of PBS before being use.

Void volume determination

To determine the void volume of the size exclusion column, 2 mg of blue dextran with molecular weight of 1000 kDa was dissolved in 5 mL of PBS and eluted in the Sepharose CL-6B size exclusion column. Five millilitres of each sample elution was collected until the blue colour solution was completely eluted out from the column. The optical density at 540 nm of the elution was measured using Multiskan Go microplate spectrophotometer (Thermo Scientific) and the chromatography profile was plotted.

Dextran standard curve preparation

The standard curve was prepared based on several sizes of dextran standards. To prepare the standard curve, 10 mg of each dextran with molecular weight of 12, 50, 80 and 150 kDa was dissolved in 5 mL of PBS and carefully eluted in the Sepharose CL-6B size exclusion column. Each size of dextran standard each size was detected by the phenol sulphuric acid method (Section 2.2.4).

Molecular weight determination of SN-ppF3

To estimate the molecular weight of SN-ppF3, it was subjected to size exclusion chromatography column. Briefly, 10 mg of SN-ppF3 was dissolved in 5 mL of PBS and loaded into the size exclusion chromatography column. The sample was eluted out with PBS as the mobile phase. Sixty fractions were collected in 5 mL aliquots using a fraction collector. The polysaccharide content of SN-ppF3 in each fraction was determined by the phenol sulphuric acid method (Section 2.2.4).

B. Monosaccharide composition analysis

Twenty milligrams of SN-ppF3 was hydrolysed with 2 mL of 4 M trifluoroacetic acid in a glass test tube for 5 hours in a 95°C water bath. The hydrolysate was then evaporated with a stream of nitrogen gas at 80°C, washed with absolute methanol and re-dissolved with 1 mL of 75% acetonitrile (ACN). It was filtered through 0.45 μM membrane filter and separated using Agilent Carbohydrate Analysis Column on Agilent HPLC 1260 (Agilent Technologies, Santa Clara CA, USA), equipped with refractive index (RI) detector. The column and detector temperature were set at 30°C and sample was eluted isocratically with 75% ACN at a flow rate of 1.4 mL/min. The possible identity of monosaccharides in the samples was determined by comparing the retention time of the peaks with those of monosaccharide standards (rhamnose, fucose, xylose, arabinose, glucose, galactose and mannose).

C. Fourier Transform Infrared (FT-IR) spectroscopy analysis

Ten milligrams of SN-ppF3 was mixed with 100 mg of potassium bromide, compressed together into approximately 1 mm pallet disc, and subjected to FT-IR spectroscopy analysis. Spectrum absorbance was recorded at 4000–400 cm^{-1} on Perkin Elmer Spectrum RX-1 FT-IR spectrophotometer (Waltham, Massachusetts, USA).

2.2.14 Oral toxicity study

Ten female BALB/c mice with the age of 8 weeks old were divided into two groups (*n*=5) and maintained in well-ventilated cages with normal food pellets and tap water, *ad libitum*. Mice in Group 1 were orally administered with 500 mg/kg/bw of SN-ppF3 daily for 14 days, while mice in the Group 2 (control) were orally administered with normal saline. Mice weight in each group was recorded for 14 days.

2.2.15 Anti-tumour potential of SN-ppF3 *in vivo*

A. Tumour induction

Twenty four female BALB/c mice with the age of 8 weeks old were maintained in well-ventilated cages with normal food pellets and tap water, *ad libitum*. The mice were subcutaneously inoculated with 1×10^6 cell/mL 4T1 mouse mammary carcinoma cell line onto the left breast line of the mice and monitored daily for tumour growth.

B. Treatment

After tumour has completely developed, approximately 14 to 21 days after tumour induction, mice with tumour were randomly selected and divided into four groups (*n*=6). Tumour-bearing mice in each group was orally administered with 200 µL of normal saline (control), 25 mg/kg of cyclophosphamide (CTX; commercial anti-cancer drug) and SN-ppF3 (250 and 500 mg/kg) daily for 10 days, respectively.

C. Treatment effect on tumour progression

Tumour progression and regression were monitored by measuring the two perpendicular dimensions (long and short) of the tumour using a calliper. The data was then integrated in the formula $Tv = (a + b^2) /2$, where a is the larger and b is the smaller tumour dimension (Zhang *et al*., 2003) for determination of the tumour volume.

D. Evaluation on tumour tissue and organ indices

Tumour, spleen and thymus were collected and weighed after the mice were sacrificed on day 11. The spleen and thymus indices were calculated according to the formula by Chen *et al*., (2010):

$$\text{Spleen and thymus index (mg/g)} = \frac{\text{weight of the spleen or thymus}}{\text{Body weight}}$$

2.2.16 Measurement of cytokines production

Blood sera collected from the sacrificed tested mice were assayed for the concentrations of TNF-α, IFN-γ, IL-4 and IL-6 by using commercially available ELISA kits (Abcam), according to procedure provided by manufacturer. All reagents and solutions required for this assay were provided in the ELISA kits. Briefly, 100 µL of mice serum was pipetted into a 96-wells microplate coated with either anti-mouse TNF-α, IFN-γ, IL-4 or IL-6 and the plate was incubated overnight at 4°C. Then, the serum was discarded, and the wells were washed four times with 300 µL washing buffer. After that, 100 µL of biotinylated anti-mouse TNF-α, IFN-γ, IL-4 or IL-6 antibody was added into each well, and the plate was incubated for an hour at room temperature with gentle shaking. The solution was discarded, and the wells were washed again before 100 µL of horseradish peroxidase-streptavidin solution was added into each well. The plate was incubated for 45 minutes at room temperature with gentle shaking. After the final wash, 100 µL TMB One-Step substrate reagent was added to each well, and the plate was incubated for another 30 minutes in the dark with gentle shaking. Lastly, 50 µL of stop solution was added into each well to stop the colour development, and the absorbance at 450 nm was immediately measured by a Multiskan Go microplate spectrophotometer (Thermo Scientific). The standard curve for the recombinant TNF-α, IFN-γ, IL-4 and IL-6 were used to determine the concentration of the cytokines (Appendix C4).

2.2.17 Histological analysis

A. Slide preparation

Tumours from the tumour-bearing mice were harvested and fixed with at least 20 mL of 10% formalin solution between 16 hours fixation periods at room temperature. Then, the fixed tumour tissues were sampled into cassettes and processed. The processed tissues were embedded into a paraffin block, sectioned into 5 μm in thickness and fixed on poly-L-lysine slides by oven dried at 37°C overnight. Slides were stored in 37°C until further use.

B. Haematoxylin and Eosin staining

Figure 2.1 shows the flow of staining procedure for the haematoxylin and eosin (H&E) staining where the process was carried out at room temperature. Briefly, the tissue sample was deparaffinised by incubating the slide into absolute xylol solution for 3 minutes, twice. The tissue was then rehydrated by incubating the slide in 95% ethanol solution, twice and 75% ethanol solution for 3 minutes each. The slide was then rinsed twice with clean distilled water before being dipped in haematoxylin solution for 15 seconds. The slide was quickly rinsed with running tap water for at least 3 minutes. Then the slide was dipped in 0.2% of HCl solution for 2 seconds and rewashed again with running tap water for another 3 minutes. Later, the slide was dipped in 0.2% sodium bicarbonate solution for 2 seconds, followed with the final washing step with running tap water for another 3 minutes. After that, the slide was rinsed with distilled water for three minutes, and then quickly dipped in eosin solution for 60 seconds, followed with a quick rinse with 95% ethanol solution. Later, the slide was incubated in absolute ethanol, twice for 3 minutes each, and xylol solution, twice, for another 3 minutes. Lastly, the slide was gently blotted on tissue paper to remove excess xylol solution before mounted with Canada balsam and covered with a glass slip. The slide

was allowed to dry in an oven at 45°C. The stained slide was observed and photographed using a light microscope attached with camera (Olympus, Shinjuku, Tokyo, Japan). The slides of the control and treated tumour tissues were observed for any cellular changes, particularly on the apoptotic features.

2.2.18 Apoptosis detection of tumour tissue by TUNEL staining

A. Slide preparation

For this assay, the tumour tissue slides were prepared as in Section 2.2.17. The tissue samples were embedded into a paraffin block, sectioned into 3 μm in thickness and then fixed on poly-L-lysine slides.

B. TUNEL staining

The TUNEL staining of the slides was carried out according to *in situ* Cell Death Detection Kit Fluorescein protocol (Roche). After the deparaffinised and dehydration processes, the slides were washed twice with PBS and incubated in a freshly prepared 0.1% Triton-X in 0.1% sodium citrate solution for 8 minutes at room temperature. The slides were then washed twice with PBS. In the staining process, 50 μL of TUNEL solution was slowly added onto the tissue sections and layered with a cover slip to avoid evaporation during incubation. The slides were then incubated in a humidified atmosphere for 1 hour at 37°C in the dark. The slides were then washed three times with PBS, slowly blotted and directly analysed under a fluorescent microscope (×200) using excitation wavelength in the range of 450–500 nm and detection wavelength in a range of 500–516 nm, with a constant exposure of blue light laser. Images were photographed using an equipped camera (Leica, Wetzlar, Germany).

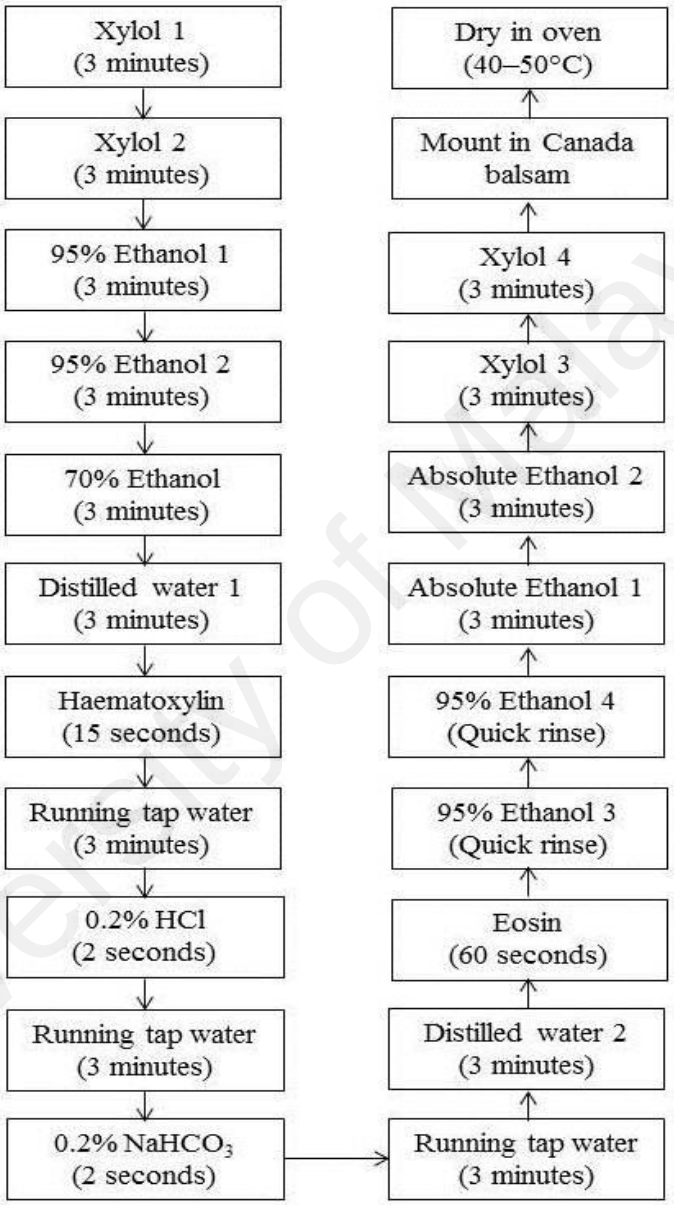

Figure 2.1: Steps for Haematoxylin and Eosin staining procedure of tumour tissue slides

2.2.19 Detection of infiltrating immune cells by immunofluorescent staining

A. Slide preparation

For this assay, tumour tissue slides were prepared as in Section 2.2.17. The tissue samples were embedded into a paraffin block, sectioned into 3 μm in thickness and then fixed on poly-L-lysine slides.

B. Immunostaining

The immunostaining procedures were carried out according to Robertson *et al*., (2008) with a slight modification. Briefly, the slides were deparaffinised and incubated in 95°C and at room temperature with sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0) for 20 and 10 minutes, respectively. Then, the tissues were blocked with PBS supplemented with 1% bovine serum albumin and 2% foetal bovine serum in room temperature for 30 minutes and washed three times with PBS for 2 minutes each. The slides were then incubated with 0.2 μg/mL anti-CD69 NK cell, anti-$CD8^+$ and anti-F4/80 macrophage antibodies conjugated with Cy5®, phycoerythrin and FITC fluorescent dyes (Abcam), respectively, in the dark for 1 hour. Then the slides were washed again three times with PBS for 2 minutes each. The slides were gently blotted on a tissue paper to remove excess buffer, observed with a constant exposure of blue light laser and photographed using a fluorescent microscope (×200) equipped with camera (Leica).

CHAPTER 3: RESULTS

The polysaccharide extracts of *S. nigrum* were isolated and tested against RAW 264.7, murine macrophage cell line to investigate its modulatory capability. Several macrophages activation assays were carried out to investigate the ability of the extract to induce nitric oxide production, pinocytosis and phagocytosis activities, and pro-inflammatory cytokines production of macrophage cells. Polysaccharide sample with the strongest immunomodulatory activity was selected to investigate its anti-tumour activity, *in vivo*. Tumour-bearing mice were oral-administered, daily with the polysaccharide sample and tumour progression was monitored for 10 days. Tumour tissues, blood and immune organs were harvested after mice were sacrificed for further evaluation. Results for this study will be further discussed in details, in this section.

3.1 Preparation of crude polysaccharide

Plant samples were divided into three parts and the weights of dried plant samples were measured. The yield of dried leaves, stems and whole plant samples obtained from 10 kg of fresh *S. nigrum* were 335.5 g, 375.5 g 725.5 g, respectively. Crude extracts of *S. nigrum* polysaccharide were prepared as described in Section 2.2.1 and the weight the crude samples obtained were measured after they were completely dried. The weight of polysaccharide from crude extracts of leaves, stems and the whole plant obtained were 7.05 g (~2.1%), 9.39 g (~2.5%), and 13.78 g (~1.9%) of the dried samples, respectively.

3.2 Cytotoxicity evaluation of crude polysaccharide extracts

Determination on the inhibition concentration (IC) of the crude polysaccharide samples, tested on the RAW 264.7 cells is necessary to ensure cells survival throughout this study and that any decrease of readings obtained is not due to cell death. Sample

concentration used to treat the cells must not cause more than 50% cells death upon 48–72 hours of incubation (Lee and Houghton, 2005). Cytotoxicity of crude polysaccharide samples on RAW 264.7 cell line was assessed using the conventional MTT assay, through the indication of cells viability by mitochondria-dependent reduction of MTT to formazan (Mosmann, 1983). Optical densities (OD) at 570 and 690 nm were measured using spectrophotometer and the percentage of inhibition (POI) was calculated. Figure 3.1 shows the dose-response curves plotted from the POI of RAW 264.7 cell line when treated with different concentrations of crude polysaccharide extracts from leaves, stems and the whole plant of *S. nigrum*. IC_{50} values of leaves, stems and the whole plant extracts were then determined by extrapolating the graph at 50% POI. The IC_{50} values of all samples evaluated were similar, which were around 50 μg/mL. This indicated that all crude polysaccharide samples did not exert cytotoxicity effect towards RAW 264.7 murine macrophage cell line. A sample which has IC_{50} value less than 30 μg/mL is deemed actively cytotoxic (Stuffness and Pezzuto, 1990). Among these samples, crude polysaccharide extracted from stem part had the lowest inhibitory effect (~60.5 μg/mL) as compared to crude polysaccharide extracts from leaves (~65.7) and the whole plant (~70.9) at the maximum concentration of 100 μg/mL (Table 3.1). Thus, only crude polysaccharide sample from stem was chosen for the next extraction procedure and subsequent macrophages activation assays.

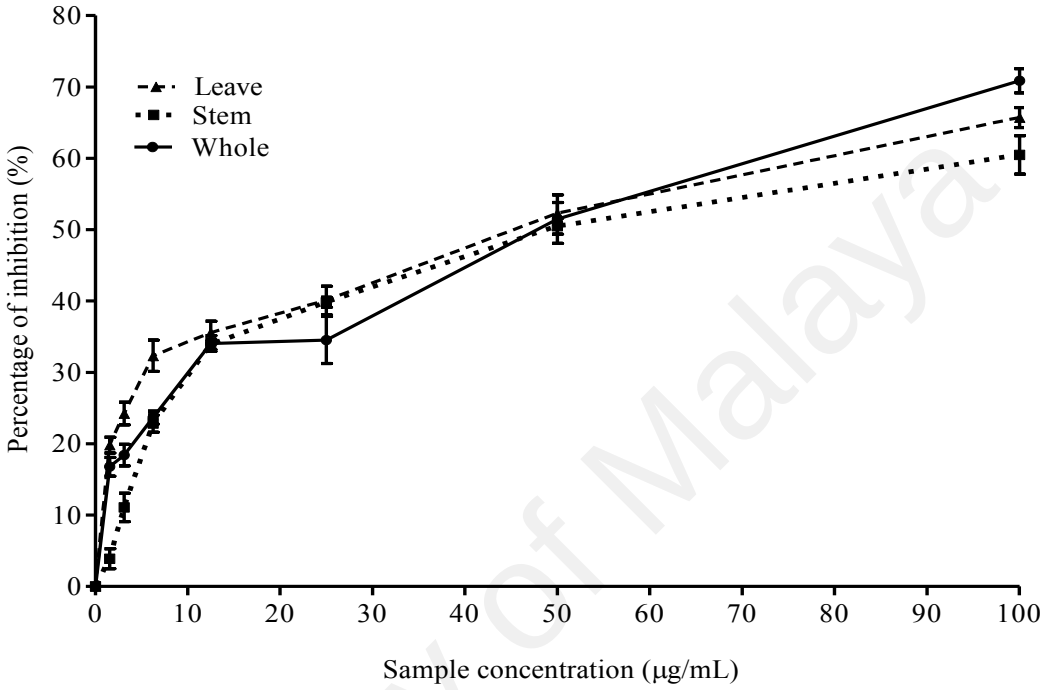

Figure 3.1: Percentage of inhibition of RAW 264.7 murine macrophage cell line treated with crude polysaccharide samples of *S. nigrum*

The crude samples extracted from (▲) leaves, (■) stems and (●) whole plant of *S. nigrum* were tested for cytotoxicity on RAW 264.7 cell line. Treatments were carried out for 72 hours and cytotoxicity was assessed using MTT assay. Data expressed were mean ± standard deviation (n=3). The IC_{50} values were determined based on cells inhibition percentage at 50%. At 100 µg/mL, the polysaccharide sample from stem showed the lowest inhibition percentage.

Table 3.1: Inhibitory activity of *S. nigrum* crude polysaccharide samples against RAW 264.7 murine macrophage cell line

Crude sample	IC_{50} value (µg/mL)	Percentage of inhibition at 100 µg/mL
Leaves	52.29 ± 1.5^{a}	65.72 ± 1.5^{b}
Stem	50.48 ± 1.1^{a}	60.48 ± 2.7^{c}
Whole plant	51.48 ± 3.4^{a}	70.88 ± 1.7^{a}

Values expressed were mean ± standard deviation (n=3). Different superscripted letters (a–c) indicate the significant differences at $p<0.05$.

3.3 Preparation of semi-purified polysaccharide sample of *S. nigrum*

Further purification of *S. nigrum* crude polysaccharide extract was carried out by ion-exchange chromatography. Based on the preliminary result of cytotoxicity evaluation on RAW 264.7 cell line, the polysaccharide sample from stem of *S. nigrum* showed the lowest inhibition percentage and thus was chosen to be further purified. The crude sample was subjected into DEAE-cellulose ion-exchange column chromatography with isocratic gradient elution of 0–1.5 M NaOH. Around 200 fractions were collected and tested with phenol sulphuric assay (Section 2.2.4). Referring to the plotted chromatography profile in Figure 3.2, the crude extract was resolved into five peaks which were high in the presence of sugar residues up until fraction number 30. No peak was observed in the fraction 31 onward. The fraction of the five peaks were pooled and labelled as SN-ppF1, SN-ppF2, SN-ppF3, SN-ppF4 and SN-ppF5 respectively.

3.4 Measurement of carbohydrate content in *S. nigrum* polysaccharide fractions

The carbohydrate content in the polysaccharide fractions was measured by phenol sulphuric method and the data was also used to estimate the purity of the samples. The carbohydrate content was determined based on the glucose standard curve with the maximum concentration of 100 mg/mL, which was the same concentration of polysaccharide samples used in this assay. Among of the five samples listed in Table 3.2, SN-ppF1 possessed the highest carbohydrate content ($p<0.05$) followed by SN-ppF4 and SN-ppF3, with approximately 98.0%, 95.0% and 80.0% of carbohydrate content, respectively. Both SN-ppF2 and SN-ppF5 have the lowest carbohydrate content ($p<0.05$), which contained approximately 74.4% and 74.1% carbohydrate, respectively. It can be concluded that the fractions were mainly composed of carbohydrate.

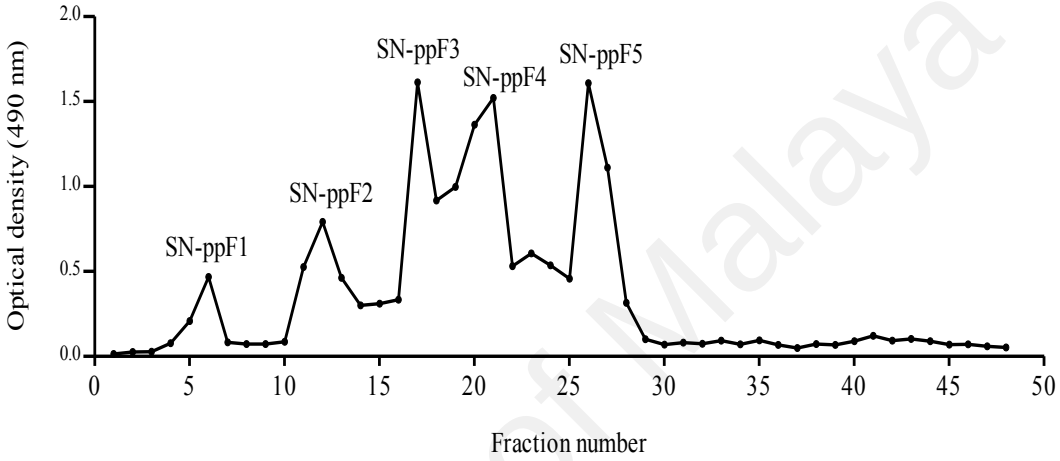

Figure 3.2: Ion-exchange chromatography profile of *S. nigrum* polysaccharide resolved through DEAE-cellulose column

Crude polysaccharide from stem was subjected to ion-exchange column chromatography eluted with 0–1.5 M NaOH. Each collected fraction was detected by phenol sulphuric reaction and monitored at absorbance of 490 nm. Detected peaks demonstrate the fractions that are high in carbohydrate residues. Resolved peaks were pooled and labelled as SN-ppF1–5. No peak was detected beyond fraction number 31.

Table 3.2: Carbohydrate content in 100 mg/mL of *S. nigrum* polysaccharide fractions

Sample	Carbohydrate content (mg/mL)
SN-ppF1	97.98 ± 1.6^{a}
SN-ppF2	74.40 ± 1.2^{c}
SN-ppF3	79.62 ± 2.8^{b}
SN-ppF4	94.88 ± 2.2^{a}
SN-ppF5	74.18 ± 2.4^{c}

Values expressed were mean ± standard deviation (n=3). Different superscripted letters indicate the significant difference at $p<0.05$.

3.5 Estimation of protein content in *S. nigrum* polysaccharide fractions

The polysaccharide fractions were assessed by Bradford assay to indicate the presence of protein in the samples. Glycoprotein, a dominant protein molecule that covalently attached with oligo- or polysaccharide chains, may also serve as a reliable immunomodulator. Thus, an indication for the presence of protein is important to deduce the effect observed was due to polysaccharide or glycoprotein in the sample. The protein content was determined based on bovine serum albumin standard curve. Referring to Table 3.3, all fractions were shown to be very low in protein content, with approximately less than 1% out total sample concentration. These values indicated that the samples were most probably polysaccharide rather than glycoprotein, which would have higher protein to carbohydrate ratio.

3.6 Cytotoxicity evaluation of *S. nigrum* polysaccharide fractions

As in Section 3.2, the IC of the polysaccharide fractions was also tested on RAW 264.7 cell line. The cytotoxicity effect of *S. nigrum* polysaccharide fractions towards the cells was evaluated by MTT assay. Based on Figure 3.3, the IC_{50} value of each fraction was determined by extrapolating the graph at POI value of 50%. However, the IC_{50} value for all fractions could not be determined (Table 3.4) where the IC_{50} of all samples were above 100 μg/mL. Hence, the inhibitory level of each fraction against RAW 264.7 cell line was determined by evaluating the POI value at the maximum concentration (100 μg/mL) of each polysaccharide fraction tested. Referring to Table 3.4, the POI caused by SN-ppF3 was significantly ($p<0.05$) lower than those by all other fractions evaluated. It inhibited only approximately 29% of RAW 264.7 macrophage cells following treatment for 72 hours. These results show that as with the crude polysaccharide extracts these polysaccharide fractions did not exert active cytotoxic effects on RAW 264.7 cells

Table 3.3: Protein content in 100 mg/mL of *S. nigrum* polysaccharide fractions

Sample	Protein content (mg/mL)
SN-ppF1	0.96 ± 0.03^{z}
SN-ppF2	0.37 ± 0.02^{y}
SN-ppF3	0.11 ± 0.05^{w}
SN-ppF4	0.15 ± 0.05^{x}
SN-ppF5	0.05 ± 0.03^{w}

Values expressed were mean ± standard deviation (n=3). Different superscripted letters indicate the significant difference at $p<0.05$.

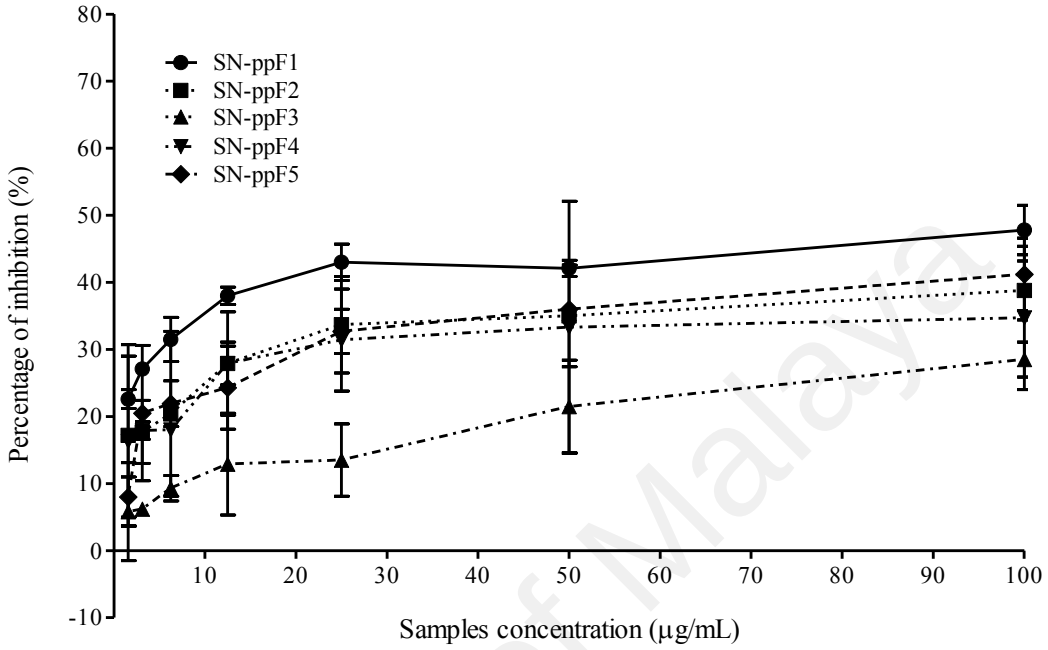

Figure 3.3: Percentage of inhibition of RAW 264.7 murine macrophage cell line treated with polysaccharide fractions SN-ppF1-F5

The polysaccharide samples (●) SN-ppF1, (■) SN-ppF2, (▲) SN-ppF3, (▼) SN-ppF4 and (♦) SN-ppF5 from ion-exchange chromatography were tested for cytotoxicity. The RAW 264.7 cells were treated with these fractions at sample concentration of 0, 1.56, 3.125, 6.25, 1.25, 25, 50 and 100 µg/mL for 72 hours. The viability of the cells after treatment was determined by MTT assay. Data presented were mean ± standard deviation (n=3). The IC_{50} values were determined based on cells inhibition percentage at 50%. The polysaccharide fraction SN-ppF3 showed the lowest inhibition percentage at 100 µg/mL where it caused only 29% cells inhibition.

Table 3.4: Inhibitory activity of *S. nigrum* polysaccharide fractions against RAW 264.7 cell line

Sample	IC_{50} value (µg/mL)	Percentage of inhibition at 100 µg/mL
SN-ppF1	> 100	48.7 ± 3.7^{c}
SN-ppF2	> 100	38.8 ± 4.4^{c}
SN-ppF3	> 100	28.5 ± 2.6^{b}
SN-ppF4	> 100	34.7 ± 10.7^{c}
SN-ppF5	> 100	41.2 ± 5.4^{c}
Non-treated*	> 100	0.0 ± 0.01^{a}

*Positive control. Values expressed were mean ± standard deviation (n=3). Different superscripted letters (a–c) indicate the significant difference at $p<0.05$.

3.7 Measurement of NO production

The production of NO by macrophages is one of the major indicators that macrophages are activated. Thus the ability of polysaccharide fraction to activate macrophages could be measured by estimating the NO production. The concentration of NO produced by RAW 264.7 cell line was determined based on the standard curve of 100 μM of nitrite solution. Figure 3.4 shows a dose-dependent pattern of NO concentration produced after 24 hours of treatment session. The treatment with the lowest dose (12 μg/mL) of all polysaccharide fractions resulted in significant increase of NO production (SN-ppF1; ~22.0 μM, SN-ppF2; ~21.0 μM, SN-ppF3; ~25.0 μM, SN-ppF4; ~16.0 μM, SN-ppF5; ~21.0, μM) as compared to non-treated cells (control; ~7.0 μM), while the use of the highest dose (100 μg/mL) indeed increased the level of NO production for all fractions as compared to the other doses. However, among of all polysaccharide fractions used, treatment with high dose of SN-ppF3 caused RAW 264.7 cell line to produce high concentration of NO (~31.0 μM), but significantly lower as compared to LPS-treated cells (~36.0 μM). Referring to both cytotoxicity and NO production findings, it clearly observed that SN-ppF3 possessed the lowest inhibitory effect but induced the highest production of NO, respectively as compared to the other fractions. Thus, only SN-ppF3 was selected to be further analysed and used in the other subsequent assays.

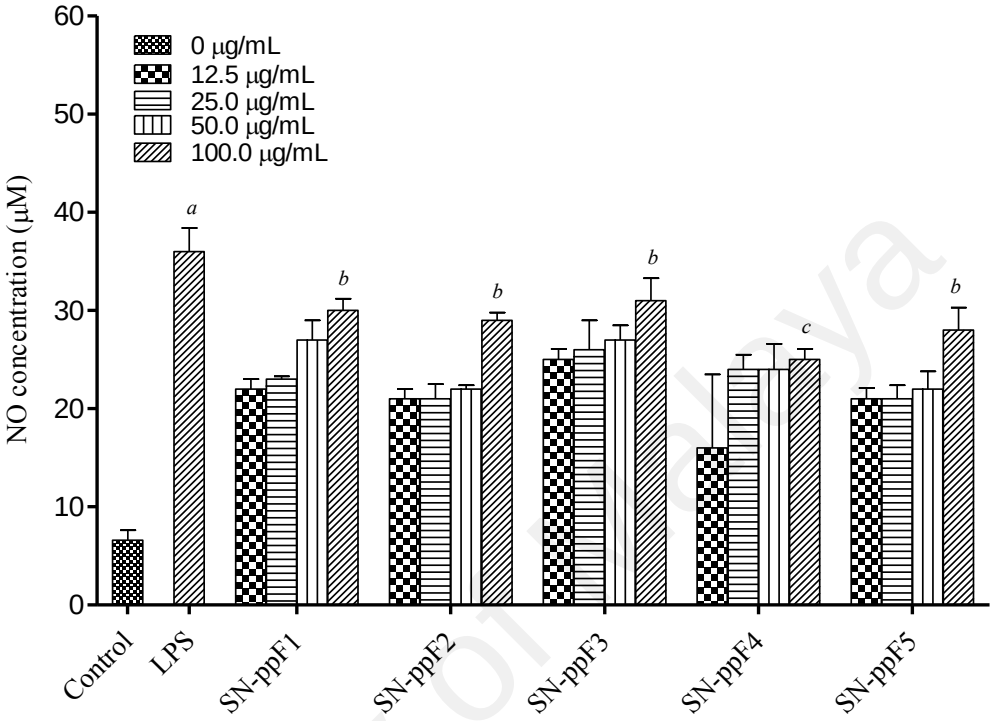

Figure 3.4: Production of nitric oxide by RAW 264.7 murine macrophage cell line treated with polysaccharide fractions SN-ppF1–F5

Cells were treated with the polysaccharide fractions at the concentrations of 12.5, 25, 50, and 100 μg/mL. Concentration of nitric oxide produced was determined based on nitrite standard solution. All polysaccharide fractions were able to induce the production of nitric oxide by RAW 264.7 cell line. (Control: Non-treated cells. LPS: Positive control, where the cells were treated with 100 μg/mL of LPS). Data presented were mean ± standard deviation (n=3). Different letters (a–c) indicate the significant difference at $p<0.05$

3.8 Chemical characterisation of SN-ppF3

3.8.1 SN-ppF3 molecular weight determination

The determination of SN-ppF3 molecular weight was carried out by subjecting it to Sepharose CL-6B size exclusion column. The molecular weight of SN-ppF3 was determined based on the dextran standard curve. Referring to Figure 3.5, only a single peak was observed at fraction number 7, 8 and 9. Based on molecular weight of dextran standards, the molecular weight of SN-ppF3 was estimated to be approximately around 109.42 kDa.

3.8.2 Monosaccharide composition analysis of SN-ppF3

The ability of polysaccharides to induce immunomodulation depends highly on its sugar composition, structure and size (Yi *et al.,* 2011; Zhou *et al.,* 2004). Therefore, a preliminary analysis was carried out to characterise the neutral monosaccharide constituent of SN-ppF3 by hydrolysing it and subjecting the hydrolysed product to HPLC analysis. Figure 3.6 shows the chromatogram of SN-ppF3 monosaccharide composition. Peaks appeared represent monosaccharides detected at specific retention time. The neutral monosaccharides composition was determined based on several neutral monosaccharide standards which were rhamnose, fucose, xylose, arabinose, mannose, glucose and galactose. As stated in Table 3.5, only rhamnose, glucose and galactose were majorly identified in SN-ppF3 sample with molarity of 8.86, 8.13 and 7.66, respectively.

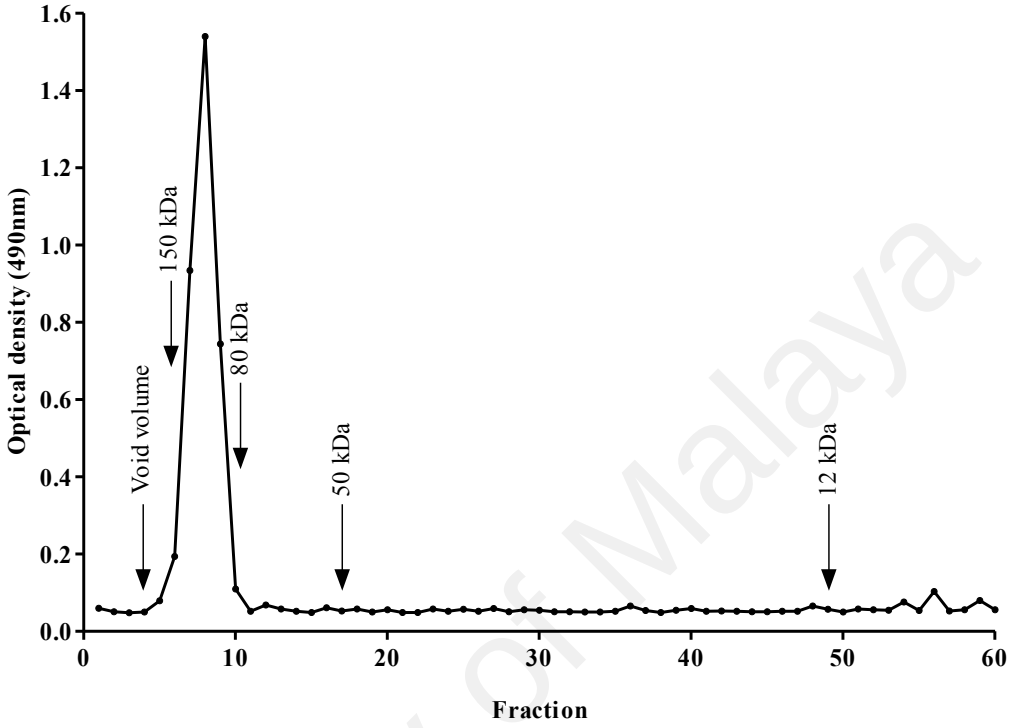

Figure 3.5: Size exclusion chromatography profile of SN-ppF3 resolved through CL-6B Sepharose column

The elution profile of SN-ppF3 was plotted after it was subjected to CL-6B size exclusion column chromatography. Each collected fraction was detected by phenol sulphuric reaction and monitored at the absorbance of 490 nm. Only single peak at fraction number 7, 8 and 9 was detected. No peak was detected beyond fraction number 11. The molecular weight of SN-ppF3 was determined based on series of dextran standards. The molecular weight estimated was 109.42 kDa. The void volume of the column was determined by using 1000 kDa of blue dextran.

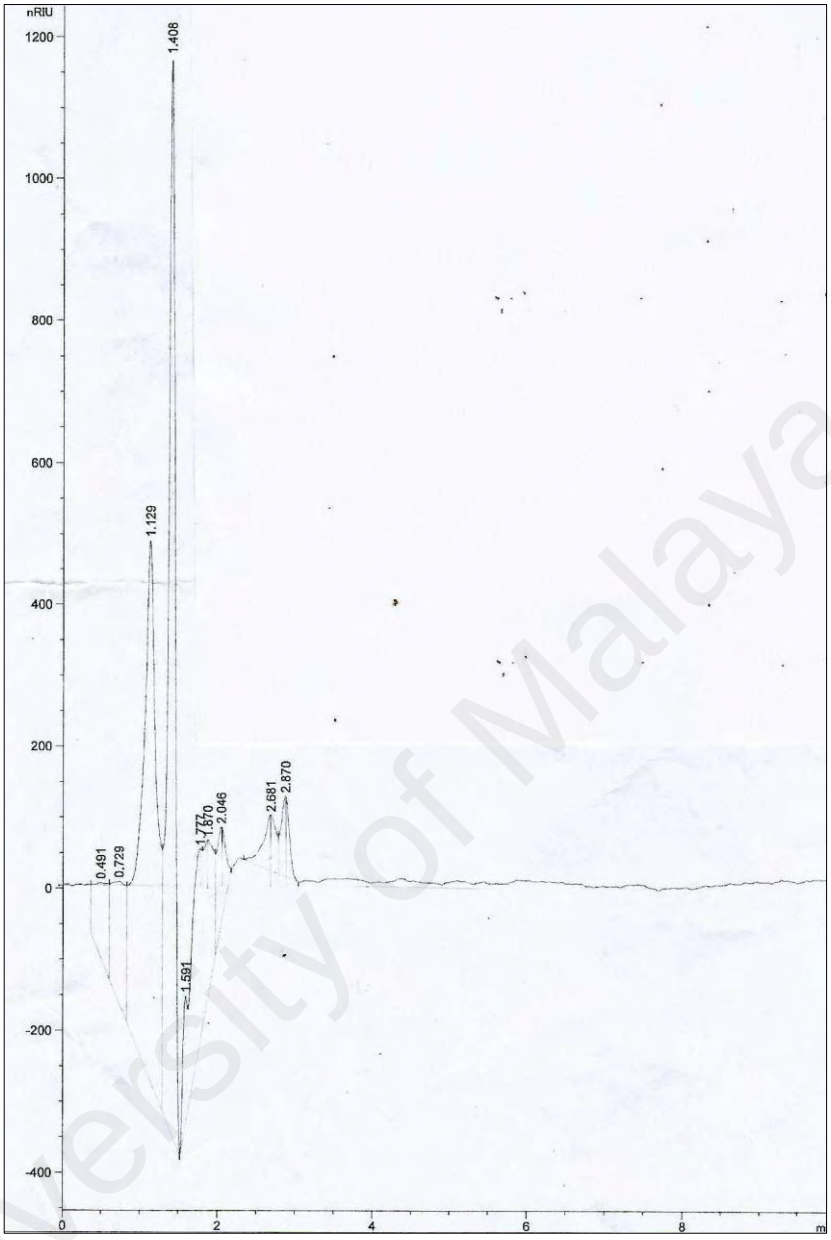

Figure 3.6: HPLC-RID chromatogram of SN-ppF3 monosaccharide composition

The polysaccharide fraction SN-ppF3 was hydrolysed, and the hydrolysate was separated through Agilent Carbohydrate Analysis Column on Agilent HPLC 1260 machine, equipped with RI detector. The detected monosaccharide peaks were identified based on retention time of several neutral monosaccharide standards.

Table 3.5: Neutral monosaccharide composition of SN-ppF3

Neutral monosaccharides composition (Molar)						
Rhamnose	**Fucose**	**Arabinose**	**Xylose**	**Glucose**	**Galactose**	**Mannose**
8.86	*n.d.*	*n.d.*	*n.d.*	8.13	7.66	*n.d.*

n.d.: Not detected.

3.8.3 FT-IR spectroscopy analysis of SN-ppF3

To further characterise the structure of SN-ppF3, the FT-IR spectroscopy analysis was carried out. Referring to the FT-IR absorbance spectrum (Figure 3.7), several peaks related to polysaccharide were detected. A broad-stretched intense peak was detected at 3424 cm^{-1}, followed by a weak peak at 2932 cm^{-1}, a relatively strong asymmetric peak at 1656 cm^{-1}, a weak peak at 1409, another strong asymmetric peak at around 1078–1034 and a weak absorption vibration at 775 cm^{-1}.

3.9 Endotoxin test

In order to rule out the contamination of endotoxin in SN-ppF3, which might result in false positivity, an endotoxin detection test was carried out. The presence of endotoxin in the sample was qualitatively evaluated by commercial Limulus amebocyte lysate (LAL) E-TOXATE^{TM} kit. The contamination of endotoxin in SN-ppF3 sample could be determined by observing the hard gel formation upon treatment with E-TOXATE reagent, Table 3.6 shows the absence of hard gel in SN-ppF3, while the hard gel was clearly observed when endotoxin standard was treated with E-TOXATE reagent. This observation indicates SN-ppF3 was free from endotoxin contamination or it may contain endotoxin at a level below the detection limit for this assay.

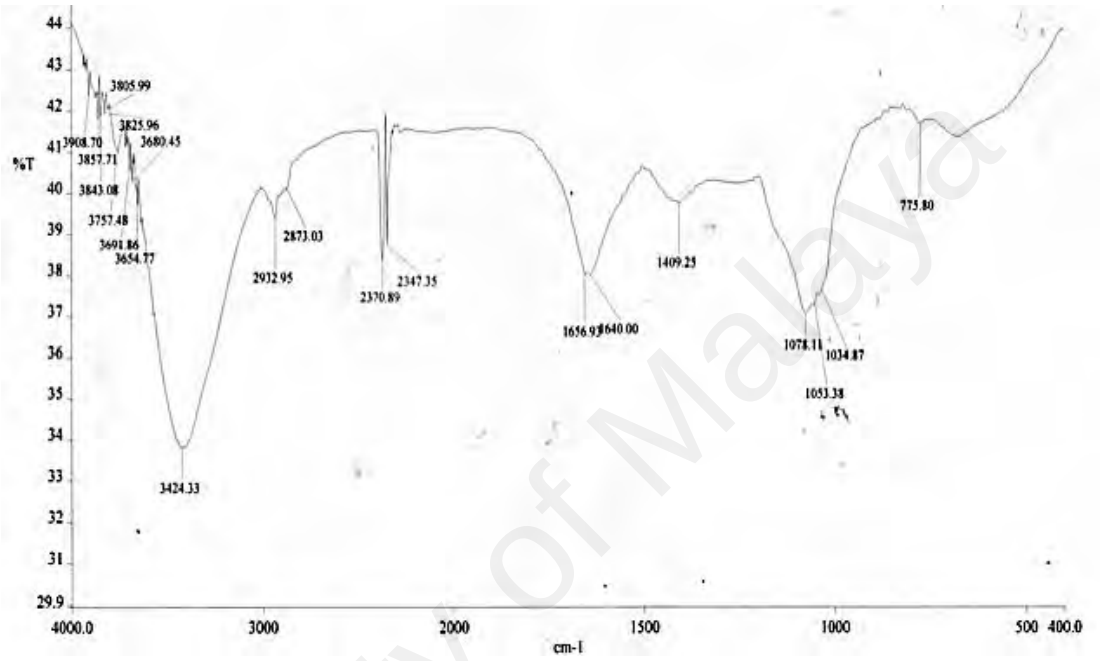

Figure 3.7: Fourier Transform Infrared (FT-IR) spectrum of SN-ppF3

The polysaccharide fraction SN-ppF3 was mixed together with potassium bromide at a ratio of 1:10, pressed into a disc and subjected to FT-IR analysis. Absorbance was recorded at 4000–400 cm^{-1}. Several related peaks in the spectrum were detected at around 3424, 2932, 1409, 1078–1034 and 775 cm^{-1}, which displayed a typical absorption peak of polysaccharide.

Table 3.6: Qualitative detection of endotoxin in SN-ppF3

Samples		
Endotoxin free water (control)	**SN-ppF3 (100 µg/mL)**	**Endotoxin standard**
-	-	+

'-' indicates the absence of hard gel. '+' indicates the presence of hard gel.

3.10 Detection of iNOS by Western blotting

iNOS is an enzyme that facilitates the conversion of arginine to citrulline during macrophages activation, releasing NO as a by-product of the reaction. In order to validate the previous NO data (Section 3.7), the presence of iNOS in the cell lysate of SN-ppF3-treated RAW 264.7 cell line was determined by Western blotting analysis. The presence of iNOS was detected in the samples as shown in Figure 3.8. The band for iNOS was clearly observed in both LPS and SN-ppF3-treated cells. However, no iNOS band was observed for non-treated cells (control). Further semi-quantitative evaluation was carried out by dividing iNOS and β-actin band intensity using ImageJ 1.48a software (National Institutes of Health). The ratios were 1.69 and 1.39 for LPS-treated and SN-ppF3-treated cells, respectively. The ratio for the control was 0 because the band intensity for iNOS could not be detected. Thus, the expression of iNOS was reasonable proxy for NO production observed previously.

3.11 Morphological observation of activated macrophages

Observation on the cellular changes is the primary evaluation of interaction between cells and treatment samples, in conjunction to the reaction of macrophages biochemical activities. As in Figure 3.9, a change of cell morphology was observed when the cells were treated with SN-ppF3 (Panel B) as compared to non-treated cells (Panel A). Formation of thread-like extension from the original individual cells in Panel B was observed and the development was increased in LPS–treated cells (Panel C). Alteration in cells shape was also observed, changing from round to asynchronous cells shape after 24 hours treatment with SN-ppF3 and LPS. These observations suggested that cells were undergoing activation in response to the treatments.

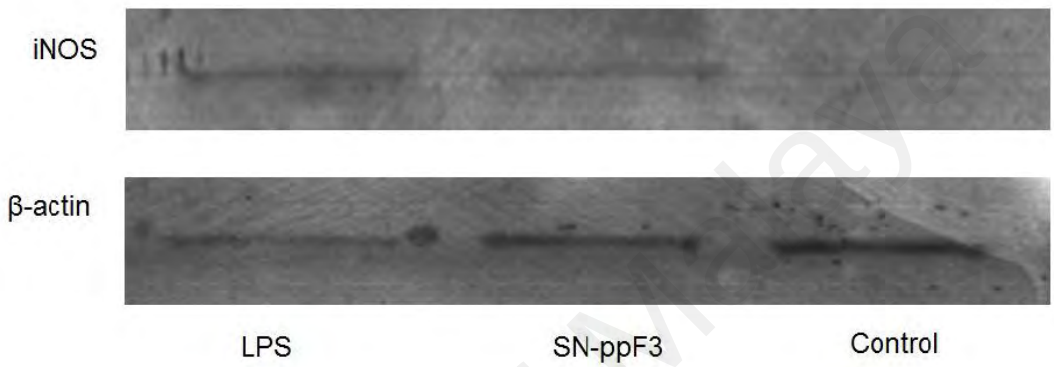

Figure 3.8: Western blot analysis for detection of iNOS and β-actin in polysaccharide-treated RAW 264.7 murine macrophage cell line

Cell lysate from control, SN-ppF3 and LPS-treated cells were subjected to 10% SDS-PAGE, followed by Western blot analysis. Top and bottom panels showed the bands developed after the membrane was incubated with respective anti-iNOS and anti-β-actin primary antibodies followed by secondary antibodies. LPS: Positive control. Control: Non-treated cells.

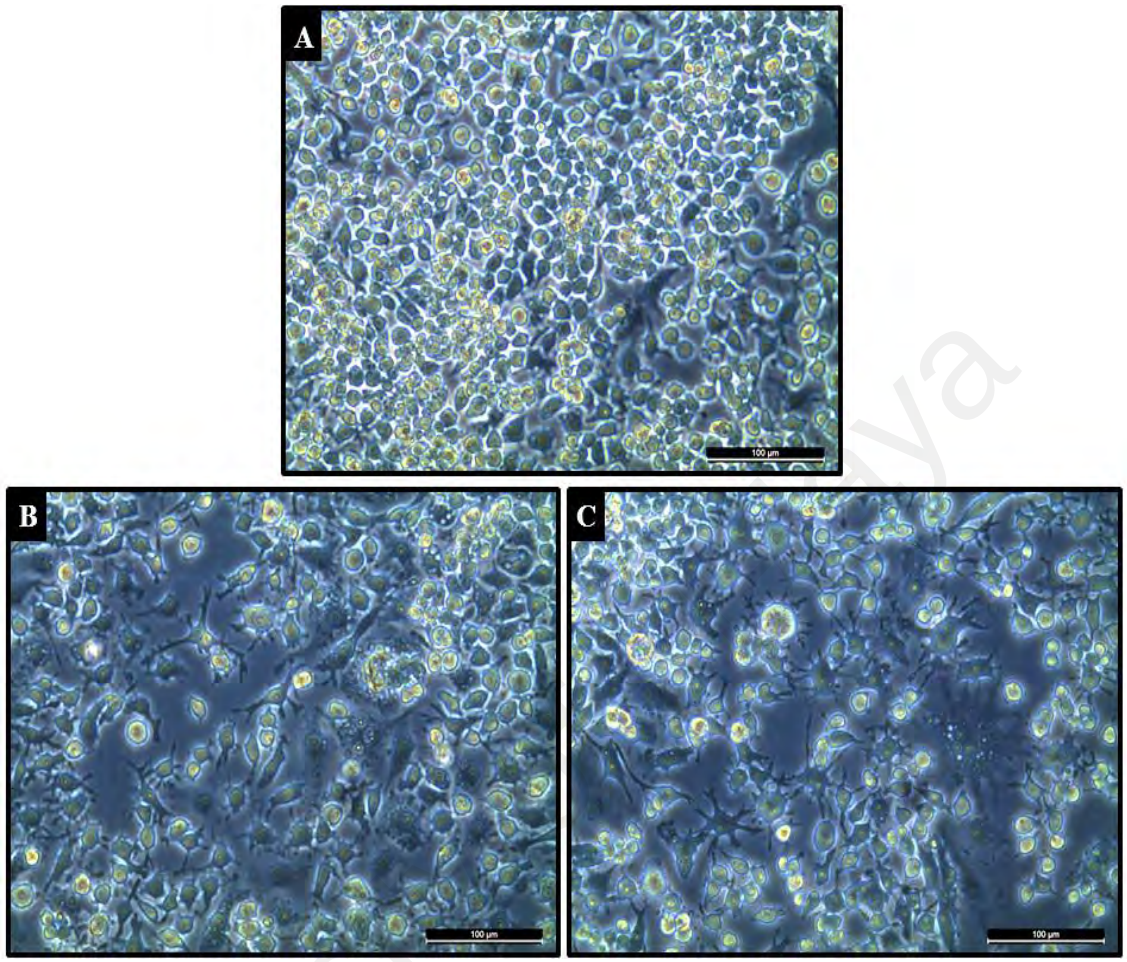

Figure 3.9: Morphological observation of (A) non-treated, (B) SN-ppF3-treated and (C) lipopolysaccharide-treated RAW 264.7 murine macrophage cell line

Cells were treated with SN-ppF3 or LPS for 24 hours, observed and photographed using a light microscope attached with a camera (Leica). Magnification power used was ×200. Observation was focus on changes of cells morphology after treatment, as compared to the non-treated cells. The formation of thread-like extension and cell shape alteration were observed after cells were treated with SN-ppF3 and LPS.

3.12 Pinocytosis analysis

One of the parameters commonly used to determine activation of macrophages is the increase in pinocytosis activity. Activated macrophages are able to uptake massive volume of extracellular fluid by endocytic activity through pinocytosis mechanism (Wang *et al.,* 2008). The ability of RAW 264.7 cells to perform pinocytosis was tested using neutral red uptake analysis. The ingested neutral red by macrophages cells was monitored at the absorbance of 540 nm. Referring to Figure 3.10, SN-ppF3-treated cells pinocytosed significantly (p<0.05) higher volume of NR solution as compared to non-treated cells, but significantly (p<0.05) lower as compared to LPS-treated cells. This NR uptake finding suggested that the macrophages were successfully activated when treated with SN-ppF3.

3.13 Phagocytosis activity

One of the main functions of macrophage is phagocytosis, which is characterised by an engulfment of large particles with diameter more than 0.5 μm. The ability of the activated RAW 264.7 cells to phagocytose foreign particles was tested using fluorescent IgG-coated latex beads. The flow cytometry analysis was carried out to indicate the phagocytic activity of SN-ppF3-treated RAW 264.7 cell line. The number of IgG-coated latex beads phagocytosed by the cells can be detected in the FITC-positive quadrant of Figure 3.11. Approximately 60.0% of cells population was phagocytosing the beads after be treated with SN-ppF3 (Figure 3.11C), significantly (p<0.05) higher from non-treated cells (~8.0%; Figure 3.11B), but significantly (p<0.05) lower as compared to LPS-treated cells (~93.0%; Figure 3.11D). This outcome could highlight the ability of SN-ppF3 in promoting macrophages phagocytosis activity by inducing the expression of Fc-γR.

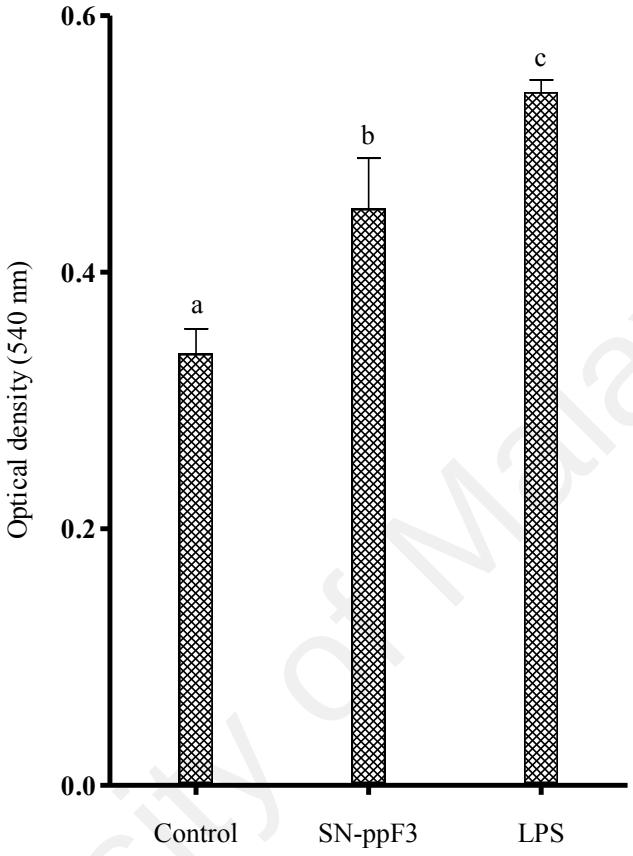

Figure 3.10: Neutral red uptake of control, SN-ppF3 and LPS-treated RAW 264.7 murine macrophage cell line for pinocytosis evaluation

Macrophages cells were treated with of SN-ppF3 and LPS at 100 µg/mL for 24 hours and incubated with neutral red solution for at least 4 hours. The pinocytosed neutral red by the cells was measured at absorbance of 540 nm. LPS-treated cells showed the highest OD followed by SN-ppF3-treated cells, indicating the activation of the cells as compared to control. Data presented were mean ± standard deviation (n=3). Different letters (a–c) indicate the significant difference at p<0.05.

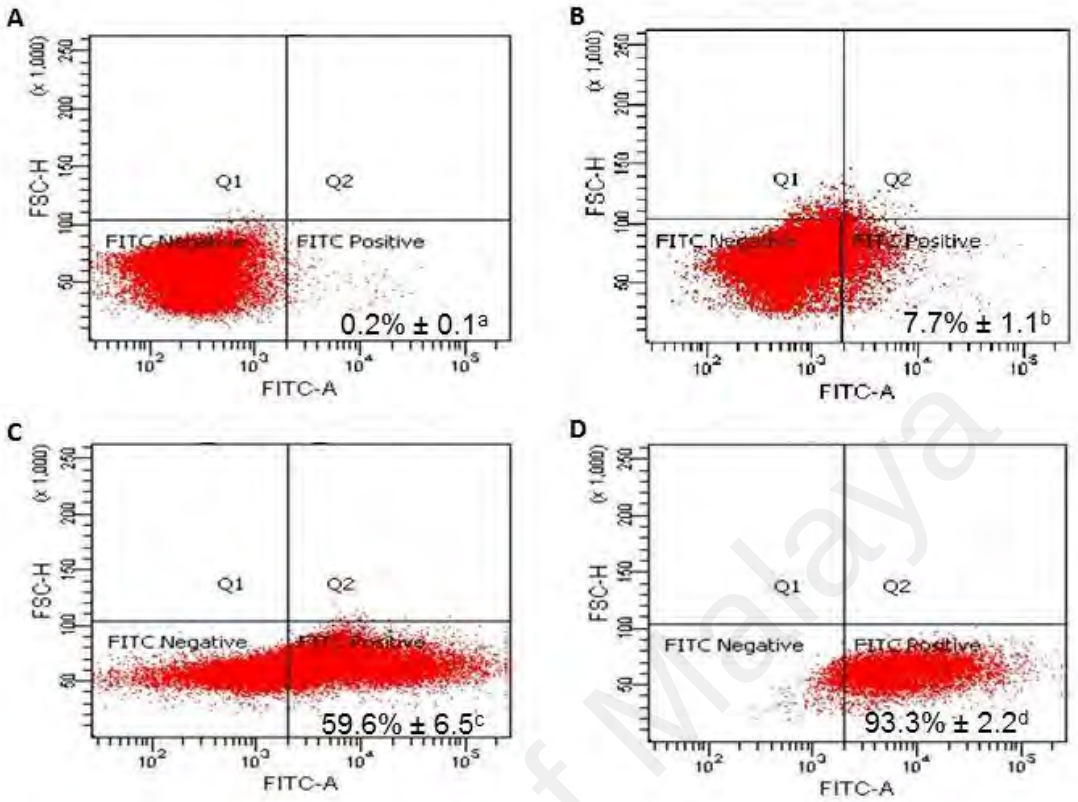

Figure 3.11: Flow cytometry analysis of IgG-coated latex beads conjugated with FITC for RAW 264.7 macrophage cells phagocytosis evaluation

Panels were (A) non-treated cells without IgG-coated latex beads, (B) non-treated cells incubated with IgG-coated latex beads, (C) SN-ppF3-treated cells incubated with IgG-coated latex beads and (D) LPS-treated cells incubated with latex IgG-coated beads. RAW 264.7 cells were treated with SN-ppF3 or LPS for 24 hours. Control and treated cells were subjected to flow cytomery analysis with at least 10,000 event numbers. Approximately 93.0% of LPS-treated cells population was phagocytosing the beads, followed by ~60.0% of SN-ppF3-treated cells, indicating the phagocytosis activity of the cells as compared to non-treated cells. Values expressed were mean ± standard deviation (n=3). Different superscripted letters (a–d) indicate the significant difference at p<0.05.

3.14 Assessment of TNF-α and IL-6 production *in vitro*

Cytokines production is often used as a measure of macrophage activation. The type of cytokine produced will also determine the effector functions of macrophages. In classically activated macrophages, TNF-α and IL-6 are the most common cytokines to be produced (Mosser and Edward, 2008). Thus, the quantification of these cytokines was important to indicate whether or not the macrophages were classically activated after SN-ppF3 treatment. In this analysis, the concentration of TNF-α and IL-6 levels were determined based on recombinant TNF-α and IL-6 standard curves, measured using ELISA. Referring to Table 3.7, the treatment of RAW 264.7 cell line with SN-ppF3 produced significantly (p<0.05) higher amount of TNF-α (~13.5 ng/mL) as compared to non-treated cells (~0.6 ng/mL). However, LPS-treated cells produced significantly (p<0.05) higher amount of TNF-α (~1443 ng/mL). The same pattern was also observed in the production of IL-6. SN-ppF3-treated cells produced significantly (p<0.05) higher amount of IL-6 (~4.0 ng/mL) as compared to non-treated cells (~2.0 ng/mL), but significantly (p<0.05) lower as compared to LPS-treated cells (4.3 ng/mL). Thus, these cytokines production results suggested that macrophages were most probably classically activated when treated with SN-ppF3.

Table 3.7: Concentration of TNF-α and IL-6 produced by RAW 264.7 cell line treated with SN-ppF3

Samples	Cytokines production (ng/mL)	
	TNF-α	IL-6
Non-treated**	0.639 ± 0.037^{a}	1.964 ± 0.024^{x}
SN-ppF3	13.461 ± 0.207^{b}	4.051 ± 0.053^{y}
LPS*	14.370 ± 0.533^{c}	4.341 ± 0.106^{z}

*Positive and **negative controls. Data expressed were mean ± standard deviation (n=3). Different superscripted letters a–c and x–z indicate the significant differences for TNF-α and IL-6 columns, respectively at $p<0.05$.

3.15 Signal transduction pathways of activated macrophage

Signal transduction pathway induced during the activation of macrophage cells varies depending on the stimuli bound to their cognate receptors expressed on the cell surface, which may lead to different effector functions of macrophages (Lawrence and Natoli, 2011). Since phosphorylation is one of the common mechanisms in activating signalling proteins (Elliott and Elliott, 2009), the identification of several phosphorylated proteins involved in inflammation signal transduction are necessary to predict the mechanism on how SN-ppF3 activates the macrophage cells. Based on the ELISA analysis monitored at 450 nm (Table 3.8), the higher absorbance value indicates the higher amount of signalling proteins expressed. In this study, SN-ppF3-treated cells expressed significantly (p<0.05) higher amount of phosphorylated-NF-κB p65 (0.5140) as compared to non-treated cells (0.455), but significantly (p<0.05) lower as compared to LPS-treated cells (0.621). Similar pattern was observed for the expression of phosphorylated-p38. SN-ppF3 and LPS-treated cells expressed significantly (p<0.05) higher amount of phosphorylated-IκB-α (0.144 and 0.175, respectively) as compared to non-treated cells (0.119). However, the expression of phosphorylated-STAT3 by SN-ppF3-treated cells was significantly (p<0.05) higher (0.487) as compared to control (0.453) and LPS-treated cells (0.415). Thus, these findings suggested that SN-ppF3 activates macrophages through signalling pathway involving phosphorylated-NF-κB p65, phosphorylated-p38, phosphorylated-IκB-α and phosphorylated-STAT3.

Table 3.8: Phosphorylation of selected signalling proteins in inflammation pathway

Protein	Absorbance value at 450 nm		
	Non-treated	**SN-ppF3**	**LPS**
NF-κB p65	3.744 ± 0.030	3.793 ± 0.020	3.745 ± 0.040
Phosphorylated NF-κB p65	0.455 ± 0.001[a]	0.514 ± 0.010[b]	0.621 ± 0.040[c]
Phosphorylated SAPK/JNK	0.089 ± 0.003	0.095 ± 0.023	0.098 ± 0.013
Phosphorylated p38	2.044 ± 0.050[a]	2.213 ± 0.090[b]	2.458 ± 0.030[c]
Phosphorylated IκB-α	0.119 ± 0.006[a]	0.144 ± 0.020[b]	0.175 ± 0.002[b]
Phosphorylated STAT3	0.453 ± 0.002[b]	0.487 ± 0.014[c]	0.415 ± 0.001[a]

LPS: Lipopolysaccharide. Data expressed were mean ± standard deviation (n=3). Different superscripted letters (a–c) in each row indicate the significant difference at p<0.05.

3.16 Oral toxicity study

Preliminary oral toxicity study was carried out to determine the toxicity effect of SN-ppF3 on healthy mice. Mice were orally administered with maximum concentration of sample treatment (500 mg/kg/bw) and normal saline (control) for 14 days, daily (Li *et al.,* 2010). Mice weight and behaviour were monitored daily. Referring to Figure 3.12, the increasing patterns in mice body weight were observed for both treatment groups from the beginning till the end of the experiment. Apparently, there is no significant (p<0.05) different between SN-ppF3-treated and control groups, which indicated that high dose of SN-ppF3 was non-toxic to the healthy mice. Moreover, all of the mice remained alive, with no abnormal behaviour and side effects commonly associated with toxicity after 14 days of the treatment session.

3.17 The effect of SN-ppF3 treatment on tumour growth

The potential of SN-ppF3 to inhibit tumour progression in tumour-bearing mice was investigated by evaluating the tumour progression before and after 10 days of treatment with doses of 250 mg/kg and 500 mg/kg. The doses of the treatment were referred according to previous documented study (Li *et al.,* 2010), accept were administered by oral not an intraperitoneal. Tumour progression was evaluated by measuring tumour volume as mentioned in Section 2.215 C. As shown in Figure 3.13, a rapid development of tumour in the tumour-bearing mice control group could be observed as early as the third day of the assay and the tumour volume was significantly (p<0.05) higher compared to the treated groups starting on day 7 (Day 3: ~0.5 cm^3; day 7: ~1.0 cm^3). As for the treated mice, whether with cyclophosphamide (CTX), low dose or high dose of SN-ppF3, it seemed that the volume of the tumours increased slightly, but the values were not statistically significant. When compared to the control group after 10 days of treatment, almost 85%, 54% and 65% of the tumour progression was inhibited in CTX, 250 and 500 mg/kg SN-ppF3 treated groups, respectively.

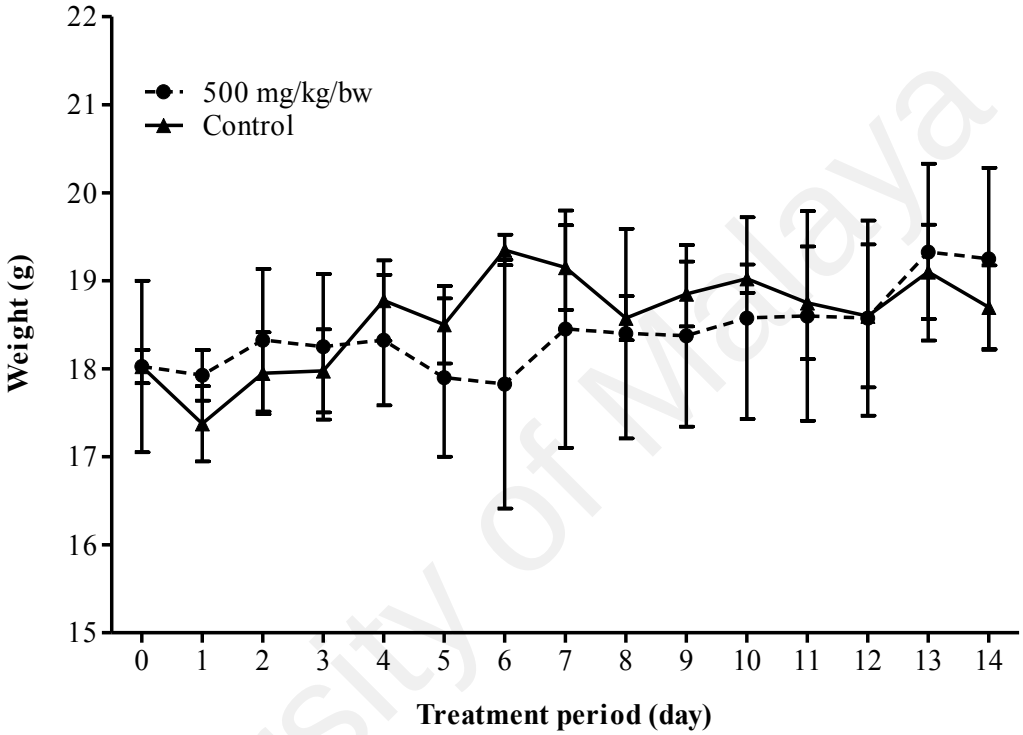

Figure 3.12: Body weight of BALB/c mice in oral toxicity study on SN-ppF3

Mice were orally administered with 200 µL of (●) 500 mg/kg/bw SN-ppF3 and (▲) normal saline (control) for 14 days, daily. Weight of each mouse was measured daily. There was no significant (p<0.05) difference in mice body weight between SN-ppF3-treated and control groups. Values presented were mean ± standard deviation (n=5).

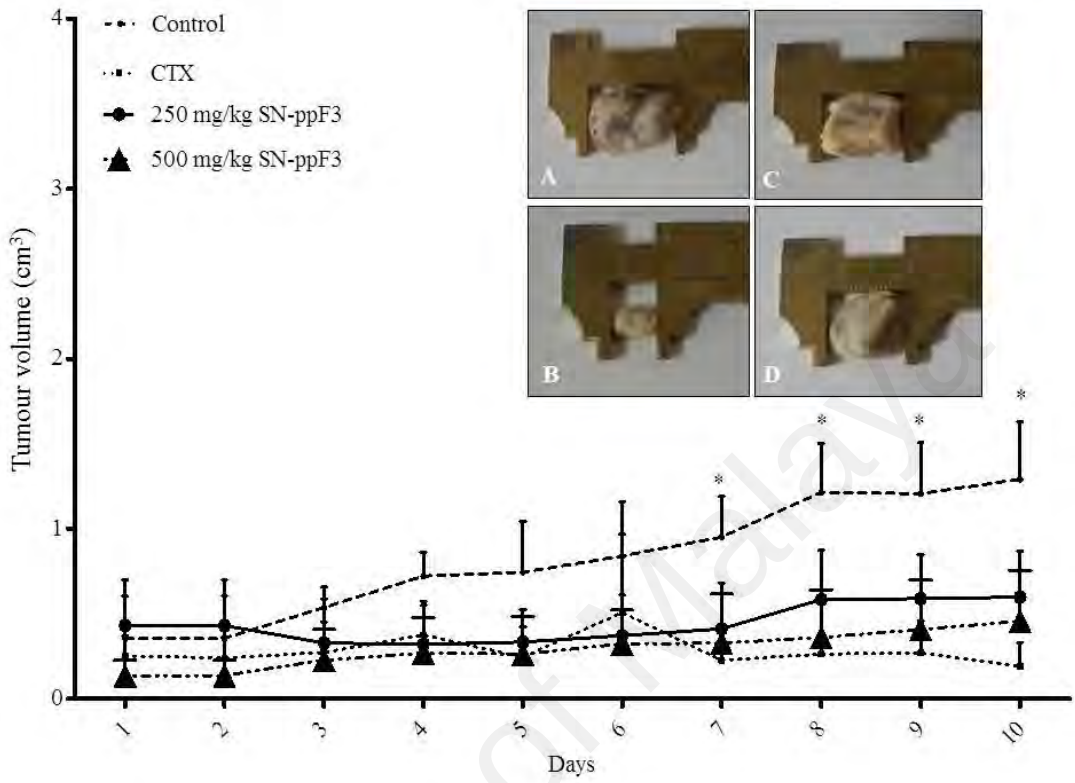

Figure 3.13: Effect of treatments with SN-ppF3 on tumour progression in tumour-bearing mice

Mice were orally administered with (●) Normal saline (control), (■) CTX, (▲) 250 mg/kg SN-ppF3, and (♦) 500 mg/kg SN-ppF3 for 10 days, daily. Tumour progression from the treated tumour-bearing mice groups were significantly inhibited as compared to control group, starting at day 7. Data presented were mean ± standard deviation (n=6). *Significant differences at p<0.05. Tumours were harvested from (A) normal saline (control), (B) CTX, (C) 250 mg/kg and (D) 500 mg/kg SN-ppF3-treated groups after 10 days of treatment.

3.18 Evaluation on tumour weight, body weight and organ indices

At the end of the treatments, the tumour, spleen, thymus and blood from control and all tumour-bearing mice treated groups were sampled. Referring to Table 3.9, the oral administration of SN-ppF3 daily significantly (p<0.05) inhibited the tumour weight with an inhibitory rate of about 38% for 250 mg/kg and 40% for 500 mg/kg treatment. Based on mice body weight after the treatments, there is no significant difference between treated groups as compared to healthy group (~19.0 g). This observation suggested that the treatments did not influence the quality life of tumour-bearing mice and as well as to conclude the side effect of SN-ppF3 consumption. The spleen weight indexes for control (~48.5 mg/g) and the treated groups (CTX: ~38.9 mg/g; 250 mg/kg SN-ppF3: ~50.0 mg/g; 500 mg/kg SN-ppF3: ~55.8 mg/g) were significantly (p<0.05) higher as compared to normal mice (~4.4 mg/g), while the thymus weight indexes for the treated groups were significantly (p<0.05) lower compared to the normal (~2.8 mg/g) and control groups (~1.0 mg/g). No significant difference was observed between CTX-treated (~0.4 mg/g) and SN-ppF3-treated groups (250 mg/kg SN-ppF3: ~0.7 mg/g; 500 mg/kg SN-ppF3: ~0.6 mg/g).

Table 3.9: Effect of SN-ppF3 treatment on tumour weight, body weight and organ indices towards tumour-bearing mice

Group	Dose (mg/kg)	Tumour weight (g)	*Body weight (g)	Spleen weight index (mg/g)	Thymus weight index (mg/g)
Normal	-	-	18.75 ± 0.64	4.39 ± 1.4^{e}	2.80 ± 0.94^{x}
Control	-	1.788 ± 0.63^{a}	20.16 ± 1.90	48.49 ± 5.72^{d}	1. 01 ± 0.11^{y}
CTX	25	0.602 ± 0.32^{c}	18.62 ± 1.90	38.89 ± 15.65^{d}	0.43 ± 0.21^{z}
SN-ppF3	250	1.117 ± 0.09^{b}	20.88 ± 0.84	50.03 ± 11.28^{d}	0.67 ± 0.19^{z}
SN-ppF3	500	1.074 ± 0.32^{b}	22.00 ± 1.55	55.78 ± 10.43^{d}	0.62 ± 0.30^{z}

Normal: healthy mice without any treatment; control: tumour-bearing mice treated with normal saline; CTX: Cyclophosphamide. Values presented were mean ± standard deviation ($n=6$). Different superscripted letters in each column indicate the significant difference at $p<0.05$. *Body weight of mice at the end of the treatment.

3.19 Measurement of cytokines production

Cytokine level in the blood serum is often used as a parameter to elucidate the induced immune responses, since cytokines play prominent roles in regulating host immune response. Thus, an investigation was carried out to see the effect of SN-ppF3 on the level of selected cytokines, namely TNF-α, IFN-γ, IL-4 and IL-6 in the serum collected from control and treated-groups of tumour-bearing mice. The concentration of these cytokines were measured based on standard curve of TNF-α, IFN-γ, IL-4 and IL-6. As shown in Figure 3.14 Panel A, a significant elevation of TNF-α level was detected in CTX and 500 mg/kg of SN-ppF3-treated groups as compared to the control group by approximately 11.8% and 12.2%, respectively. While in Panel B, the treatment of tumour-bearing mice with both concentrations of SN-ppF3 showed a significant increase of IFN-γ level in the serum by approximately 19.6% and 20.0%, respectively, while a significantly elevation of IFN-γ level (~50.7%) was detected in CTX-treated group, as compared to control group. As in Panel C, a different pattern of IL-4 level was observed. The treatment with 500 mg/kg of SN-ppF3 significantly induced the increase in IL-4 level in mice blood serum (~11.8%) as compared to the control group. However, the level of IL-4 in CTX and 250 mg/kg treated groups were significantly decreased by approximately 10.6% and 11.2%, respectively as compared to non-treated group. The level of IL-6 (Panel D) was highly elevated in control mice as compared to the healthy mice with approximately seven folds increase. Interestingly, a significant reduction of IL-6 level was detected in CTX (~60.8%) and 250 mg/kg (~62.9%) treated group as compared to control group. However, the level of IL-6 for 500 mg/kg of SN-ppF3-treated mice was significantly higher as compared to CTX and 250 mg/kg of SN-ppF3, but significantly lower (~52.4%) as compared to the non-treated group. Overall, the treatments were able to decrease the IL-6 level. These findings indicated that the

treatments could influence the secretion of these cytokines, where the changes on their concentration could be detected in the serum of the treated mice.

3.20 Histological analysis

Haematoxylin and Eosin (H&E) is a common staining procedure which clinically performed to observe cells morphology. The H&E slides of tumour tissues harvested from control and treated groups were microscopically analysed on the architecture and morphology of the tumour cells. Panel A in Figure 3.15 showed the normal cellular morphology of 4T1, mouse mammary carcinoma cell line. However, the treated tumour cells for tissues in panel B, C and D showed features of apoptosis with cellular shrinkage, the condensation of nuclear chromatin and the formation of apoptotic bodies were clearly observed. These findings suggested that cellular morphology of the treated mice tissues were altered due to the treatment. The histologic findings were confirmed by a pathologist expert, Dr. Huzlinda Hussin (MD., MPath.) from Department of Pathology, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, Serdang, Selangor, Malaysia.

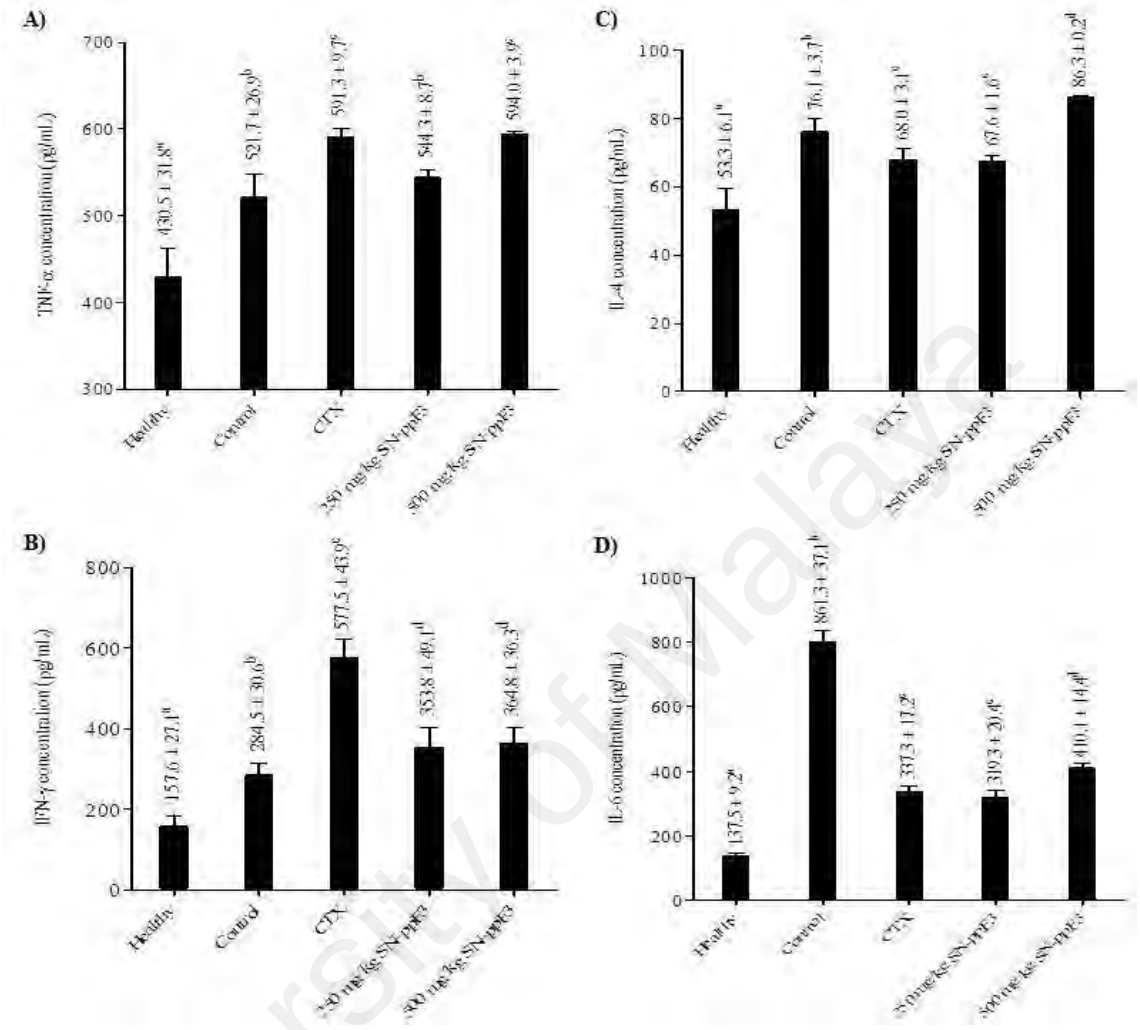

Figure 3.14: Level of selected cytokines detected in healthy and treated tumour-bearing mice

Tumour-bearing mice were orally administered with normal saline (control), CTX, 250 and 500 mg/kg of SN-ppF3 for 10 days, daily. Serum was sampled after the mice were sacrificed. The sera were subjected to ELISA analysis for detection of (A) TNF-α, (B) IFN-γ, (C) IL-4 and (D) IL-6. The treatment caused the increase in TNF-α, IFN-γ and IL-4 levels as compared to the control group, while the treatment caused the decreased in IL-6 level. Data presented were mean ± standard deviation (n=3). Different superscripted letters (a–d) indicate the significant difference at p<0.05.

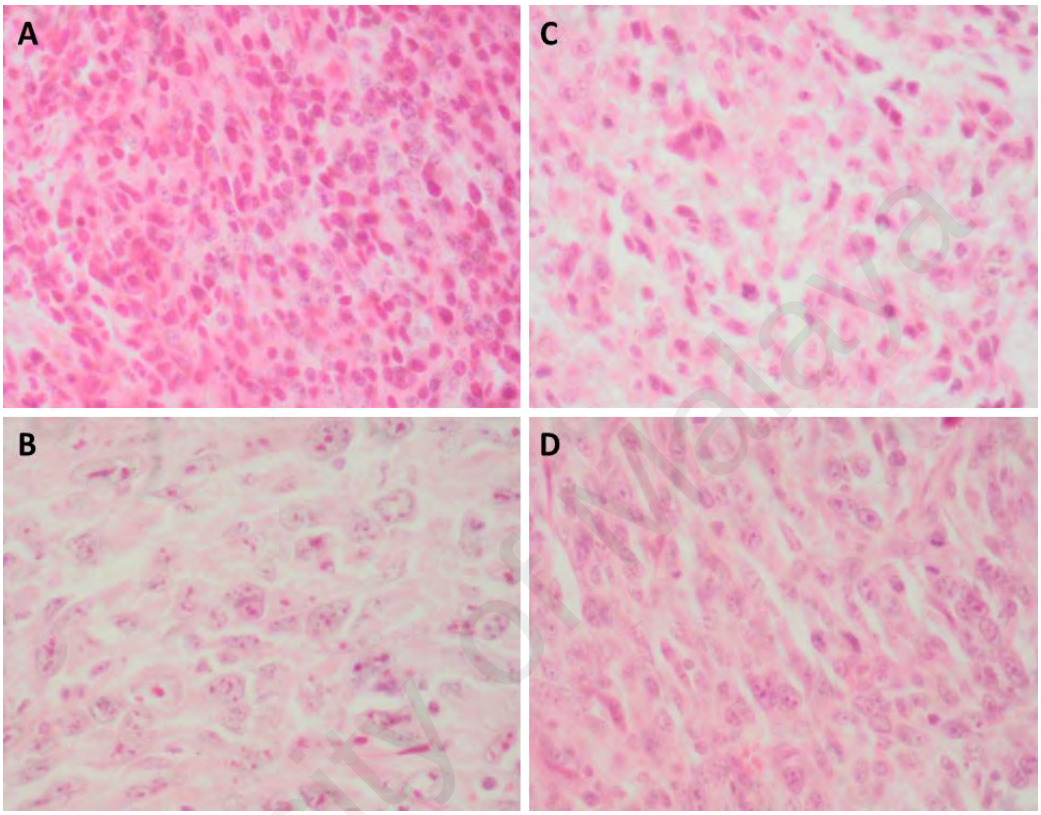

Figure 3.15: Representative of tumour tissues morphology

The tissue samples were from (A) normal saline (control), (B) CTX-treated, (C) 250 mg/kg and (D) 500 mg/kg SN-ppF3-treated groups were stained using H&E staining method and photographed. Magnification power used was ×400. Alterations of cells morphology due to apoptosis were observed in tumour tissues from treated groups as compared to the control group.

3.21 Apoptosis detection of tumour tissue by TUNEL assay

The previous histological observation via H&E staining (Figure 3.15) indicated that the tumour tissues from CTX and SN-ppF3-treated groups were morphologically distorted most probably due to apoptosis. In order to validate this observation, a specific apoptosis detection procedure was carried out by TUNEL assay. Referring to Figure 3.16, a highly significant (p<0.05) fluorescein intensity in the tumour tissue from (B) CTX, (D) 250 mg/kg and (C) 500 mg/kg of SN-ppF3-treated groups as compared to the normal saline-treated group (control: tissue A) was observed. However, the mean fluorescent intensity of tissue sample from 500 mg/kg of SN-ppF3-treated group was significantly higher (p<0.05) as compared to 250 mg/kg of SN-ppF3, but not significant as compared to CTX-treated group. The mean fluorescent intensity (E) in slides of treated groups was significantly higher as compared to the control group. However, the mean fluorescent intensity of tumour tissue from 500 mg/kg SN-ppF3-treated group was significantly higher as compared to 250 mg/kg SN-ppF3-treated group, but non-significant as compared to the CTX-treated group. These findings strongly suggested that the treatments of SN-ppF3 were able to suppress tumour progression via apoptosis, which supported the H&E analysis.

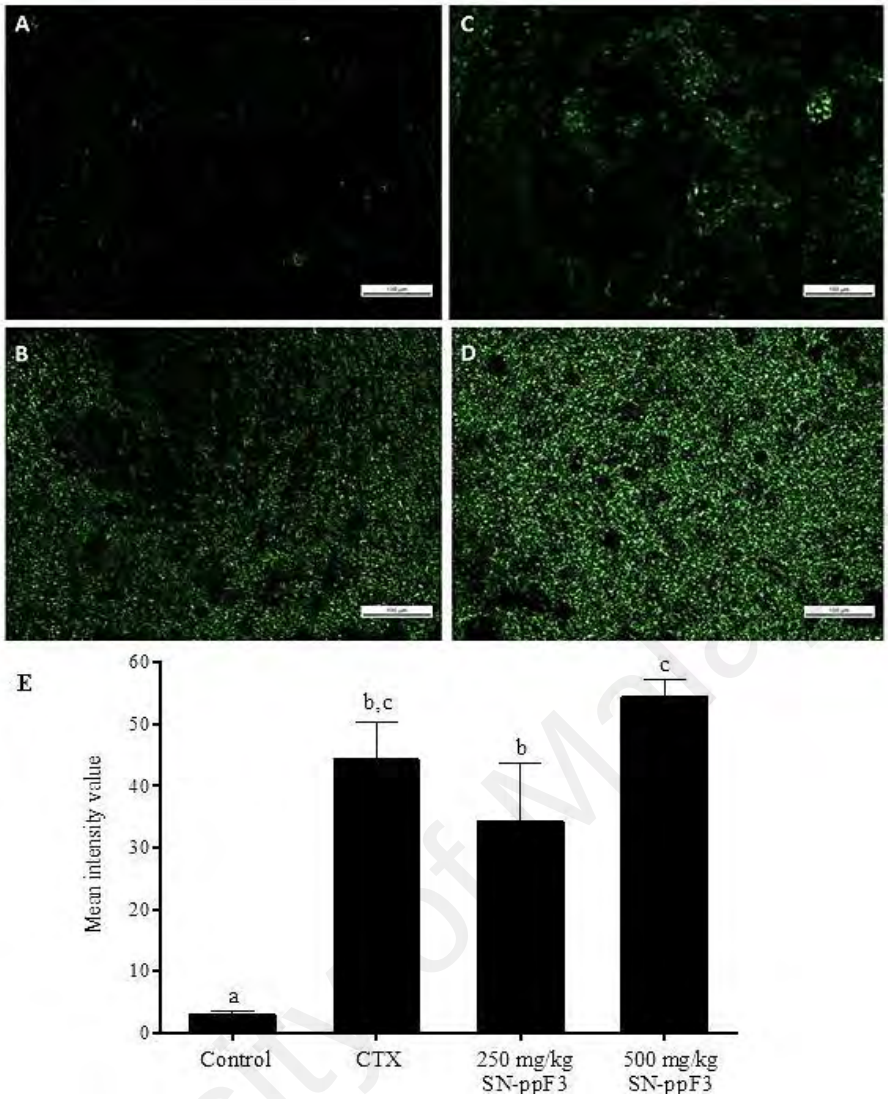

Figure 3.16: Cell death detection by TUNEL assay on representative tumour tissue

The tissue samples from (A) normal saline-treated, (B) CTX-treated, (C) 250 mg/kg and (D) 500 mg/kg SN-ppF3-treated groups were stained with TUNEL staining solution and photographed. Magnification power used was ×200. Scale showed 1 cm = 100 μm. The fluorescent intensity (E) for each slide were quantified and statistically analysed using ImageJ 1.49 software (National Institutes of Health) and 2-tailed T-test, respectively. The mean fluorescent intensities in slides of treated groups were significantly higher as compared to the control group. However, the mean fluorescent intensity of tumour tissue from 500 mg/kg SN-ppF3-treated group was significantly higher as compared to 250 mg/kg SN-ppF3-treated group, but not significant as compared to the CTX-treated group. Different letters a–c in E indicate the significant difference at $p<0.05$ ($n=3$).

3.22 Detection of infiltrating immune cells by immunofluorescent

Further evaluation was done on the tumour tissues harvested from control, CTX and SN-ppF3-treated tumour-bearing mice to detect the infiltration of immune cells such as NK cells, $CD8^+$ T cells and macrophages into the solid tumour. In this experiment, the paraffin blocks of the tumour tissues from control and treated mice groups were sectioned and fixed on slides and then stained with anti-69 NK cell, anti-$CD8^+$ and anti-F4/80 macrophage antibodies conjugated with fluorescent dye and observed (×200) under a fluorescent microscope. The intensities of fluorescent dye on the tissue slides were assessed qualitatively. In the first column panels of Figure 3.17, 500 mg/kg of SN-ppF3 treated group (D1) showed the most intense staining of NK cells in the tumour tissues followed by CTX (B1) and 250 mg/kg SN-ppF3 (C1) treated groups as compared to the control tumour tissue (A1). In the second column panels, tumour tissues from the CTX (B2), 500 mg/kg SN-ppF3 (D2) and 250 mg/kg SN-ppF3 (C2) treated groups showed most intense staining of $CD8^+$ T cells as compared to the control tumour tissue (A2). The presence of macrophages was also detected as high FITC intensity in tumour tissue from 500 mg/kg SN-ppF3-treated group (D3), followed by the CTX-treated group (B3). However, low fluorescent intensity was observed in tumour tissue treated with 250 mg/kg SN-ppF3 (C3) as compared to the control (A3) group. These results suggested the increase in infiltration of immune cells into the solid tumour.

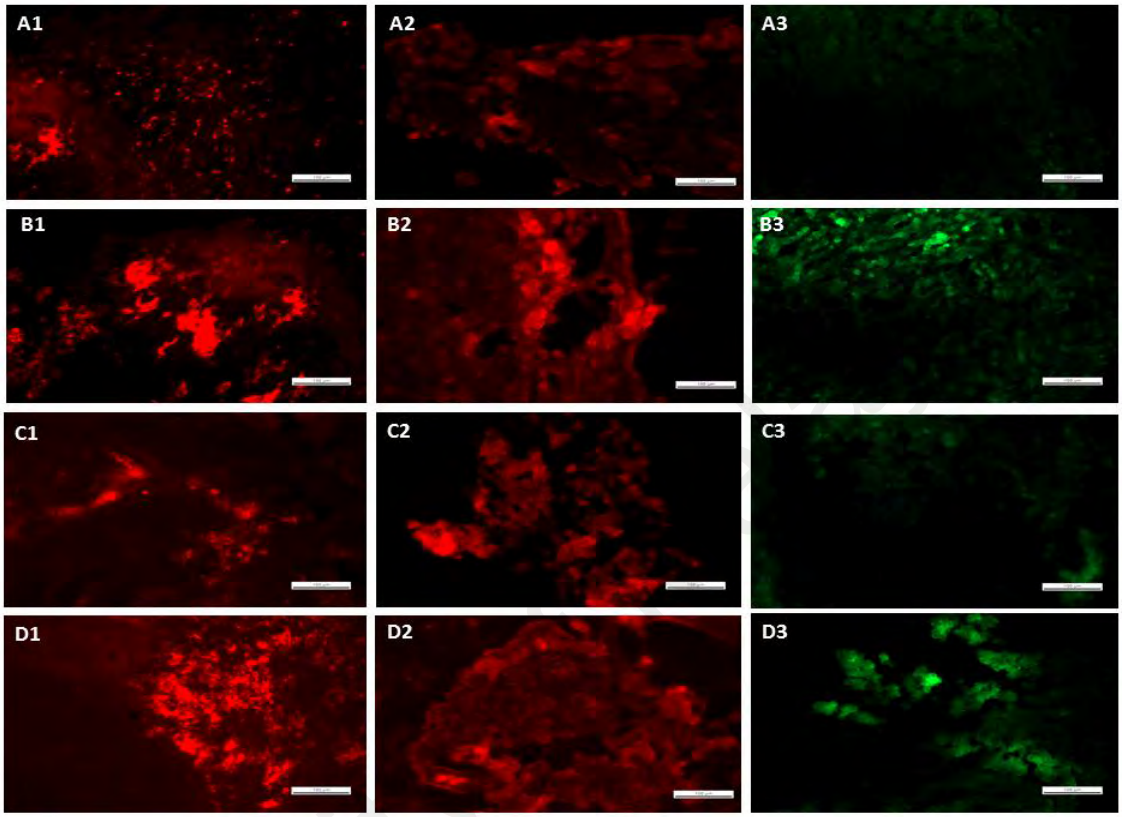

Figure 3.17: Immunofluorescent staining of representative tumour tissues

The tissue samples from non-treated (A1, A3 and A3), CTX-treated (B1, B2 and B3), 250 mg/kg (C1, C2 and C3) and 500 mg/kg of SN-ppF3 (D1, D2 and D3)-treated groups were stained with antibodies conjugated with anti-NK cells (first column, 1), anti-$CD8^{+}$ T cells (second column, 2) and anti-macrophage cells (third column, 3) and photographed. Higher fluorescent intensity in each slide (column 1, 2 and 3) of treated groups (B, C, and D) was observed as compared to control group (A). Magnification power used was ×200. Scale showed 1 cm = 100 μm.

3.23 Cytotoxicity evaluation *in vitro*

In order to confirm whether SN-ppF3 has a direct cytotoxicity effect towards 4T1, mouse mammary carcinoma cell line, an MTT assay was carried out. As shown by the dose-response curve (Figure 3.18), the IC_{50} value of SN-ppF3 could not be determined where the IC_{50} value of the sample was above 100 μg/mL. According to the U.S National Cancer Institute plant screening program, the plant crude extract with IC_{50} value of 20 μg/mL or less was considered to have cytotoxicity effect on tested cancer cells upon 48–72 hours of incubation (Lee and Houghton, 2005). This result suggested that SN-ppF3 has no active cytotoxicity effect, directly towards 4T1 cell line after 72 hours incubation period.

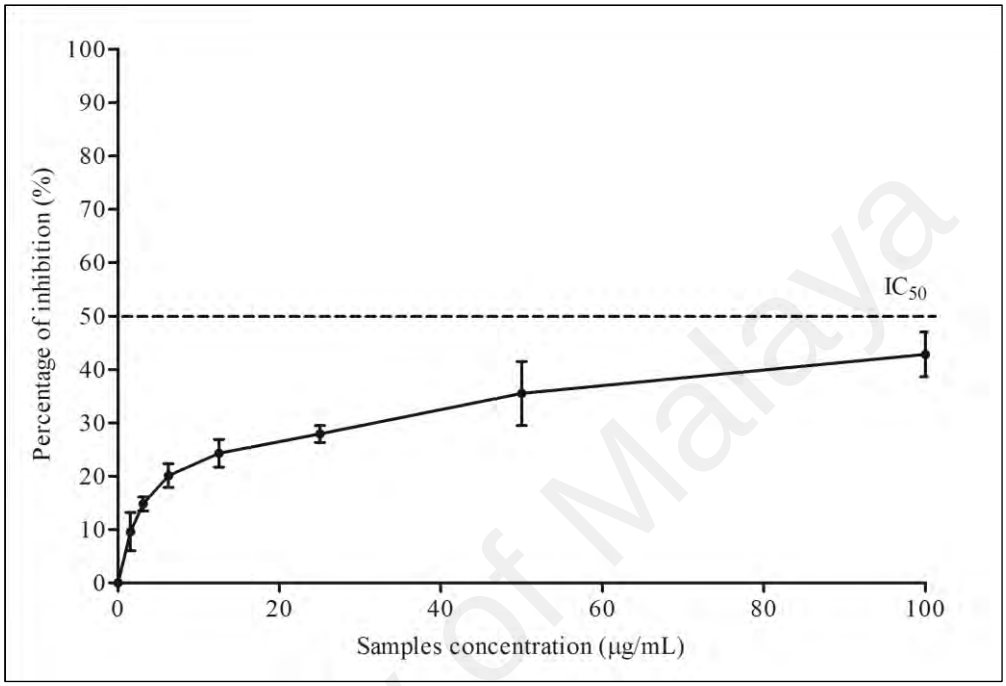

Figure 3.18: Percentage of inhibition of 4T1, mouse mammary carcinoma cell line treated with SN-ppF3

Cells were treated with SN-ppF3 at sample concentration of 0, 1.56, 3.125, 6.25, 1.25, 25, 50 and 100 µg/mL for 72 hours. The viability of the cells after treatment was determined by MTT assay. The IC_{50} value was determined based on cells inhibition percentage at 50% (dashed line). The IC_{50} value of SN-ppF3 could not be determined where the IC_{50} value of the sample was above 100 µg/mL. Data presented were mean ± standard deviation (*n*=3).

CHAPTER 4: DISCUSSION

Referring to Chapter 3, five polysaccharide fractions were successfully isolated. All polysaccharide fractions were proven to have the ability to induce nitric oxide (NO) production. Due to its ability to induce highest NO production and possessed the lowest inhibitory effect, only polysaccharide fraction 3, SN-ppF3 was selected to be further analysed and used in the other subsequent macrophages activation assays. Based on results obtained, SN-ppF3 was shown to be able to activate macrophages due to its ability to induce pinocytosis, phagocytosis and the production of pro-inflammatory cytokines. Based on the *in vivo* study, the treatment was able to inhibit tumour progression in breast tumour-bearing mice, most probably by inducing host immune system. These findings will be further discussed in details, in this section.

4.1 Preparation of crude polysaccharide from *S. nigrum*

Crude polysaccharide was extracted out by using hot water extraction method. In this extraction procedure, *S. nigrum* sample was immersed in distilled water at 95°C for at least 5 hours. As high energy was continuously applied, crude polysaccharide was extracted out from the plant sample into the solution. Commonly, there are several other polar compounds that can be extracted out from the plant as well if water was used as a solvent, such as amino acids, monosaccharides, disaccharides and polysaccharides (Houghton and Raman, 1998). In the extraction method described in Section 2.2.1, the sample further underwent double-solvent extraction process first with petroleum ether and followed by 80% ethanol using soxhlet apparatus. Petroleum ether, a solvent with low polarity, is chemically used in extracting several classes of chemical compounds, such as waxes, fats, fixed oils and volatile oils. Next, slightly higher polar solvent, an aqueous ethanol, was used as a solvent to extract out proteins, monosaccharide and disaccharide from the plant sample (Houghton and Raman, 1998). After the solvent

extractions, it was expected that only polysaccharide was mainly present in the sample, and thus, the hot water extraction was used to harvest out the remaining polysaccharide residues.

4.2 Cytotoxicity evaluation of *S. nigrum* polysaccharide samples

The common method to study the toxicity effect of a given sample is through conventional MTT cytotoxicity study against a cell line. In this study, RAW 264.7 cell line was tested with series of crude polysaccharide sample concentrations. Cytotoxicity potential of the samples was determined by looking at the IC_{50} value, a sample concentration that causes 50% of cell inhibition or cell death (Lai *et al.,* 2015).

The IC_{50} value for crude polysaccharide samples extracted from leaves, stem and combination between fruit, leaves and stem were 52, 50 and 51 μg/mL, respectively. According to the U.S. National Cancer Institute plant screening program, the plant crude extract with IC_{50} value of 20 μg/mL or less was considered to have cytotoxicity effect on tested cancer cells upon incubation for 48–72 hours (Lee and Houghton, 2005). As the IC_{50} values seen in this present study are more than 20 μg/mL, it could therefore be concluded that the crude polysaccharides samples are not actively cytotoxic against RAW 264.7 cells

However, looking at inhibition percentage at the maximum concentration of 100 μg/mL tested, the crude polysaccharide isolated from stem induced the least cell inhibition as compared to that of leave and combination of fruits, leave and stem crude polysaccharide. It was previously reported that *S. nigrum* contained glycoalkaloids compound such as solanine, which is toxic even in small quantity, and was recently reported responsible for inducing acute interstitial nephirtis in kidney (Oh *et al*., 2016).

Alkaloid derivatives which are conjugated with polysaccharide chain might possibly co-purified during the isolation process. Alkaloids are mostly concentrated and can naturally be found in leaves and fruits of the plant, which function as major natural defences against herbivores (Jain *et al.,* 2011). However, the extraction processes which favours high molecular weight polysaccharide isolation were most probably able to eliminate these alkaloid compounds, which resulted in stem sample with the least inhibitory effect. Sample with the least inhibitory activity to the cell will ensure adequate number of cells during the treatment session.

The evaluations of cytotoxic activity of semi-purified polysaccharide fractions were also carried out against RAW 264.7 cell line. Findings show that none of the polysaccharide fractions had cytotoxic effects on RAW 264.7 cells after 72 hours incubation period, thus strengthening the fact that most polysaccharides derived from higher plant were mainly non-toxic (Schepetkin and Quinn, 2006). Unlike microbial-derived polysaccharides, the negative effects due to the interaction between plant polysaccharide and immune cells have not yet been reported. The interaction is only limited to the immune cells activation or differentiation. However, negative implications of microbial-derived polysaccharides towards immune cells are well studied. The exposure of selected immune cells to microbial polysaccharides may result in apoptosis (Pericolini *et al.,* 2006; Robinet *et al.,* 2014).

However, by looking at the killing percentage the maximum concentration of 100 μg/mL, SN-ppF3 had the lowest inhibitory effect. Referring to the ion-exchange profile, SN-ppF3 was mildly charged as compared to the other fractions. In several cases, polysaccharides with too strong positively or negatively charged were able to interact with variety of proteins (Zhang *et al.,* 2013), thus lead to the dysfunctional of

protein-based components such as cell membrane. However, the charges for SN-ppF1-5 were not strong enough to exhibit the cytotoxicity action towards RAW 264.7 cell line.

4.3 Preparation of semi-purified polysaccharide sample from *S. nigrum*

The semi-purified polysaccharide sample was obtained by subjecting the crude polysaccharide of S. *nigrum* stem into DEAE ion-exchange chromatography column. The plotted chromatography profile indicates that crude polysaccharide was resolved into five peaks high in sugar residues within the first 30 fractions. No peak was detected beyond fraction 30. Those peaks were pooled and labelled as SN-ppF1, SN-ppF2, SN-ppF3, SN-ppF4 and SN-ppF5.

Previously, Li *et al.,* (2010) also performed ion-exchange chromatography on crude polysaccharide extract of *S. nigrum*. However, the profile obtained in our study was not comparable with the one reported by Li's group, as they obtained only three major peaks. This difference could be explained based on the sample used, where the extract in our study was prepared only from the stem part of *S. nigrum* instead of the whole plant. Moreover, the plant was grown in a different location and climates. Li's group obtained their sample from Taihang Mountain in Hebei province located in China, whereas the samples for this study was grown by local farmer at a lower ground in tropical climate, located in Raub, Pahang, Malaysia. It is well documented that the differences in environment, season and climate will affect the plant composition (Batovska *et al*., 2008; Cartea *et al*., 2010; Farah and Donangelo, 2006).

4.4 Characterisation of *S. nigrum* semi-purified polysaccharide samples

4.4.1 Carbohydrate and protein content estimation

The importance of this analysis was to detect the purity level of polysaccharide fraction where sample with high carbohydrate content indicates a highly purified polysaccharide fraction. An established phenol-sulphuric acid method was used to measure the carbohydrate content in polysaccharide fractions. Basically, a concentrated sulphuric acid was used to break down polysaccharides, oligosaccharides and disaccharides into monosaccharides. Then, the 5-carbons sugars (pentoses) were dehydrated to form a furfural ring, while 6-carbons sugars (hexoses) to form a hydroxyl furfural. These compounds reacted with phenol at high temperature and produce yellowish solution which can be quantitatively measured at 490 nm (Masuko *et al.,* 2005).

SN-ppF1 has the highest carbohydrate content among the five samples followed by SN-ppF4, SN-ppF3, SN-ppF2 and then SN-ppF5. All five samples did not show a 100% carbohydrate content and thus indicated the fractions were not purely polysaccharide. It is possible contaminant presents in the polysaccharide fractions such as glycoprotein, a protein molecule that covalently attached with oligo- or polysaccharide chains, which might have precipitated during the purification process. Some glycoproteins are known to have an immunomodulatory activity (Chen *et al.,* 2010; Park *et al.,* 2012). To rule out that the samples are not glycoproteins, protein estimation was carried out.

The determination of protein content in all polysaccharide fractions was carried out by using Bradford assay. Basically, the Bradford assay relies on the binding of Coomassie blue G250 dye to protein. A very low protein concentration was detected,

which was less than 1% of total protein in each polysaccharide fraction. The small amount of proteins detected in each fraction was expected not to contribute significantly in the outcome for the subsequence immune cells activation assays. The low ratio of protein to high carbohydrate content suggested the fractions were not glycoproteins.

Referring to both carbohydrate and protein content results, though polysaccharide was the major component, none of the five samples can be considered purely polysaccharide. Even though the extraction process favours polysaccharide isolation, the other glycoconjugates compound beside glycoprotein such as glycolipids, maybe present in those polysaccharide fractions. Another possible explanation would be the sugar content of the polysaccharide. Those polysaccharide fractions compose other sugar beside glucose. Thus the estimation might not as accurate since the standard used was glucose alone. Taken together, the total of polysaccharide content obtained in the fractions were between 74–98%. The carbohydrate content in those polysaccharide fractions were comparable with some previously reported purified plant polysaccharides, who managed to extract out 58–81% (Jia *et al.,* 2015), 39–58% (Sanandiya *et al.,* 2014) and 80% (Han *et al.,* 2016) of carbohydrate content.

4.5 Measurement of NO production

The release of NO by macrophages is one of the hallmarks of macrophage activation. The reaction between NO and hydrogen peroxide or superoxide produces a peroxynitrite radical, a killing substance for phagocytosed microbes in macrophages. Other than eliminating microbes, NO is also considered to be one of the most important mediators directly involved in tumour-cell killing (Klimp *et al.,* 2002). Thus, the ability of polysaccharide fractions to activate macrophage could be measured by estimating the NO production. Fundamentally, the reaction between sulphanilamide and NED (Griess

reagent) with nitrite ions presence in culture media under the acidic condition will produce an azo-compound, which could be detected by measuring the OD value at 520 nm (Promega, 2009).

Significant increases (p<0.05) in NO production were detected, suggesting that the polysaccharide fractions were able to induce NO production by activating macrophages in a dose-dependent manner. Although the NO concentration produced were significantly lower than that of LPS-stimulated cells, the amount were comparable to NO production from 100 µg/mL of Jew's ear purified polysaccharide (Yu *et al.,* 2009), red ginseng acid crude polysaccharide (Byeon *et al.,* 2012) and Changsong-I mushroom β-glucan (Bae *et al.,* 2013). However, crude polysaccharide samples isolated from the other *Solanaceae* species namely *S. melongena*, *S. macrocarpon*, and *S. torvum* did not significantly stimulate NO production in RAW 264.7 cell line (Ng, 2015). Although NO is important in pathogen elimination, excessive production is undesirable. NO concentration beyond the physiological level in humans may cause systemic acute inflammatory or septic shock and possibly lead to some other health complications such as pathologic vasodilatation and tissue damage (Stoclet, 1999; Titheradge, 1999).

4.6 Detection of iNOS by Western blotting

Macrophage cell is one of innate immune cells that are majorly involved in modulating inflammation and immune response to maintain a defensive reaction (Kang *et al.,* 2011) against various antigens. Briefly, the binding of activator such LPS to CD14 triggers the signalling cascade through Toll-like receptor 4 presented on the macrophages cell membrane (Lowenstein and Padalko, 2004) which in turn activates NF-κB signalling pathway. Upon activation, phosphorylated NF-κB-p65/p50 subunit is

translocate from cytosol into the nucleus and acts as a major transcription factor for regulation of iNOS expression (Guha and Mackman, 2001; Konkimalla *et al.,* 2010). The binding of the heterodimer subunits to its DNA motif modulates a transcription of several targeted genes, especially pro-inflammatory mediator such iNOS, COX-2 and various of pro-inflammatory cytokines (Chao *et al.,* 2010). In resting macrophages, the NF-κB subunits, p65 and p50 are forming a complex with inhibitory factor, IκB-α causing it to remain inactive in cell cytosol.

NO is a short-lived radical species synthesised during natural enzymatic conversion of L-arginine to L-citruline facilitated by iNOS (Kerwin and Heller, 1994). The level of iNOS expressed is related to the degree of inflammation and reflected to the amount of NO released by classically activated macrophages. In order to validate the result in NO production assay, a Western blot analysis was carried out to detect the presence of iNOS in the cell lysate. The intensity of iNOS bands in SN-ppF3 and LPS-treated macrophage cells proved the correlation between iNOS expression and the secretion of NO. iNOS is commonly absent in resting macrophages and only be synthesised when the cells are activated. Thus, the amount of iNOS expressed in the cell matrix is comparable to the production of NO.

4.7 Chemical characterisation of SN-ppF3

The biological activity of an immunomodulatory polysaccharide depends on many factors including chemical composition, glycosidic linkages, branching, solubility and molecular weight. Therefore, some characterisations were carried out on SN-ppF3 in order to understand how the biological activity was induced.

4.7.1 Molecular weight determination

Several of physicochemical characteristics, especially the molecular weight of polysaccharide can influence its bioactivity (Zhou *et al.,* 2004). Therefore, determining the molecular weight was the first step to characterize the polysaccharide. A previous study reported that the bioactive polysaccharides with very high molecular weight would create an unfavourable interaction or membrane barriers penetration that will affects the pharmacological activity (Alban and Franz, 2000). Conversely, polysaccharides with lower molecular weight can produce interaction and bioactivity towards targeted organisms (Li *et al.,* 2015).

Various sizes of immunomodulatory polysaccharides have been reported, ranging from about 1–7000 kDa (Ramberg *et al*., 2010). A study carried out on partially cellulose digested Aloe polysaccharide revealed that polysaccharide fractions within the size of 5–400 kDa had the most potent anti-cancer activity, indicating size of polysaccharide may contributes to its biological activity (Im *et al*., 2005). In this study, the molecular weight of SN-ppF3 was estimated approximately 109.42 kDa. SN-ppF3 was considered to have an average molecular weight and believed to be able to exert strong pharmacological effects towards tested subjects. However, considering the recognition of polysaccharides by macrophages receptor, the relationship between ligand and receptor is very important to initiate activation of macrophages.

4.7.2 Monosaccharide compositional analysis

To determine the monosaccharide composition, SN-ppF3 was hydrolysed with a mild acid at high temperature for a few hours. At this state, hydrogen bonds that connect between monosaccharides will be broken, producing separate single monosaccharides in the solution. A HPLC analysis was carried out to separate those monosaccharides based

on their molecular weight and structure. The types of monosaccharide were determined by comparing the retention time with a set of neutral monosaccharide standards. At least 3 types of monosaccharide were detected in hydrolysed SN-ppF3, which were rhamnose, glucose and galactose. However, several other monosaccharides might be present in SN-ppF3, since only neutral monosaccharide standards were used in this experiment. Based on detected sugars, it was predicted that SN-ppF3 belonged to either rhamnogalacturonan or rhamnose hexose types of pectic polysaccharide group (Khoo *et al*., 2014).

A rhamnogalacturonan isolated from leaves of *Panax ginseng* CA. Mayer (Shin *et al*., 1997) was reported to possess an immunomodulatory property where it elevated immune response activity of macrophage cells by enhancing the expression of Fc receptor on macrophage surface (Sun *et al*., 1994). However, more in-depth analysis needs to be carried out in determining the polysaccharide structure and how it interacts with macrophage, considering the structural factors may influence macrophage activation activity (Lin *et al*., 2015).

4.7.3 FT-IR spectroscopy analysis

Aside from molecular weight and monosaccharide composition determination, SN-ppF3 was also subjected to FT-IR spectroscopy analysis for further characterisation in term of its structural information. Based on the FT-IR spectrum, several related peaks were detected, which displayed a typical absorption of polysaccharide peaks (Zheng *et al*., 2016; Zhu *et al*., 2013). The broadly stretched intense peak at 3424 cm^{-1} was designated to hydroxyl (–OH) stretching vibration, followed by a weak C–H peak at 2932 cm^{-1} (Zhao *et al.*, 2015). The relatively strong asymmetric peak at around 1656 cm^{-1} indicated the characteristic of deprotonated carboxylic group (COO^{-}) (Wu *et al.*,

2015), suggesting the presence of uronic acid in this polysaccharide (Wu *et al.*, 2016), which could not be determined by HPLC analysis (Section 4.7.2). A weak absorption peak at 1409 cm^{-1} correspondent to the vibration of CH_2 and C–OH groups (Zheng *et al.*, 2016; Zhao *et al.*, 2010). The absorption peaks at range 1078–1034 cm^{-1} indicated as aldehyde (C–O–H) and ketone (C–O–C) groups (Zhao *et al.*, 2010). The absorption peak at about 775 cm^{-1} suggested the rings vibration due to α-configuration of rhamnose units (Yi *et al.*, 2011). Overall, the data further supported the observed sugar residues by HPLC analysis (Section 4.7.2). With the detection of uronic acid, it strengthens the prediction that SN-ppF3 was rhamnogalacturonan. Based on the FT-IR data, it was suggested that SN-ppF3 possessed potential unique binding features that allow to be recognise by certain immune cells, and subsequently stimulates immune responses (Plato *et al.*, 2013; Takeuchi and Akira, 2010).

4.8 Activation of murine macrophage cell line

4.8.1 Morphological observation

Morphological alteration of macrophage cells was observed when cells were treated with LPS and SN-ppF3. One of the common morphological changes of classically activated macrophages is the cells shape alteration, where larger and irregular cell shape was observed instead of smaller round or circular shape of resting macrophage cells (Kang *et al*., 2011). The morphological alteration is mediated by the increase in actin filaments, as a response towards pro-inflammatory factor such as LPS or IFN-γ (Shinji *et al*., 1991). As a result, an extension formation of filopodia, lamellipodia and membrane ruffles was observed in this study, reflecting the actin cytoskeletal reorganisation (Williams and Ridley, 2000) as preparation for macrophages function enhancement, especially for phagocytosis (Kress *et al*., 2007)

4.8.2 Pinocytosis analysis

One of the well-known macrophages characteristics is the capability of the cells to uptake large volume of fluid phase materials by pinocytosis and antigen ingestion by phagocytosis. Commonly, macrophages are able to infuse extracellular fluids with small particles from surrounding environment into the cytoplasm by non-specifically endocytic process. However, it was reported that the activity of pinocytosis remarkably increases when cells were treated with inflammatory factor such as LPS (Wang *et al*., 2008). In this analysis, the result suggested that the treatment of RAW 264.7 cells with SN-ppF3 significantly increased the pinocytosis activity as compared to that of non-treated cells, although not as high as LPS-treated cells. The constitutive process of pinocytosis could continuously occur and it is enhanced with activator such as LPS. A previous research stated that pinocytosis activity in macrophage is significantly enhanced only the cell was exposed to LPS with concentration of 100 ng/mL or higher. Since phagocytosis prefer complementary C3b or IgG appropriate binding, pinocytosis can act as an additional support to phagocytosis in eliminating various types of antigens (Peppelenbosch *et al*., 1999).

4.8.3 Phagocytosis activity

The other hallmark to indicate the activation of macrophage is to measure the phagocytosis activity of macrophage cells, which is an important physiological process, characterised by a specific engulfment of large particles with diameters of more than 0.5 μm. The process begins with the binding of the particles via receptors on the macrophage surface, leading to the formation of phagosome, and eventually to the death of the invading cell or ingestion of the foreign particles. In this current study, the treated RAW 264.7 cell line was introduced with rabbit-IgG coated latex beads conjugated with FITC, as a probe for the identification factor. The conjugated rabbit-IgG on the surface

of latex beads helps in opsonisation process, made the bead easily be recognised by Fc-γ receptor (Fc-γR) on the surface of the activated macrophage.

Observations show that the degree of phagocytosis activity of SN-ppF3-treated cells was significantly higher as compared to the non-treated cells, but significantly lower as compared to LPS-treated cells. The pre-treatment of cells with the sample and LPS was to initiate the activation of the macrophages. Prior the activation, PRRs such TLRs and G protein-coupled receptor, expressed by the macrophages can recognise the sample and LPS. The binding induces the expression of antibody Fc and complement receptors 3 (CRs3) for phagocytosis and initiate the other killing mechanisms to eliminate pathogen molecules. Even though the percentage of phagocytosis activity for SN-ppF3-treated cell was not as high as LPS-treated, there is a possibility that both treated cells went through similar activation pathway due to the similar biological reaction in macrophages. The ability of LPS in triggering the immune complex clearance through Fc-γR is well documented. Fc-γR is a specific receptor for recognizing Fc portion of IgG. An opsonised LPS by IgG will be recognised by Fc-γR expressed on macrophage cells surface and will lead to the phagosome formation.

On the other hands, plant polysaccharides may bind to macrophages specifically with the other receptors such as Toll-like receptor 4, CD14, scavenger receptor, dectin-1, mannose binding side or with the same CD11b/CD18 Fc-γR co-transducer for LPS recognition sides (Schepetkin and Quinn, 2006). Fc-γR is constitutively expressed on macrophage cells surface and can be up-regulated by activators such as LPS or IFN-γ (Mosser and Zhang, 2011). Thus, the up-regulation of Fc-γR expression will directly improve the phagocytosis capability of macrophages. Researches previously reported that rhamnose-contained and peptic type of plant polysaccharides are able to bind to one

of the receptors and up-regulate the expression of Fc-γR on macrophage cells surface (Matsumoto and Yamada, 1995; Shin *et al*., 1997). Besides, the binding of plant polysaccharide to either of these receptors will initiate cytokines or chemokines production, reactive oxygen species production and also cells proliferation (Schepetkin and Quinn, 2006). In this study, the increase in phagocytosis activity of activated macrophages by LPS or SN-ppF3 was assumed to be associated with the increase in expression of Fc-γR and CR as compared to non-treated cells.

4.8.4 Assessment of TNF-α and IL-6 production *in vitro*

The interaction between either SN-ppF3 or LPS to the receptors presented on macrophages cell surface activated specific signal transduction that leads to production of cytokines. Cytokines are important mediators to regulate immune responses, and often used as a parameter for macrophage activation. The type of cytokine released will determine the effector function of macrophages. Classically activated macrophages induced by LPS will release TNF-α and IFN-γ, which later will act as a pro-inflammatory factor of other cytokines production such as IL-1, IL-6 and IL-23 (Mosser and Edward, 2008).

In this study, the amount of TNF-α and IL-6 secreted by RAW 264.7 cells was measured in order to elucidate the capability of SN-ppF3 in activating the macrophage cells classically. The concentrations of TNF-α secreted in the culture medium by both SN-ppF3- and LPS-treated cells were significantly higher as compared to the non-treated cells. TNF-α is commonly released by macrophages upon activation by microbes to prolong the inflammation by inducing positive feedback on activated macrophages, causing more efficient attack on the infected cells. Aside from maintaining the inflammation, activated macrophages are able to migrate to the infected tissues and

release TNF-α, which plays an important role in inducing apoptosis (Parameswaran and Patial, 2010).

Macrophages commonly secrete IL-6 as a further response to cytokines such as TNF-α and IL-4. The result in this study showed that the concentration of IL-6 present in culture medium of SN-ppF3-treated cells was significantly higher as compared to non-treated cells, but significantly lower as compared to LPS-treated cells. IL-6 is a cytokine with many important roles for both innate and adaptive immune responses. This cytokine commonly induces inflammation in innate immune response. Cells such as macrophages and neutrophils express IL-6 receptor (IL-6R) which allows continuous activation of these cells through IL-6 classic signalling pathways (Scheller *et al.,* 2011). IL-6 produced by macrophages plays an important role as one of the stimulators for production of acute phase proteins, a type of plasma proteins involved in acute inflammation at infection site. On the other hand, IL-6 can contributes in the intermediate phase changes from acute to chronic inflammation, in purpose to extent the inflammation process (Gabay, 2006). While in adaptive immune response, IL-6 is needed by B cells for antibody production, and as well as for T cell proliferation and differentiation. Previously, it has been reported that *S. nigrum* polysaccharide was able to increase the lymphocytes population by suppressing its apoptosis signal (Li *et al*., 2010). It is possible that the apoptosis inhibition of the lymphocytes is not directly induced by the polysaccharide treatment, but might possibly due to the cytokines produced by innate immune response, induced by the polysaccharide.

4.9 Prediction of signalling pathway induced by SN-ppF3 in RAW 264.7 cells

The determination of specific phosphorylated protein associated with inflammation is one of the mechanisms to predict the possible signalling pathways

triggered by SN-ppF3 to induce classically activated macrophages. Based on the previous NO and cytokines production results, it is a hint that the treatment of macrophage cells with SN-ppF3 triggered NF-κB signalling pathway downstream, as closely similar to LPS-triggered pathway. The present study shows that the phosphorylation of certain signalling proteins such as p38 MAP kinase, IκB-α, STAT3 and NF-κB p65 were significantly higher to that of SN-ppF3-treated cells as compared to non-treated cells.

In a well-outlined inflammation signalling pathway, LPS was able to be recognised by most mammalian macrophage cells membrane molecules through CD14 and TLRs, especially TLR4. (Hoshino *et al*., 1999). Signal transduction by TLR4 initiates the activation of IL-1R-associated kinase (IRAK) through an adaptor myeloid differentiation protein 88 (MyD88). This subsequently activates TRAF6 and IKK, leading to phosphorylation of IκB-α. Phosphorylated IκB-α detaches from NF-κB p65/p50 subunit and is degraded. The degradation of IκB-α allows the phosphorylated-p65/p50 dimeric NF-κB transcription factor, translocates into the nucleus and induced the expression of several pro-inflammatory mediators such as cytokines, iNOS and COX-2 (Newton *et al*., 2012; Kang *et al*., 2011). Higher expression of phosphorylated p65 NF-κB in SN-ppF3 and LPS treated cells suggested the induction of NF-κB signalling pathway.

The treatment of macrophage cells with SN-ppF3 also triggered a significant phosphorylation of p38 MAPK, as compared to non-treated cells. Phosphorylated p38 MAPK is commonly detected in LPS stimulated macrophages (Chen *et al*., 2001) and responsible signal for IL-1 and TNF-α production in most mammalian monocytes, and actively participates in a signalling cascade, which controls the cellular response to pro-

inflammatory cytokines and other cellular stresses (Raman *et al*., 2007). Thus, expression of phosphorylated p38 MAPK reflects the result of TNF-α production in the treated macrophage cells.

The expression of phosphorylated STAT3 protein in SN-ppF3 and LPS-treated were significantly up-regulated and down-regulated, respectively as compared to non-treated cells. In other words, the pre-treatment of macrophages with SN-ppF3 induced the phosphorylation of STAT3 while LPS inhibited the phosphorylation. The STAT3 pathway is commonly mediated by IL-6, which leads to the phosphorylation of STAT3 and subsequently induces diverse biological responses (Abroun *et al*., 2015). If referring back to the IL-6 production data (Section 3.14), the expression of phosphorylated STAT3 was expectedly comparable to the IL-6 production. However, lower expression of phosphorylated STAT3 in LPS-treated macrophages cell lysate was observed. Since signalling of STAT3 involves with the translocation of phosphorylated STAT3 into the nucleus, different time frame analysis would give a better picture to determine the lower number of the phosphorylated STAT3. This is because lower detection of phosphorylated STAT3 in the cell lysate may due to lower amount of the phosphorylation inhibition or it may also due to translocation of STAT3 into the nucleus and binds to its promoter element. More detailed studies are required in order to deduce a better understanding on the signalling pathway.

4.10 Endotoxin test

Endotoxin is a lipopolysaccharide constituent, originated from the wall of gram-negative bacteria which can be recognised by macrophage and induce its activation. The presence of endotoxin in polysaccharide samples will contribute to a false positive analysis for the experiment. Contamination of endotoxin might be co-precipitated with

the polysaccharide samples during the purification process. Thus, a test for endotoxin contamination is necessary to be carried out prior to macrophage activation assay to ensure the result obtained specifically due to polysaccharide treatment, not from the microbial endotoxin. Result suggested that SN-ppF3 sample was not contaminated with endotoxin or else contained endotoxin at a level below the detection limit of this assay.

4.11 Oral toxicity study *in vivo*

In vitro study has shown that SN-ppF3 possessed the immunomodulatory activity where it was able to activate macrophages. Since cancer can be inhibited through immunomodulation, further analysis on SN-ppF3 ability in inhibiting cancer in animal model was carried out. The cytotoxicity potential of SN-ppF3 was determined prior to the treatment session, just to ensure that the SN-ppF3 concentration used in the treatment was harmless to the tested healthy mice. Cytotoxicity assay performed in this study shows that SN-ppF3 is not actively cytotoxic and therefore is in agreement with findings from other studies which states that most of polysaccharides which derived from higher plant were mainly nontoxic (Schepetkin and Quinn, 2006).

4.12 Anti-tumour potential of SN-ppF3 *in vivo*

4.12.1 Effect of SN-ppF3 treatment on tumour progression

One of the common indicators for anti-tumour activity *in vivo* is to evaluate the tumour volume of tumour-bearing animal model, before and after the treatment period. The tumour volume measurement represents the actual model of cancer condition; either it will increase progressively, regress or suppress over time. Successful anti-tumour treatments are either reducing the tumour volume (regress) or slowing down and stop the rate of tumour cell proliferation (suppress) after the treatment session. In this study, the ability of SN-ppF3 to inhibit tumour progression in tumour-bearing mice was

demonstrated by evaluating the tumour progression before and after 10 days of oral treatment with doses of 250 and 500 mg/kg.

After 10 days of the treatment session, the tumour volume for CTX, 250 and 500 mg/kg SN-ppF3-treated mice were significantly suppressed, as the tumour progression had gotten slower as compared to control group starting at day 7. A similar study was carried out by Li *et al*., (2009), where U14 cervical cancer cell line was used in the analysis. The group reported 58% and 49% inhibition when 180 mg/kg and 360 mg/kg of the polysaccharide were used in the treatment, respectively. The difference in inhibition percentage at the end of the assay as compared to the one in this study might be due to different polysaccharide fraction and different cancer cell line was used.

The other indicator to determine the anti-tumour activity of the sample treatment is through the measurement of tumour weight. In certain cases, the tumour shrinkage was hardly observed within a short treatment period, but the tumour cells were actually dying due to the treatment. An active anti-tumour agent normally causes the dead of tumour cell, hence leading to decreasing in tumour density. Thus, the reliable parameter for this case is to measure the tumour weight rather than the size. Even though the dead tumour cells residues still remain on the solid tissue, the residues will be cleared up by macrophages. The only drawback for this method is that it could only be done once the animal model is sacrificed. However, by looking at both parameters, it still can be concluded that the treatment inhibited the tumour progression.

The oral administration of SN-ppF3 daily could significantly ($p<0.05$) inhibit the tumour weight at about 38% for 250 mg/kg and 40% for 500 mg/kg treatment. The tumour inhibition rates by SN-ppF3 were comparable to 200 mg/kg of neutral

polysaccharide fraction extracted from *Gynostemma pentaphyllum*, which was able to suppress about 50% of tumour progression in H22 tumour-bearing mice after 10 days of treatment (Liu *et al*., 2014).

Instead of *i.p.* administration, an oral treatment was performed in this study. An oral treatment was preferred due to target accuracy of immune cells activation, especially towards macrophages. Studies have reported that an oral treatment of immunomodulatory polysaccharides daily, can increase the spreading rate of activated macrophages and induce their phagocytic activity (Nanba *et al*., 1987), elevate the plasma macrophage chemotactic activity (Kurashige *et al*., 1997), and thus reduce the tumour burden of tumour-bearing mice (Ramberg *et al*., 2010). Early part of this study demonstrated the ability of SN-ppF3 to activate macrophages *in vitro*, and the main target of oral administration is to introduce SN-ppF3 to tissue-based macrophages, or other immune cells present on alimentary lining. It was assumed that daily treatment using *i.p.* or *i.v*. may contribute to false positive analyses due to subject stress and unavoidable sterility. Furthermore, high molecular weight polysaccharides may interact directly to gut microbiota instead of immune cells during an *i.p*. administration (Ramberg *et al*., 2010). Plus, in the effort to develop SN-ppF3 as anti-cancer drug, an oral medication is preferable due to easy yet safe drug administration (Ramberg *et al*., 2010) for a patient to consume, independently.

4.12.2 Evaluation of immune organ indices

An additional analysis was done for the relative weight indexes of spleen and thymus. During microbial infection or cancer, these immune organs will actively involve. Therefore, the relative sizes and densities of immune organs usually increase caused by massive production and maturation of immune cells during the infection.

Thus, measuring the relative organ weight indexes would give some insight regarding the correlation between anti-tumour activities and immune response induced by an immunomodulator.

Spleen is the major site for initiation of immune responses against blood-borne antigens. The spleen weight indexes for control and the treated groups were significantly (p<0.05) higher as compared to that of normal mice. Previous study on H22 tumour-bearing mice reported that spleen weight of mice with hepatoma was much higher than normal mice. The H22 tumour-bearing mice showed a significant increase in spleen size compared to normal mice, which correlated with the increase in cell number in this organ (Fang *et al*., 2014). The increase in spleen weight indexes suggested the initiation of immune responses, leading to massive production of immune cells that is responsive to the tumour (Cao *et al*., 2011).

Thymus is an exclusive site for T lymphocyte maturation. In general, most immature cells of the T cells lineage undergo various maturation stages in this organ. Mature T cells exit the thymus and enter the blood stream and peripheral lymphoid tissues. During infection, thymus significantly increases in relative size or density due to the massive production and maturation of T cells. However, thymus weight indexes for the treated groups in this study were significantly (p<0.05) lower compared to the normal and controlled mice. No significant difference was observed between CTX-treated and SN-ppF3-treated groups. Thymic involution was reported earlier in mouse mammary tumour model, which reported a dramatic decreases in both weight and thymocytes number after 2 to 3 weeks tumour implantation (Carrio and Lopez, 2013). The cells count from the thymus decreased to 2–5% from normal control thymus (Fu *et al*., 1989). In comparison to the other similar studies, the thymus relative weight of the

tumour-bearing group was significantly lower than that of the normal group (Sharp *et al*., 1976; Toge *et al*., 1988). In addition, a closely similar pattern in the thymus weight indexes were also been observed when 5-Fluorouracil and *G. pentaphylum* polysaccharide were used to treat H22 hepatocarcinoma-bearing mice (Liu *et al*., 2014), comparable to the current finding. In late stage cancer, more mature T cells were progressively activated, associated and degraded along with tumour cells. Due to the event, the maturation rate of T cells increased, forcing matured T cells to leave thymus and migrate to the peripheral tissues or blood vessel in maintaining the T cells homeostasis (Abbas *et al*., 2012). Besides, the abnormal recruitment of immature subset of thymocytes to periphery lymphoid organ such spleen may explain the thymic involution and relatively increase the spleen weight indexes (Carrio and Lopez, 2013).

4.12.3 Measurement of cytokines production

Cytokine level in blood serum samples is often used as a parameter to elucidate the immunomodulatory effect, since cytokines play a prominent role in regulating host immune responses. Commonly, cytokines regulate both cellular and humoral immune responses by affecting immune cell proliferation, differentiation and functions. The type of cytokines produced depends on the mechanism of interaction between immune cells and their activator. For example, the *in vitro* analysis showed that classically activated macrophages secreted TNF-α and IL-6, as a response to the polysaccharide treatment.

In this *in vivo* study, a highlight was given to the level of TNF-α, IFN-γ, IL-4 and IL-6 in the serum of the tumour-bearing mice. TNF-α plays a pivotal role in the host defence system by inducing the expression of other immunoregulatory and inflammatory mediators to battle against infection (Baugh and Bucala, 2001), and depending on the cellular context, TNF-α may play a role in eliminating tumour cells

directly (Wajant, 2009). It was reported that elevation of TNF-α in blood serum may contribute to apoptosis induction via TNFR-1 receptor present on the variety of cell types, especially cancer cells leads to the expression of caspase-8 and caspase-3 activation (Wang and Lin, 2008). Since TNF-α is produced by immune cells such as macrophage and T helper cells, the elevation observed in SN-ppF3-treated mice indicated the induction of immune response upon treatment (Figure 3.13, Panel A). This increase was comparable to that of CTX treatment, which is known to induce immune response at low dosage (Sistigu *et al*., 2011). In the host defence system, TNF-α is also necessary as a primary regulator to boost other cytokines production by phagocytes, which may explain the increment of IFN-γ and IL-4 in the serum of treated mice as compared to the control group (Figure 3.13, Panel B and C), after 10 days of oral administration with a high dose of SN-ppF3.

The presence of IFN-γ and IL-4 will help to prolong inflammation and responsible to promote the activation of macrophages against infection (Mosser and Edward, 2008). IFN-γ has many functions and plays a crucial role in the activation of Th1 immune response. Apart from that, IFN-γ has been known to play an important role in eliminating cancer cells by increasing the expression of MHC class I on the cancer cells leading to the elimination of the cells by CTLs. Interestingly, a study by Critchley-Thorne *et al.* (2009) has shown that the impairment of IFN-γ signalling could be associated with breast cancer (BrCa), indicating the importance of this cytokine on anti-tumour activity of T and B lymphocytes. The elevation of IFN-γ in the serum of treated groups suggested that this cytokine plays physiologically important roles in promoting both innate and adaptive immunes responses. The presence of IFN-Rβ on the most epithelial-derived tumour cells (Garcia-Tunon *et al*., 2007) allow IFN-γ to associate and initiate signal transductions, causing direct anti-proliferative effects on human BrCa by

up-regulating apoptotic members of the bcl-2 family (Zhang *et al*., 2003).

Although it is known as an important cytokine in activating Th2 immune response and as an antagonist for IFN-γ, several evidences have shown that IL-4 plays a role in the production of IFN-γ in NK and DCs (Bream *et al*., 2003). Apart from promoting adaptive immune response, IL-4 can also interact directly towards certain cancer cells, including BrCa through IL-4R expressed on human BrCa cells. Previously, IL-4 was reported to be responsible for human BrCa inhibition and apoptosis via STAT6 signalling pathway (Gooch *et al.,* 2002). Significant elevation of IL-4 in CTX and SN-ppF3-treated mice blood serum may suggest the tumour suppression mechanism by this cytokine as compared to the control group.

Intriguingly, the level of IL-6 for CTX-treated and a high dose of SN-ppF3-treated group were significantly (p<0.05) lower as compared to the control group by ~58% and ~49%, respectively. IL-6 is needed for B cell proliferation but it is commonly elevated in patients with cancer. It was previously reported that there was an elevation of IL-6 concentration (~48%) in patient serum that was unresponsive to any therapy agent. The results suggested that the elevated IL-6 level in the serum may reflect to the poorer prognostic predictor in metastatic BrCa (Zhang and Adachi, 1999), which also suggested the metastases capability of 4T1 carcinoma cell line (Lou *et al*., 2014). The level of IL-6 observed in this study may indicate the malignancy of the tumour, rather than the decrease in immune response. Regulation of IL-6 was previously reported as responsible for tumour promotion in human breast and lung cancer. The binding of IL-6 to its receptor, IL-6R activates JAK tyrosine kinase, leading to the activation of STAT3, which responsible in mediating cell growth, differentiation and survival. IL-6 also can induce signalling of Ras, MAPK, Cox-2 and PI3K/Akt pathways, explaining the

tumorigenic and anti-apoptotic activities of this cytokines (Guo *et al*., 2012). Inhibition of IL-6 production in CTX and SN-ppF3-treated groups could be one of the proxies for tumour suppression mechanisms.

4.12.4 Histological analysis

The histological analysis was carried out to observe the condition of tumour tissue before and after treatment with SN-ppF3 and CTX. The solid tumour tissues were harvested, fixed on slides and stained with H&E staining. This method was commonly used in histopathology to differentiate and indicate the changes which occur on a given tissue. The pathological observation was made by Dr. Huzlinda Hussin (MD., MPath.), a pathologist expert from the Department of Pathology, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, Serdang, Selangor, Malaysia. Histological evaluation showed that cellular morphology of the treated mice tissues were altered, and features of apoptosis were clearly observed. It was believed that the tumour tissues were disrupted most probably because of immune responses.

As earlier discussed, the elevation of cytokines in blood serum of treated mice, especially TNF-α can initiate apoptosis signalling pathway, thus leading to tumour killing process. This potent cytokine was produced by many cell types, but mainly by macrophages in response to inflammation or other environmental changes such cancer. Interaction of TNF-α with TNFR1 initiates a pathway that triggers activation signal of effector caspases that are responsible for the proteolytic cleavage, which ultimately leading to cell death (Baud and Karin, 2001; Shearwin-Whyatt *et al.,* 2000). Aside from the cytokine, T cells and NK cells also contribute to the major killing mechanisms in tumour cells. These lymphocytes express the death ligand CD95L on the cell surface and have affinity towards death receptor CD95, thus triggering apoptosis mechanism of

the targeted cells (Igney and Krammer, 2002). During immune surveillance for tumours and metastases, NK cells use the death TNF-related apoptosis-inducing ligand (TRAIL) to trigger apoptosis signal in targeted cancer cells through TRAIL receptors (Takeda *et al*., 2001). The death signal is similar to the death receptor of TNF-superfamily, which leads to apoptosis activation. Hypothetically, these two immune cells are commonly present in the tumour tissues. However, these cells are hardly observed by H&E staining procedure. Hence, a specific immunostaining technique is required to detect the presence of these cells in the solid tumour tissues.

4.12.5 Detection of infiltrating immune cells

Some immune cells such as NK cells, T cells and macrophages are able to infiltrate into solid tumour and induce killing mechanism on the tumour cells specifically, causing destruction to the tumour tissue. Direct and specific interaction of immune cells with the tumour cells is probably the best approach to eliminate tumour without causing too much damage to the adjacent healthy tissues. In this study, further evaluation was carried out on tumours from control and treated mice to detect the infiltration of NK cells, $CD8^+$ T cells and macrophages into the solid tumour.

The NK cells, CD8 T cells and macrophages possess a potent anti-cancer ability by invading several types of solid tumour (Giannakakis *et al*., 2014), producing chemokines in inducing inflammatory responses (Wu and Lanier, 2003), exerting direct damages to tumour tissues (Tannock *et al*., 2013) and releasing cancer-related cytokines as well as phagocytosing apoptotic particles (Klimp *et al*., 2002). Interestingly, it seemed that the intensity for NK cells was the highest in the tissue of mice treated with 500 mg/kg SN-ppF3. Many studies have shown the importance of NK cells in eliminating cancer where without it, cancer cells will become more aggressive and

metastasize (Waldhauer and Steinle, 2008). Although NK cells are inefficient in infiltrating tumour, treatment with cytokines such as IL-2 and IFNs can markedly increase the infiltration (Albertsson *et al.*, 2003). The most important function of NK cells are exerting direct killing mechanisms onto targeted tumour by (i) releasing cytoplasmic granules containing perforin and granzymes, which later leads to apoptosis induction via dependent and independent-caspase pathways, (ii) initiating apoptosis-mediated receptors such Fas and TRAIL-R onto tumour cells, (iii) secreting various anti-tumour effector molecules such cytokines and NO, and (iv) through ADCC by expressing CD16 to destroy tumour cells (Cheng *et al*., 2013).

As for $CD8^+$ T cells, the fluorescent intensities were the highest in samples from CTX-treated mice and followed by both SN-ppF3-treated mice as compared to the control. The ability of $CD8^+$ T cells to infiltrate into many solid tumours is well documented (Tannock *et al.*, 2013) and was shown as a major contributor in eliminating cancer. Unlike NK cells, T cells require specific activation by antigen presenting cells, which later promote the $CD8^+$ T cells differentiation. In cancer microenvironment of immune surveillance, cancer cells are commonly expressing MHC class I molecules, which are recognised by $CD8^+$ T cells that possess the ability to kill the targeted tumour cells. Differentiation of $CD8^+$ T cells from naive T cells allows the formation of cytolytic granules. Perforin and granzymes are released from the cytolytic granules once $CD8^+$ T cells came into contact with tumour cells membrane via MHC I-$CD8^+$ complex. Perforin causes pores formation and allows granzyme proteases to enter and initiates apoptosis to the tumour cells (Nguyen *et al.,* 2013). Referring to the results, it was suggested that the treatment with SN-ppF3 somehow was able to activate adaptive immune response in combating tumour proliferation by enhancing the $CD8^+$ T cells population to infiltrate the target tumour.

Slight increase in FITC intensities for both CTX and 500 mg/kg of SN-ppF3-treated mice represent the increase in macrophages population in the tumour tissues. One of the main functions of macrophages is to phagocytose apoptotic tumour cells caused by apoptosis-mediated action by immune system and it is important for maintaining tissue homeostasis (Poon *et al*., 2014). Mechanism on how tumour cells may activate macrophages still remains unclear. However, there is a possibility that the activation of macrophages is due to IFN-γ released by tumour-specific T cells, which provides direct recognition to the surface antigens of tumour cells. Classically activated macrophages can serve in direct killing of tumour cells by releasing lysosomal enzymes, ROS and NO (Abbas *et al*., 2012). However, macrophages may also be alternatively activated upon interaction with an activator such as IL-4, which act antagonistically from classically activated macrophage (Mosser, 2003). Further analysis has to be carried out to identify the paradigm of this cell, since alternatively activated macrophages were known to cause poor prognosis of tumour as compared to classically activated macrophages which can cause tumour destruction (Hao *et al*., 2012).

4.12.6 Detection of apoptosis in tumour tissue

As discussed in Section 4.12.4, the treatment of SN-ppF3 and CTX induced apoptotic activity on the tumour cells. Apoptosis is one of the typical biological hallmarks for tumour suppression in the tumour tissues, which were confirmed in this study by TUNEL assay. During apoptosis, the cellular morphology underwent changes as described in Section 4.12.4. Nuclear changes during apoptosis are due to extensive damage to the chromatin where the DNA was cleaved and fragmented (Cohen *et al*., 1992). The DNA cleavage produced double-stranded DNA fragments and can be specifically identified by labelling the free 3'-OH termini with modified nucleotides conjugated with fluorescent dye (Gorczyca *et al*., 1993).

Through fluorescent microscopic observation, (Figure 3.16) the treatments of tumour-bearing mice with SN-ppF3 and CTX managed to elevate the apoptosis activity in tumour cells as compared to untreated mice. These findings strongly suggested that the treatment of SN-ppF3 was able to suppress tumour progression via the mechanism of apoptosis, which supports the H&E analysis. Furthermore, it may reflect the tumour elimination mechanism by the infiltrating immune cells. A preliminary study done by Huang (2013) also showed that the intraperitoneal treatment of 50, 100 and 200 mg/kg polysaccharide isolated from *Tupistra chinensis* towards H22-bearing Kumning mice for 15 days were able to induce apoptosis as compared to the control group by electron microscopy observation. A similar outcome was observed when EAC-bearing mice were treated with galactomannan polysaccharide extracted from *Punica granatum* (Joseph *et al*., 2013) through host immune system improvement.

4.13 *In vitro* cytotoxicity evaluation of SN-ppF3 to 4T1 cell line

In the previous results, it was clearly demonstrated that the tumour progression was inhibited by mice immune responses when treated with SN-ppF3 through immunomodulation. However, there is a possibility that it was due to the direct cytotoxicity effect of SN-ppF3 towards 4T1 tumour cells, leading to tumour cells destruction. To confirm whether SN-ppF3 has a direct cytotoxicity effect towards 4T1 tumour cells, MTT assay was carried out. By referring to the cytotoxicity dose response curve (Figure 3.18), the IC_{50} value of SN-ppF3 could not be determined (IC_{50} > 100 µg/mL). According to the U.S National Cancer Institute plant screening program, the plant crude extract with IC_{50} value of 20 µg/mL or less is deemed actively cytotoxic after 48–72 hours of incubation (Lee and Houghton, 2005). Thus, it could be concluded that SN-ppF3 has no active cytotoxicity effect on 4T1 cells after a 72 hours incubation period.

Although many reports have shown direct cytotoxicity effect of purified polysaccharides towards cancer cell lines, indirect cytotoxicity is not uncommon. Polysaccharides purified from *Innotus obliguus*, *Pananx ginseng*, *Schisandra chinensis* and a few others did not show significant cytotoxicity on cell lines tested, but significantly inhibit the tumour growth *in vivo*. The treatment with those polysaccharides caused significant improvement in host immune responses. Elevation of anti-tumour-related cytokines (TNF-α, IFN-γ IL-2, IL-12), expression of Bax apoptosis protein, NK cells activity, $CD4^+/CD8^+$ ratio and phagocytosis activity of macrophage cell were observed (Chen *et al*., 2015; Wang *et al*., 2015; Zhao *et al*., 2013). The anti-cancer activity of these polysaccharides observed *in vivo* was most probably due to their ability to modulate the immune system, which in turn eliminates the cancer cells.

CHAPTER 5: CONCLUSION

5.1 Conclusion

The ability of certain plant polysaccharides to modulate immune responses is well studied and has the potential therapeutic properties to be used as alternative, effective and non-toxic treatment for immune-associated diseases including cancer. With the immunomodulatory property, it is possible to use the polysaccharide of *S. nigrum* to enhance the host immune response in fighting cancer. Thus this study was carried out to evaluate the possible mechanism of SN-ppF3 polysaccharide inhibiting tumour progression on breast tumour-bearing mice, by enhancing the immune responses of the host.

Crude polysaccharides were isolated from leaves, stem and the combination between leaves, stem and fruit of *S. nigrum* herbal plant. These crude polysaccharide samples were not actively cytotoxic (IC_{50} > 100 µg/mL) towards RAW 264.7 murine macrophage cell line. Out of these three samples, crude polysaccharide extracted from stem was shown to have the least inhibitory effect at the maximum concentration tested. Thus, only crude polysaccharide sample from stem was selected to be further purified. Five polysaccharide fractions were obtained from the ion-exchange chromatography which was proven to have high percentage of carbohydrate content with minimal amount of protein present. All five polysaccharide fractions were not actively cytotoxic (IC_{50} > 100 µg/mL) towards RAW 264.7 cells. Among these fractions, fraction 3, SN-ppF3 exerted the least inhibitory effect at maximum concentration tested.

Based on the data obtained, it could be concluded that classically activated macrophages could be induced by polysaccharide fractions isolated from *S. nigrum*. All five fractions managed to elevate the NO production, which is one of the indications of

macrophage activation. The highest concentration of NO was produced when macrophage cells were treated with maximum concentration (100 μg/mL) of SN-ppF3. Based on this result, only SN-ppF3 was selected to be tested in the subsequent of macrophage activation assays, *in vitro*. Furthermore, SN-ppF3 was shown to have the least cytotoxicity effect as compared to the other fractions. To validate the NO production data, a significant amount of iNOS was detected by Western blot analysis, suggesting the correlation between iNOS expression and NO production.

The ability of macrophage to perform pinocytosis and phagocytosis are the major hallmarks of macrophage activation. In this investigation, it was proven that the treatment of RAW 264.7 cells with SN-ppF3 was able to significantly (p<0.05) induce both pinocytosis and phagocytosis activities. Other than that, the presence of selected cytokines such as TNF-α and IL-6 was successfully detected in the culture medium by ELISA analysis. These findings suggested that the macrophage cells were classically activated when it was treated with SN-ppF3.

For the *in vivo* analysis of this experiment, the progression of tumour tissues were successfully inhibited when tumour-bearing mice were orally administered with SN-ppF3. The tumour weight and volume were suppressed as compared to that of control-treated mice at the end of treatment session. One of the inhibition mechanisms was most probably due to the regulation of cytokines production which was detected in the mice blood serum. It is possible that to some extent, cytokines such as TNF-α can directly involve in tumour killing mechanism, apart from regulating the immune system.

According to the histological analyses, the disruption of tumour cells morphology was observed in tumour tissue of mice treated groups. It was suggested that

the treatment of tumour-bearing mice with SN-ppF3 promoted apoptosis in tumour cells. The observation was validated with the increase of DNA fragmentation which detected by TUNEL assay. Besides, the elevation of NK cells, $CD8^+$ T cells and macrophages population were detected in the solid tumour tissues which most probably responsible in inducing apoptosis on the tumour cells.

Through the data obtained, it was clearly shown that the polysaccharide fraction SN-ppF3 was able to activate macrophage cells, which possibly acts as an important proxy against cancer. SN-ppF3 was also proven to have anti-tumour activity when it was orally administered to tumour-bearing mice. These results suggested that the *in vivo* tumour regression mechanism by SN-ppF3 might occur through inducing host immune responses thus potentially be developed as novel anti-cancer agent.

5.2 Future study

Physicochemical properties of SN-ppF3 such as structural characteristic, branching type and the presence of additional ligand should be determined to provide a better understanding in term of interaction between SN-ppF3 and those immune cells. SN-ppF3 needs to be characterised more specifically in term of its accurate composition to avoid any variability due to plant materials and sources, for results in the experiment to be reproduced. In addition to toxicity study of SN-ppF3 (Section 4.2), those physicochemical studies are also necessary to validate the absence of glycoalkaloid compounds in the sample.

The immunomodulatory activity of SN-ppF3 could be tested towards human macrophage cells, or other human immune cells, since testing the polysaccharide sample to human cells is more clinically relevant for a drug development. More in-depth underlying anti-tumour mechanism of SN-pF3 in breast cancer could be carried out.

Further analyses on the effect of SN-ppF3 treatment on cancer progression towards other human breast cancer cells such as MCF-7, or human hormone-dependent cancer such as prostate cancer, could also be carried out to see the effect on human tumour cells. Bigger sample size would be used in order to obtain statistically reliable data (statistical relevant sample size, n=6). However, performing similar analyses in nude mice might give incomparable data, considering that the mice have an impaired immune system.

In the effort to be developed as an anti-cancer medication, the toxicity of SN-ppF3 is important to be carried out before it can be pharmaceutically consumed. The *in vivo* evaluation on liver and kidney condition is important to ensure the SN-ppF3 did not exert any severe complication to the vital organs after a longer period of treatment session. In addition to that matter, the final concentration of SN-ppF3 is necessary to be determined based on clinical procedure.

REFERENCES

Abbas, A. K., Lichtman, A. H., & Pillai, S. (2012). *Cellular and molecular immunology* (7th Ed.). Philadelphia, PA, USA: Saunders Elsevier.

Abdullah-Soheimi, S. S., Lim, B. K., Hashim, O. H., & Shuib, A. S. (2010). Patients with ovarian carcinoma excrete different altered levels of urine CD59, kininogen-1 and fragments of inter-alpha-trypsin inhibitor heavy chain H4 and albumin. *Proteome Science*, *8*(1), 58.

Abroun, S., Saki, N., Ahmadvand, M., Asghari, F., Salari, F., & Rahim, F. (2015). STATs: An old story, yet mesmerizing. *Cell Journal (Yakhteh)*, *17*(3), 395–411.

Afkarian, M., Sedy, J. R., Yang, J., Jacobson, N. G., Cereb, N., Yang, S. Y., ... Murphy, K. M. (2002). T-bet is a STAT1-induced regulator of IL-12R expression in naive CD4+ T cells. *Nature Immunology, 3*, 549–557.

Agarwal, S. S., & Singh, V. K. (1999). Immunomodulators: A review of studies on Indian medicinal plants and synthetic peptides. Part 1: Medicinal plants. *Proceedings of the Indian National Science Academy,* 179–204.

Akhtar, M. S., & Munir, M. (1989). Evaluation op the gastric antiulcerogenic effects of *Solanum nigrum*, *Brassica oleracea* and *Ocimum basilicum* in rats. *Journal of Ethnopharmacology*, *27*(1), 163–176.

Alban, S., & Franz, G. (2000). Characterization of the anticoagulant actions of a semisynthetic curdlan sulfate. *Thrombosis Research*, *99*(4), 377–388.

Albertsson, P. A., Basse, P. H., Hokland, M., Goldfarb, R. H., Nagelkerke, J. F., Nannmark, U., & Kuppen, P. J. (2003). NK cells and the tumour microenvironment: implications for NK-cell function and anti-tumour activity. *Trends in Immunology*, *24*(11), 603–609.

Antonarakis, E. S., & Drake, C. G. (2010). Current status of immunological therapies for prostate cancer. *Current Opinion in Urology, 20*(3), 241–246.

Atherton, P. (2002). Aloe vera: magic or medicine with antidiabetic potential. *Journal of Ethnopharmacology*, *81*, 81–100.

Batovska, D. I., Todorova, I. T., Bankova, V. S., Parushev, S. P., Atanassov, A. I., Hvarleva, T. D., ... Popov, S. S. (2008). Seasonal variations in the chemical composition of wine-grape leaf surface. *Natural Product Research*, *22*(14), 1231–1236.

Bae, I. Y., Kim, H. W., Yoo, H. J., Kim, E. S., Lee, S., Park, D. Y., & Lee, H. G. (2013). Correlation of branching structure of mushroom β-glucan with its physiological activities. *Food Research International*, *51*(1), 195–200.

Baud, V., & Karin, M. (2001). Signal transduction by tumor necrosis factor and its relatives. *Trends in Cell Biology, 11*(9), 372–377.

Baugh, J. A., & Bucala, R. (2001). Mechanisms for modulating TNF alpha in immune and inflammatory disease. *Current Opinion in Drug Discovery & Development*, *4*(5), 635–650.

Bevilacqua, M. P., Pober, J. S., Majeau, G. R., Fiers, W., Cotran, R. S., & Gimbrone, M. A. Jr. (1986). Recombinant tumor necrosis factor induces procoagulant activity in cultured human vascular endothelium: characterization and comparison with the actions of interleukin 1. *Proceedings of the National Academy of Sciences, 83*(12), 4533–4537.

Billiau, A., & Matthys, P. (2001). Modes of action of Freund's adjuvants in experimental models of autoimmune diseases. *Journal of Leukocyte Biology*, *70*(6), 849–860.

Bowman, S. M., & Free, S. J. (2006). The structure and synthesis of the fungal cell wall. *Bioessays*, *28*(8), 799–808.

Boyer, R. F. (1999). *Concept in Biochemistry* (1st Ed.). Pacific Grove, CA: Brooks/Cole Publishing.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, *72*(1-2), 248–254.

Bradley, J. R. (2008). TNF-mediated inflammatory disease. *The Journal of Pathology*, *214*(2), 149–160.

Bream, J. H., Curiel, R. E., Yu, C. R., Egwuagu, C. E., Grusby, M. J., Aune, T. M., & Young, H. A. (2003). IL-4 synergistically enhances both IL-2 and IL-12 induced IFN-γ expression in murine NK cells. *Blood*, *102*(1), 207–214.

Brunton, L., Chabner, B., & Knollman, B. (2011). *Goodman & Gilman's pharmacological basis of therapeutics* (12th ed.). New York, USA: McGraw-Hill.

Byeon, S. E., Lee, J., Kim, J. H., Yang, W. S., Kwak, Y. S., Kim, S. Y., ... Cho, J. Y. (2012). Molecular mechanism of macrophage activation by red ginseng acidic polysaccharide from Korean red ginseng. *Mediators of Inflammation*, *2012*.

Campbell, L. L., & Polyak, K. (2007). Breast tumor heterogeneity: cancer stem cells or clonal evolution?. *Cell Cycle*, *6*(19), 2332–2338.

Campbell N. A., & Reece, J. B. (2005). *Biology* (7th Ed.). San Francisco, CA: Benjamin Cummings.

Campo, V. L., Kawano, D. F., da Silva, D. B., & Carvalho, I. (2009). Carrageenans: Biological properties, chemical modifications and structural analysis - A review. *Carbohydrate Polymers*, *77*(2), 167–180.

Cao, M., Xu, Y., Youn, J. I., Cabrera, R., Zhang, X., Gabrilovich, ... Liu, C. (2011). Kinase inhibitor Sorafenib modulates immunosuppressive cell populations in a murine liver cancer model. *Laboratory Investigation*, *91*(4), 598–608.

Carrio, R., & Lopez, D. M. (2013). Insights into thymic involution in tumor-bearing mice. *Immunologic Research*, *57*(1–3), 106–114.

Cartea, M. E., Francisco, M., Soengas, P., & Velasco, P. (2010). Phenolic compounds in Brassica vegetables. *Molecules*, *16*(1), 251–280.

Chao, M. P., Majeti, R., & Weissman, I. L. (2012). Programmed cell removal: a new obstacle in the road to developing cancer. *Nature Reviews Cancer*, *12*(1), 58–67.

Chao, W. W., Hong, Y. H., Chen, M. L., & Lin, B. F. (2010). Inhibitory effects of *Angelica sinensis* ethyl acetate extract and major compounds on NF-κB trans-activation activity and LPS-induced inflammation. *Journal of Ethnopharmacology*, *129*(2), 244–249.

Chen, J. R., Yang, Z. Q., Hu, T. J., Yan, Z. T., Niu, T. X., Wang, L., Cui, D. A., & Wang, M. (2010). Immunomodulatory activity *in vitro* and *in vivo* of polysaccharide from *Potentilla anserina*. *Fitoterapia*, 81(8), 1117–1124.

Chen, Y., Huang, Y., Cui, Z., & Lui, J. (2015). Purification, characterization and biological activity of a novel polysaccharide from *Inonotus obliquus*. *International Journal of Biological Macromolecules, 79*, 587–594.

Chen, Z., Gibson, T. B., Robinson, F., Silvestro, L., Pearson, G., Xu, B. E., ... Cobb, M. H. (2001). MAP kinases. *Chemical Reviews*, *101*(8), 2449–2476.

Cheng, M., Chen, Y., Xiao, W., Sun, R., & Tian, Z. (2013). NK cell-based immunotherapy for malignant diseases. *Cellular and Molecular Immunology*, *10*(3), 230–252.

Chevrier, M. R., Ryan, A. E., Lee, D. Y. W., Zhongze, M., Wu-Yan, Z., & Via, C. S. (2005). *Boswellia carterii* extract inhibits TH1 cytokines and promotes TH2 cytokines *in vitro*. *Clinical and Diagnostic Laboratory Immunology*, *12*(5), 575–580.

Cohen, G. M., Sun, X. M., Snowden, R. T., Dinsdale, D., & Skilleter, D. N. (1992). Key morphological features of apoptosis may occur in the absence of internucleosomal DNA fragmentation. *Biochemical Journal*, *286*(2), 331–334.

Critchley-Thorne, R. J., Simons, D. L., Yan, N., Miyahira, A. K., Dirbas, F. M., Johnson, D. L., ... Holmes, S. (2009). Impaired interferon signaling is a common immune defect in human cancer. *Proceedings of the National Academy of Sciences USA*, *106*(22), 9010–9015.

Croxford, A. L., Kulig, P., & Becher, B. (2014). IL-12 and IL-23 in health and disease. *Cytokine and Growth Factor Reviews*, *25*(4), 415–421.

Dash, S., Nath, L. K., Bhise, S., Kar, P., & Bhattacharya, S. (2006). Stimulation of immune function activity by the alcoholic root extract of *Heracleum nepalense* D. Don. *Indian Journal of Pharmacology*, *38*(5), 336–340.

Duque, G. A., & Descoteaux, A. (2014). Macrophage cytokines: involvement in immunity and infectious diseases. *Frontiers in Immunology*, *5*.

Dutt, P., & Stambolic, V. (2013). Oncogenes and Tumor-supressor gene. Oncogenes and Tumor-Suppressor Genes. In Tannock, I., Hill, R., Bristow, R. & Harrington, L. (Eds.), *The basic science of oncology* (5th Ed.) (pp. 147–171). New York, USA: McGraw-Hill.

Edge, S. B., & Compton, C. C. (2010). The American Joint Committee on Cancer: the 7th edition of the AJCC cancer staging manual and the future of TNM. *Annals of Surgical Oncology*, *17*(6), 1471–1474.

Edmonds, J. M., & Chweya, J. A. (1997). *Black nightshades: Solanum nigrum L. and related species, in: Promoting the conservation and use of underutilized and neglected crops.* (Vol. 15). Rome, Italy: Institute of Plant Genetics and Crop Plant Research, Gatersleben / International Plant Genetic Resources Institute.

El-Enshasy, H. A., & Hatti-Kaul, R. (2013). Mushroom immunomodulators: unique molecules with unlimited applications. *Trends in Biotechnology*, *31*(12), 668–677.

Elliott, W. H., & Elliott, D. C. (2009). *Biochemistry and molecular biology* (4th Ed.). New York, USA: Oxford University Press.

Fang, J. J., Zhu, Z. Y., Hui, D. O. N. G., Zheng, G. Q., Teng, A. G., & Liu, A. J. (2014). Effect of spleen lymphocytes on the splenomegaly in hepatocellular carcinoma-bearing mice. *Biomedical and Environmental Sciences*, *27*(1), 17–26.

Farah, A., & Donangelo, C. M. (2006). Phenolic compounds in coffee. *Brazilian Journal of Plant Physiology*, *18*(1), 23–36.

Farrar, M. A., & Schreiber, R. D. (1993). The molecular cell biology of interferon-gamma and its receptor. *Annual Review of Immunology*, *11*(1), 571–611.

Fahey, J. M., & Girotti, A. W. (2015). Accelerated migration and invasion of prostate cancer cells after a photodynamic therapy-like challenge: Role of nitric oxide. *Nitric Oxide*, *49*, 47–55.

Flannagan, R. S., Jaumouille, V., & Grinstein, S. (2012). The cell biology of phagocytosis. *Annual Review of Pathology: Mechanisms of Disease*, *7*, 61–98.

Fu, Y. A. N. G. X. I. N., Paul, R. D., Wang, Y. A. N. G., & Lopez, D. M. (1989). Thymic involution and thymocyte phenotypic alterations induced by murine mammary adenocarcinomas. *The Journal of Immunology*, *143*(12), 4300–4307.

Gabay, C. (2006). Interleukin-6 and chronic inflammation. *Arthritis Research and Therapy*, *8*(2), S3.

Galon, J., Mlecnik, B., Bindea, G., Angell, H. K., Berger, A., Lagorce, C., ... Nagtegaal, I. D. (2014). Towards the introduction of the 'Immunoscore' in the classification of malignant tumours. *The Journal of Pathology*, *232*(2), 199–209.

Gao, S. P., Mark, K. G., Leslie, K., Pao, W., Motoi, N., Gerald, W. L., ... Bromberg, J. F. (2007). Mutations in the EGFR kinase domain mediate STAT3 activation via IL-6 production in human lung adenocarcinomas. *The Journal of Clinical Investigation*, *117*(12), 3846–3856.

Garcia-Tunon, I., Ricote, M., Ruiz, A., Fraile, B., Paniagua, R., & Royuela, M. (2007). Influence of IFN-gamma and its receptors in human breast cancer. *BMC Cancer*, *7*(1), 158.

GBI Research. (2012) Biobanks: Yearbook. *Survey Report*. GBI Research.

Gea-Banacloche, J. C. (2006). Immunomodulation. In Runge, M. S., & Patterson, C. (Eds.), *Principles of molecular medicine* (pp. 893–904). Totowa, New Jersey: Humana Press.

Giannakakis, A., Karapetsas, A., Dangaj, D., Lanitis, E., Tanyi, J., Coukos, G., & Sandaltzopoulos, R. (2014). Overexpression of SMARCE1 is associated with CD8+ T-cell infiltration in early stage ovarian cancer. *The International Journal of Biochemistry & Cell Biology*, *53*, 389–398.

Gooch, J. L., Christy, B., & Yee, D. (2002). STAT6 mediates interleukin-4 growth inhibition in human breast cancer cells. *Neoplasia, 4*(4), 324–331.

Gorczyca, W., Gong, J., & Darzynkiewicz, Z. (1993). Detection of DNA strand breaks in individual apoptotic cells by the in situ terminal deoxynucleotidyl transferase and nick translation assays. *Cancer Research*, *53*(8), 1945–1951.

Guha, M., & Mackman, N. (2001). LPS induction of gene expression in human monocytes. *Cellular Signalling*, *13*(2), 85–94.

Guo, Y., Xu, F., Lu, T., Duan, Z., & Zhang, Z. (2012). Interleukin-6 signaling pathway in targeted therapy for cancer. *Cancer Treatment Reviews, 7*, 904–910.

Haller-Jr, J. S. (1990). A drug for all seasons. Medical and pharmacological history of aloe. *Bulletin of the New York Academy of Medicine*, *66*(6), 647.

Han, Y., Wu, J., Liu, T., Hu, Y., Zheng, Q., Wang, B., ... Li, X. (2016). Separation, characterization and anticancer activities of a sulfated polysaccharide from *Undaria pinnatifida*. *International Journal of Biological Macromolecules*, *83*, 42–49.

Hao, N. B., Lu, M. H., Fan, Y. H., Cao, Y. L., Zhang, Z. R., & Yang, S. M. (2012). Macrophages in tumor microenvironments and the progression of tumors. *Clinical and Developmental Immunology*, *2012*.

Heo, K. S., Lee, S. J., Ko, J. H., Lim, K., & Lim, K. T. (2004). Glycoprotein isolated from *Solanum nigrum* L. inhibits the DNA-binding activities of NF-κB and AP-1, and increases the production of nitric oxide in TPA-stimulated MCF-7 cells. *Toxicology In Vitro*, *18*(6), 755–763.

Hofseth, L. J., Hussain, S. P., Wogan, G. N., & Harris, C. C. (2003). Nitric oxide in cancer and chemoprevention. *Free Radical Biology and Medicine*, *34*(8), 955–968.

Hoshino, K., Takeuchi, O., Kawai, T., Sanjo, H., Ogawa, T., Takeda, Y., ... Akira, S. (1999). Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. *The Journal of Immunology*, *162*(7), 3749–3752.

Houghton, P. J., & Raman, A. (1998). *A laboratory manual for the fractionation of natural extracts*. London, England: Chapman & Hall.

Howad, M., Farrar, J., & Hilfiker, M. (1982). Identification of a T cell-derived B cell stimulatory factor distinct from interleukin 2. *Journal of Experimental Medicine*, *155*(3), 914–923.

Hirano, T., Taga, T., Yasukawa, K., Nakajima, K., Nakano, N., Takatsuki, F., ... Sakiyama, F. (1987). Human B-cell differentiation factor defined by an anti-peptide antibody and its possible role in autoantibody production. *Proceedings of the National Academy of Sciences*, *84*(1), 228–231.

Hu, K., Kobayashi, H., Dong, A., Jing, Y., Iwasaki, S., & Yao, X. (1999). Antineoplastic agents. III: Steroidal glycosides from *Solanum nigrum*. *Planta Medica*, *65*(1), 35–38.

Huang, H. C., Syu, K. Y., & Lin, J. K. (2010). Chemical composition of *Solanum nigrum* linn extract and induction of autophagy by leaf water extract and its major flavonoids in AU565 breast cancer cells. *Journal of Agricultural and Food Chemistry*, *58*(15), 8699–8708.

Huang, W. (2013). Anticancer effect of plant-derived polysaccharides on mice. *Journal of Cancer Therapy, 4*(2), 500.

Hulka, B. S., & Stark, A. T. (1995). Breast cancer: cause and prevention. *The Lancet*, *346*(8979), 883–887.

Hume, D. A. (2012). The biology of macrophages – An online review. Retrieved from http://www.macrophages.com/macrophage-review

Igney, F. H., & Krammer, P. H. (2002). Immune escape of tumors: apoptosis resistance and tumor counter-attack. *Journal of Leukocyte Biology*, *71*(6), 907–920.

Im, S. A., Oh, S. T., Song, S., Kim, M. R., Kim, D. S., Woo, S. S., ... Lee, C. K. (2005). Identification of optimal molecular size of modified Aloe polysaccharides with maximum immunomodulatory activity. *International Immunopharmacology, 5*(2), 271–279.

Itokawa, H., Morris-Natschke, S. L., Akiyama, T., & Lee, K. H. (2008). Plant-derived natural product research aimed at new drug discovery. *Journal of Natural Medicines*, *62*(3), 263–280.

Jain, R., Sharma, A., Gupta, S., Sarethy, I. P., & Gabrani, R. (2011). *Solanum nigrum*: current perspectives on therapeutic properties. *Alternative Medicine Review*, *16*(1), 78–85.

Jaiswal, S., Jamieson, C. H., Pang, W. W., Park, C. Y., Chao, M. P., Majeti, R., ... Weissman, I. L. (2009). CD47 is up-regulated on circulating hematopoietic stem cells and leukemia cells to avoid phagocytosis. *Cell*, *138*(2), 271–285.

Jemal, A., Bray, F., Center, M. M., Ferlay, J., Ward, E., & Forman, D. (2011). Global cancer statistics. *CA: A Cancer Journal for Clinicians*, *61*(2), 69–90.

Jia, X., Zhang, C., Qiu, J., Wang, L., Bao, J., Wang, K., ... Han, J. (2015). Purification, structural characterization and anticancer activity of the novel polysaccharides from *Rhynchosia minima* root. *Carbohydrate Polymers*, *132*, 67–71.

Jing, L., Cui, G., Feng, Q., & Xiao, Y. (2009). Evaluation of Hypoglycemic Activity of the Polysaccharides Extracted from *Lycium Barbarum*. *African Journal of Traditional, Complementary, and Alternative Medicines*, *6*(4), 579–584.

Joseph, M. M., Aravind, S. R., George, S. K., Varghese, S., & Sreelekha, T. T. (2013). A galactomannan polysaccharide from *Punica granatum* imparts *in vitro* and *in vivo* anticancer activity. *Carbohydrate Polymers*, *98*(2), 1466–1475.

Kang, S. R., Han, D. Y., Park, K. I., Park, H. S., Cho, Y. B., Lee, H. J., ... Kim, J. A. (2011). Suppressive effect on lipopolysaccharide-induced proinflammatory mediators by *Citrus aurantium* L. in macrophage RAW 264.7 cells via NF-κB signal pathway. *Evidence-Based Complementary and Alternative Medicine*, *2011*.

Karp, G. (2008). *Cell and cellular biology* (5th ed.). Hoboken, New Jersey: John Wiley & Sons Inc.

Keegstra, K. (2010). Plant cell walls. *Plant Physiology*, *154*(2), 483–486.

Kerwin, J. F., & Heller, M. (1994). The arginine-nitric oxide pathway: A target for new drugs. *Medicinal Research Reviews*, *14*(1), 23–74.

Khajuria, A., Gupta, A., Garai, S., & Wakhloo, B. P. (2007). Immunomodulatory effects of two sapogenins 1 and 2 isolated from Luffa cylindrica in Balb/C mice. *Bioorganic and Medicinal Chemistry Letters*, *17*(6), 1608–1612.

Khoo, L. T., Abas, F., Abdullah, J. O., Mohd Tohit, E. R., & Hamid, M. (2014). Anticoagulant activity of polyphenolic-polysaccharides isolated from *Melastoma malabathricum* L. *Evidence-Based Complementary and Alternative Medicine*, *2014*.

Kidd, P. (2003). Th1/Th2 balance: the hypothesis, its limitations, and implications for health and disease. *Alternative Medicine Review*, *8*(3), 223–246.

Kiessling, L. L., & Grim, J. C. (2013). Glycopolymer probes of signal transduction. *Chemical Society Reviews*, *42*(10), 4476–4491.

Klimp, A. H., De Vries, E. G. E., Scherphof, G. L., & Daemen, T. (2002). A potential role of macrophage activation in the treatment of cancer. *Critical Reviews in Oncology/Hematology, 44*(2), 143–161.

Konkimalla, V. B., Blunder, M., Bauer, R., & Efferth, T. (2010). Inhibition of inducible nitric oxide synthase by bis (helenalinyl) glutarate in RAW264.7 macrophages. *Biochemical Pharmacology*, *79*(11), 1573–1580.

Kuan, G. L. K., Papapreponis, P., & Hin, Y. M. (2015). State level variation of breast cancer cases in 2007 among Malaysian women. *Malaysian Journal of Public Health Medicine*, *15*(1), 62–68.

Kumar, S., Gupta, P., Sharma, S., & Kumar, D. (2011). A review on immunostimulatory plants. *Journal of Chinese Integrative Medicine*, *9*(2), 117–128.

Kurashige, S., Akuzawa, Y., & Endo, F. (1997). Effects of *Lentinus edodes*, *Grifola frondosa* and *Pleurotus ostreatus* administration on cancer outbreak, and activities of macrophages and lymphocytes in mice treated with a carcinogen, N-butyl-N-butanolnitrosoamine. *Immunopharmacology and Immunotoxicology*, *19*(2), 175–183.

Kress, H., Stelzer, E. H., Holzer, D., Buss, F., Griffiths, G., & Rohrbach, A. (2007). Filopodia act as phagocytic tentacles and pull with discrete steps and a load-dependent velocity. *Proceedings of the National Academy of Sciences*, *104*(28), 11633–11638.

Lawrence, T. & Natoli, G. (2011). Transcriptional regulation of macrophage polarization: enabling diversity with identity. *Nature Reviews Immunology*, *11*(11), 750–761.

Lee, C. C., & Houghton, P. (2005). Cytotoxicity of plants from Malaysia and Thailand used traditionally to treat cancer. *Journal of Ethnopharmacology*, *100*(3), 237–243.

Leung, M. Y. K., Liu, C., Koon, J. C. M., & Fung, K. P. (2006). Polysaccharide biological response modifiers. *Immunology Letters*, *105*(2), 101–114.

Lai, Y. P., Jeng, C. J., & Chen, S. C. (2011). The Roles of $CD4^+$ T cells in tumor immunity. *ISRN Immunology*, *2011*.

Lai, Y. S., Chen, J. Y., Tsai, H. J., Chen, T. Y., & Hung, W. C. (2015). The SUV39H1 inhibitor chaetocin induces differentiation and shows synergistic cytotoxicity with other epigenetic drugs in acute myeloid leukemia cells. *Blood Cancer Journal*, *5*(5), e313.

Li, J., Li, Q. W., Gao, D. W., Han, Z. S., & Lu, W. Z. (2009). Antitumor and immunomodulating effects of polysaccharides isolated from *Solanum nigrum* Linne. *Phytotherapy Research*, *23*(11), 1524–1530.

Li, J., Li, Q., Peng, Y., Zhao, R., Han, Z., & Gao, D. (2010). Protective effects of fraction 1a of polysaccharides isolated from *Solanum nigrum* Linne on thymus in tumor-bearing mice. *Journal of Ethnopharmacology*, *129*(3), 350–356.

Li, S., Xiong, Q., Lai, X., Li, X., Wan, M., Zhang, J., ... Zhang, D. (2015). Molecular Modification of Polysaccharides and Resulting Bioactivities. *Comprehensive Reviews in Food Science and Food Safety*, 237–250.

Lighvani, A. A., Frucht, D. M., Jankovic, D., Yamane, H., Aliberti, J., Hissong, B. D., ... O'Shea, J. J. (2001). T-bet is rapidly induced by interferon-γ in lymphoid and myeloid cells. *Proceedings of the National Academy of Sciences*, *98*(26), 15137–15142.

Lin, P. C., Wu, D. T., Xie, J., Zhao, J., & Li, S. P. (2015). Characterization and comparison of bioactive polysaccharides from the tubers of *Gymnadenia conopsea*. *Food Hydrocolloids*, *43*, 199–206.

Lin, Y., Choksi, S., Shen, H. M., Yang, Q. F., Hur, G. M., Kim, Y. S., ... Liu, Z. G. (2004). Tumor necrosis factor-induced nonapoptotic cell death requires receptor-interacting protein-mediated cellular reactive oxygen species accumulation. *Journal of Biological Chemistry*, *279*(11), 10822–10828.

Liu, J., Zhang, L., Ren, Y., Gao, Y., Kang, L., & Qiao, Q. (2014). Anticancer and immunoregulatory activity of *Gynostemma pentaphyllum* polysaccharides in H22 tumor-bearing mice. *International Journal of Biological Macromolecules*, *69*, 1–4.

Lopez Jr, J. L. (2007). Use of *Opuntia cactus* as a hypoglycemic agent in managing type 2 diabetes mellitus among Mexican American patients. *Nutrition Bytes*, *12*(1).

Lou, C., Takahashi, K., Irimura, T., Saiki, I., & Hayakawa, Y. (2014). Identification of Hirsutine as an anti-metastatic phytochemical by targeting NF-κB activation. *International Journal of Oncology*, *45*(5), 2085–2091.

Lowenstein, C. J., & Padalko, E. (2004). iNOS (NOS2) at a glance. *Journal of Cell Science*, *117*(14), 2865–2867.

Mark, K. S., Trickler, W. J., & Miller, D. W. (2001). Tumor necrosis factor-α induces cyclooxygenase-2 expression and prostaglandin release in brain microvessel endothelial cells. *Journal of Pharmacology and Experimental Therapeutics*, *297*(3), 1051–1058.

Masuko, T., Minami, A., Iwasaki, N., Majima, T., Nishimura, S. I., & Lee, Y. C. (2005). Carbohydrate analysis by a phenol sulfuric acid method in microplate format. *Analytical Biochemistry*, *339*(1), 69–72.

Matsumoto, T., & Yamada, H. (1995). Regulation of immune complexes binding of macrophages by pectic polysaccharide from *Bupleurum falcatum* L.: pharmacological evidence for the requirement of intracellular calcium/calmodulin on Fc receptor up-regulation by Bupleuran 2IIb. *Journal of Pharmacy and Pharmacology*, *47*(2), 152–156.

McChesney, J. D., Venkataraman, S. K., & Henri, J. T. (2007). Plant natural products: back to the future or into extinction?. *Phytochemistry*, *68*(14), 2015–2022.

Miao, S., Mao, X., Pei, R., Miao, S., Xiang, C., Lv, Y., ... Liu, Y. (2013). Antitumor activity of polysaccharides from *Lepista sordida* against laryngocarcinoma *in vitro* and *in vivo*. *International Journal of Biological Macromolecules*, *60*, 235–240.

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods*, *65*(1–2), 55–63.

Mosser, D. M. (2003). The many faces of macrophage activation. *Journal of Leukocyte Biology*, *73*(2), 209–212.

Mosser, D. M., & Edwards, J. P. (2008). Exploring the full spectrum of macrophage activation. *Nature Reviews Immunology*, *8*(12), 958–969.

Mosser, D. M., & Zhang, X. (2011). Measuring Opsonic Phagocytosis via Fcγ Receptors and Complement Receptors on Macrophages. *Current Protocols in Immunology*, *95*. Retrieved from http://doi.org/10.1002/0471142735.im1427s95

Nagai, S., & Toi, M. (2000). Interleukin-4 and breast cancer. *Breast Cancer*, *7*(3), 181–186.

Naka, T., Narazaki, M., Hirata, M., Matsumoto, T., Minamoto, S., Aono, A., ... Kishimoto, T. (1997). Structure and function of a new STAT-induced STAT inhibitor. *Nature, 387*, 924–929.

Nanba, H., Mori, K., Toyomasu, T., & Kuroda, H. (1987). Antitumor action of shiitake (*Lentinos edodes*) fruit bodies orally administered to mice. *Chemical and Pharmaceutical Bulletin*, *35*(6), 2453–2458.

Navard, J., & Navard, P. (2012). Introduction: challenges and opportunities in building a multinational, interdisciplinary research and education network on polysaccharides. In Navard, P. (Ed.) *The european polysaccharide network of excellence* (pp. 1–12). New York, USA: Springer.

Ng, R. F. L. (2015). *Immunomodulatory activity of selected Solanaceae extracts on macrophage RAW 264.7 cell line*. (Unpublished master's thesis). University of Malaya, Kuala Lumpur, Malaysia.

Nguyen, L. T., Lind, E. F., & Ohashi, P. S. (2013). The Immune System and Immunotherapy. In Tannock, I., Hill, R., Bristow, R., & Harrington, L. (Eds.). *The basic science of oncology* (5th Ed.) (pp. 501–528). New York, USA: McGraw-Hill.

Newton, K., & Dixit, V. M. (2012). Signaling in innate immunity and inflammation. *Cold Spring Harbor Perspectives in Biology*, *4*(3), a006049.

Nie, S., Zhang, H., Li, W., & Xie, M. (2013). Current development of polysaccharides from Ganoderma: Isolation, structure and bioactivities. *Bioactive Carbohydrates and Dietary Fibre*, *1*(1), 10–20.

Nurnberger, W., Platonov, A., Stannigel, H., Beloborodov, V. B., Michelmann, I., Von-Kries, R., ... Gobel, U. (1995). Definition of a new score for severity of generalized *Neisseria meningitidis* infection. *European Journal of Pediatrics, 154*(11), 896–900.

Obeid, E., Nanda, R., Fu, Y. X., & Olopade, O. I. (2013). The role of tumor-associated macrophages in breast cancer progression (review). *International Journal of Oncology*, *43*(1), 5–12.

Oh, S. S., Choi, M. W., Choi, M. R., Lee, J. H., Yang, H. J., Choi, Y. J., ... Sun, I. O. (2016). Acute interstitial nephritis induced by *Solanum nigrum*. *Kidney Research and Clinical Practice*, *35*(4), 252–254.

Pages, F., Galon, J., Dieu-Nosjean, M. C., Tartour, E., Sautes-Fridman, C., & Fridman, W. H. (2010). Immune infiltration in human tumors: a prognostic factor that should not be ignored. *Oncogene*, *29*(8), 1093–1102.

Palumbo, M. O., Kavan, P., Miller Jr., W. H., Panasci, L., Assouline, S., Johnson, N., ... Batist, G. (2013). Systemic cancer therapy: achievements and challenges that lie ahead. *Frontiers in Pharmacology*, *4*, 57–56.

Parameswaran, N., & Patial, S. (2010). Tumor necrosis factor-α signaling in macrophages. *Critical Reviews in Eukaryotic Gene Expression*, *20*(2), 87–103.

Park, H. Y., Choi, H., Yu, A., Choi, I., & Kim, Y. (2012). Immunomodulating effects of glycoprotein fraction isolated from rice bran on a RAW 264.7 macrophage cell line. *The FASEB Journal*, *26*(1), lb323.

Parnham, M. J., & Nijkamp, F. P. (2005). *Principles of immunopharmacology* (2nd Ed.). Basel, Switzerland: Birkhauser Verlag

Patil, U. S., Jaydeokar, A. V., & Bandawane, D. D. (2012). Immunomodulators: A pharmacological review. *International Journal of Pharmacy Pharmaceutical Sciences*, *4*(1), 30–36.

Peppelenbosch, M. P., DeSmedt, M., ten-Hove, T., van-Deventer, S. J., & Grooten, J. (1999). Lipopolysaccharide regulates macrophage fluid phase pinocytosis via CD14-dependent and CD14-independent pathways. *Blood*, *93*(11), 4011–4018.

Pericolini, E., Cenci, E., Monari, C., De-Jesus, M., Bistoni, F., Casadevall, A., & Vecchiarelli, A. (2006). *Cryptococcus neoformans* capsular polysaccharide component galacto xylomannan induces apoptosis of human T cells through activation of caspase 8. *Cellular Microbiology*, *8*(2), 267–275.

Plato, A., Willment, J. A., & Brown, G. D. (2013). C-type lectin-like receptors of the dectin-1 cluster: ligands and signaling pathways. *International Reviews of Immunology*, *32*(2), 134–156.

Poon, I. K., Lucas, C. D., Rossi, A. G., & Ravichandran, K. S. (2014). Apoptotic cell clearance: basic biology and therapeutic potential. *Nature Reviews Immunology*, *14*(3), 166–180.

Promega (2009). Griess reagent system. *Manufacture Protocol*. Madison, WI, USA. Retrieved from https://worldwide.promega.com/product/cell-health-assays/oxidative-stress-assays/griess-reagent-system/?catNum=G2930

Raman, M., Chen, W., & Cobb, M. H. (2007). Differential regulation and properties of MAPKs. *Oncogene*, *26*(22), 3100–3112.

Ramani, T., Auletta, C. S., Weinstock, D., Mounho-Zamora, B., Ryan, P. C., Salcedo, T. W., & Bannish, G. (2015). Cytokines: The good, the bad, and the deadly. *International Journal of Toxicology*, *34*(4), 355–365.

Ramberg, J. E., Nelson, E. D., & Sinnott, R .A. (2010). Immunomodulatory dietary polysaccharides: a systematic review of the literature. *Nutrition Journal, 9*(1), 54.

Ranjan, D., Chen, C., Johnston, T. D., Jeon, H., & Nagabhushan, M. (2004). Curcumin inhibits mitogen stimulated lymphocyte proliferation, NFκB activation, and IL-2 signaling. *Journal of Surgical Research*, *121*(2), 171–177.

Robak, T., Gladalska, A., & Stepien, H. (1998). The tumour necrosis factor family of receptors/ligands in the serum of patients with rheumatoid arthritis. *European Cytokine Network, 9*(2), 145–154.

Robertson, D., Savage, K., Reis-Filho, J. S., & Isacke, C. M. (2008). Multiple immunofluorescence labelling of formalin-fixed paraffin-embedded (FFPE) tissue. *BMC Cell Biology*, *9*(1), 13.

Robinet, P., Baychelier, F., Fontaine, T., Picard, C., Debré, P., Vieillard, V., ... Elbim, C. (2014). A polysaccharide virulence factor of a human fungal pathogen induces neutrophil apoptosis via NK cells. *The Journal of Immunology*, *192*(11), 5332–5342.

Sanandiya, N. D., & Siddhanta, A. K. (2014). Chemical studies on the polysaccharides of *Salicornia brachiata*. *Carbohydrate Polymers*, *112,* 300–307.

Sansone, P., Storci, G., Tavolari, S., Guarnieri, T., Giovannini, C., Taffurelli, M., ... Chieco, P. (2007). IL-6 triggers malignant features in mammospheres from human ductal breast carcinoma and normal mammary gland. *The Journal of Clinical Investigation*, *117*(12), 3988–4002.

Sarma, D. N. K., & Khosa, R. L. (1994). Immunomodulators of plant origin - A review. *Ancient Science of Life*, *13*(3–4), 326–331.

Saroj, P., Verma, M., Jha, K. K., & Pal, M. (2012). An overview of immunomodulation. *Journal of Advanced Scientific Research, 3*(1), 7–12.

Scheller, J., Chalaris, A., Schmidt-Arras, D., & Rose-John, S. (2011). The pro-and anti-inflammatory properties of the cytokine interleukin-6. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*, *1813*(5), 878–888.

Schepetkin, I. A., Kirpotina, L. N., Jakiw, L., Khlebnikov, A. I., Blaskovich, C. L., Jutila, M. A., & Quinn, M. T. (2009). Immunomodulatory activity of oenothein B isolated from *Epilobium angustifolium*. *The Journal of Immunology*, *183*(10), 6754–6766.

Schepetkin, I. A., & Quinn, M. T. (2006). Botanical polysaccharides: macrophage immunomodulation and therapeutic potential. *International Immunopharmacology*, *6*(3), 317–333.

Schmitz, J., Weissenbach, M., Haan, S., Heinrich, P. C., & Schaper, F. (2000). SOCS3 exerts its inhibitory function on interleukin-6 signal transduction through the SHP2 recruitment sites of gp130. *Journal of Biological Chemistry, 275*, 12848–12856.

Schroder, K., Hertzog, P. J., Ravasi, T., & Hume, D. A. (2004). Interferon-γ: an overview of signals, mechanisms and functions. *Journal of Leukocyte Biology, 75*(2), 163–89.

Shaha, D., Londhe, V., Mazumder, R., & Parikh, R. (2011). Can levamisole alone maintain the immunity?. *International Journal of Pharmacy and Pharmaceutical Sciences*, *3*, 161–164.

Sharma, H. L., & Sharma, K. K. (2007). *Principles of pharmacology* (1st Ed.). Hyderabad, India: Paras Medical Publisher

Sharp, J. G., Riches, A. C., Littlewood, V., & Thomas, D. B. (1976). The incidence, pathology and transplantation of hepatomas in CBA mice. *The Journal of Pathology*, *119*(4), 211–220.

Shearwin-Whyatt, L. M., Harvey, N. L., & Kumar, S. (2000). Subcellular localization and CARD-dependent oligomerization of the death adaptor RAIDD. *Cell Death and Differentiation, 7*(2), 155–165.

Shin, K. S., Kiyohara, H., Matsumoto, T., & Yamada, H. (1997). Rhamnogalacturonan II from the leaves of *Panax ginseng* CA Meyer as a macrophage Fc receptor expression-enhancing polysaccharide. *Carbohydrate Research*, *300*(3), 239–249.

Shinji, H., Kaiho, S., Nakano, T., & Yoshida, T. (1991). Reorganization of microfilaments in macrophages after LPS stimulation. *Experimental Cell Research*, *193*(1), 127–133.

Siegel, R. L., Miller, K. D., & Jemal, A. (2015). Cancer statistics, 2015. *CA: A Cancer Journal for Clinicians*, *65*(1), 5–29.

Sikdar, M., & Dutta, U. (2008). Traditional phytotherapy among the Nath people of Assam. *Ethno-Medicine*, *2*, 39–45.

Sistigu, A., Viaud, S., Chaput, N., Bracci, L., Proietti, E., & Zitvogel, L. (2011). Immunomodulatory effects of cyclophosphamide and implementations for vaccine design. *Seminars in Immunopathology, 33*(4), 369–383.

Slauch, J. M. (2011). How does the oxidative burst of macrophages kill bacteria? Still an open question. *Molecular Microbiology*, *80*(3), 580–583.

Snijders, A., Kalinski, P., Hilkens, C. M., & Kapsenberg, M. L. (1998). High-level IL-12 production by human dendritic cells requires two signals. *International Immunology, 10*, 1593–1598.

Song, J. Y., Han, S. K., Son, E. H., Pyo, S. N., Yun Y. S., & Yi S. Y. (2002). Induction of secretory and tumoricidal activities in peritoneal macrophages by ginsan. *International Immunopharmacology, 2,* 857–865.

Stoclet, J. C., Martinez, M. C., Ohlmann, P., Chasserot, S., Schott, C., Kleschyov, A. L., ... Andriantsitohaina, R. (1999). Induction of nitric oxide synthase and dual effects of nitric oxide and cyclooxygenase products in regulation of arterial contraction in human septic shock. *Circulation*, *100*(2), 107–112.

Stuffness, M., & Pezzuto, J. M. (1990). Assays related to cancer drug discovery. In Hostettmann, K. (Ed.), *Methods in plant biochemistry: Assays for bioactivity* (pp. 71–133). London, UK. Academic Press.

Sultana, S., Perwaiz, S., Iqbal, M., & Athar, M. (1995). Crude extracts of hepatoprotective plants, *Solanum nigrum* and *Cichorium intybus* inhibit free radical-mediated DNA damage. *Journal of Ethnopharmacology*, *45*(3), 189–192.

Sun, X. B., Matsumoto, T., & Yamada, H. (1994). Purification of immune complexes clearance enhancing polysaccharide from the leaves of Panax ginseng, and its biological activities. *Phytomedicine*, *1*(3), 225–231.

Szabo, S. J., Kim, S. T., Costa, G. L., Zhang, X., Fathman, C. G., & Glimcher, L. H. (2000). A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell, 100*, 655–669.

Tan, L., Eberhard, S., Pattathil, S., Warder, C., Glushka, J., Yuan, C., ... Baldwin, D. (2013). An Arabidopsis cell wall proteoglycan consists of pectin and arabinoxylan covalently linked to an arabinogalactan protein. *The Plant Cell*, *25*(1), 270–287.

Takeuchi, O., & Akira, S. (2010). Pattern recognition receptors and inflammation. *Cell*, *140*(6), 805–820.

Tanaka, T., Narazaki, M., & Kishimoto, T. (2014). IL-6 in inflammation, immunity, and disease. *Cold Spring Harbor Perspectives in Biology*, *6*(10), a016295.

Tannock, I., Hill, R., Bristow, R., & Harrington, L. (2013). *The basic science of oncology* (5th Ed.). New York, USA: McGraw Hill Education

Takeda, K., Hayakawa, Y., Smyth, M. J., Kayagaki, N., Yamaguchi, N., Kakuta, S., ... Okumura, K. (2001). Involvement of tumor necrosis factor-related apoptosis-inducing ligand in surveillance of tumor metastasis by liver natural killer cells. *Nature Medicine*, *7*(1), 94–100.

Teixeira, L. K., Fonseca, B. P., Barboza, B. A., & Viola, J. P. (2005). The role of interferon-gamma on immune and allergic responses. *Memorias do Instituto Oswaldo Cruz*, *100*, 137–144.

Thakur, M., Connellan, P., Deseo, M. A., Morris, C., & Dixit, V. K. (2011). Immunomodulatory polysaccharide from *Chlorophytum borivilianum* roots. *Evidence-Based Complementary and Alternative Medicine*, *2011*.

Titheradge, M. A. (1999). Nitric oxide in septic shock. *Biochimica et Biophysica Acta (BBA)-Bioenergetics*, *1411*(2), 437–455.

Toge, T., Kuroi, K., Kuninobu, H., Yamaguchi, Y., Kegoya, Y., Baba, N., & Hattori, T. (1988). Role of the spleen in immunosuppression of gastric cancer: predominance of suppressor precursor and suppressor inducer T cells in the recirculating spleen cells. *Clinical and Experimental Immunology*, *74*(3), 409–412.

Tortora, G. J., Funke, B. R., & Case, C. L. (2007). *Microbiology: An introduction – International student edition* (9th Ed.). San Francisco, USA: Pearson Benjamin Cummings.

Underhill, D. M., & Ozinsky, A. (2002). Phagocytosis of microbes: complexity in action. *Annual Review of Immunology*, *20*(1), 825–852.

Veronesi, U., Boyle, P., Goldhirsch, A., Orecchia, R., & Viale, G. (2005). Breast cancer. *The Lancet, 365*, 1727–1741.

Waage, A., Halstensen, A., & Espevik, T. (1987). Association between tumour necrosis factor in serum and fatal outcome in patients with meningococcal disease. *Lancet*, 1(8529), 355–357.

Waldhauer, I., & Steinle, A. (2008). NK cells and cancer immunosurveillance. *Oncogene*, *27*(45), 5932–5943.

Wajant, H. (2009). The role of TNF in cancer. *Death Receptors and Cognate Ligands in Cancer,* 1–15.

Wajant, H., Pfizenmaier, K., & Scheurich, P. (2003). Tumor necrosis factor signaling. *Cell Death and Differentiation*, *10*(1), 45–65.

Wang, K. S., Frank, D. A., & Ritz, J. (2000). Interleukin-2 enhances the response of natural killer cells to interleukin-12 through up-regulation of the interleukin-12 receptor and STAT4. *Blood*, *95*(10), 3183–3190.

Wang, S. Y., Tai, G. X., Zhang, P. Y., Mu, D. P., Zhang, X. J., & Liu, Z. H. (2008). Inhibitory effect of activin A on activation of lipopolysaccharide-stimulated mouse macrophage RAW 264.7 cells. *Cytokine*, *42*(1), 85–91.

Wang, X., & Lin, Y. (2008). Tumor necrosis factor and cancer, buddies or foes?. *Acta Pharmacologica Sinica*, *29*(11), 1275–1288.

Wang, Y., Huang, M., Sun, R., & Pan, L. (2015). Extraction, characterization of a Ginseng fruits polysaccharide and its immune modulating activities in rats with Lewis lung carcinoma. *Carbohydrate Polymer, 127*, 215–221.

Williams, L. M., & Ridley, A. J. (2000). Lipopolysaccharide induces actin reorganization and tyrosine phosphorylation of Pyk2 and paxillin in monocytes and macrophages. *The Journal of Immunology*, *164*(4), 2028–2036.

Wu, J., & Lanier, L. L. (2003). Natural killer cells and cancer. *Advances in Cancer Research, 90*, 127–156.

Wu, J., Zheng, J., Xia, X., & Kan, J. (2015). Purification and structural identification of polysaccharides from bamboo shoots (*Dendrocalamus latiflorus*). *International Journal of Molecular Sciences*, *16*(7), 15560–15577.

Wu, S., Fu, X., Brennan, M. A., Brennan, C. S., & Chun, C. (2016). The Effects of different purifying methods on the chemical properties, in vitro anti-tumor and immunomodulatory activities of *Abrus cantoniensis* polysaccharide fractions. *International Journal of Molecular Sciences*, *17*(4), 511.

Xu, H. S., Wu, Y. W., Xu, S. F., Sun, H. X., Chen, F. Y., & Yao, L. (2009). Antitumor and immunomodulatory activity of polysaccharides from the roots of *Actinidia eriantha*. *Journal of Ethnopharmacology*, *125*(2), 310–317.

Yang, X., Guo, D., Zhang, J. & Wu, M. (2007). Characterization and anti-tumor activity of pollen polysaccharide. *International Immunopharmacology*, *7*(3), 401–408

Yokota, T., Arai, N., Vries, J. D., Spits, H., Banchereau, J., Zlotnik, A., ... Lee, F. (1988). Molecular biology of interleukin 4 and interleukin 5 genes and biology of their products that stimulate B cells, T cells and hemopoietic cells. *Immunological Reviews, 102*(1), 137–87.

Yi, Y., Liao, S. T., Zhang, M. W., Shi, J., Zhang, R. F., Deng, Y. Y., & Wei, Z. C. (2011). Physicochemical characteristics and immunomodulatory activities of three polysaccharide-protein complexes of longan pulp. *Molecules*, *16*(7), 6148–6164.

Yip, C. H., Bhoo, P. N., & Teo, S. H. (2014). A review of breast cancer research in malaysia. *The Medical Journal of Malaysia*, *69*, 8–22.

Yip. C. H., Taib, N. A., & Mohamed, I. (2006). Epidemiology of breast cancer in Malaysia. *Asian Pacific Journal of Cancer Prevention*, *7*(3), 369–374.

Yu, M., Xu, X., Qing, Y., Luo, X., Yang, Z., & Zheng, L. (2009). Isolation of an anti-tumor polysaccharide from *Auricularia polytricha* (jew's ear) and its effects on macrophage activation. *European Food Research and Technology*, *228*(3), 477–485.

Yucel, A. A., Gulen, S., Dincer, S., Yucel, A. E., & Yetkin, G. I. (2012). Comparison of two different applications of the Griess method for nitric oxide measurement. *Journal of Experimental and Integrative Medicine*, *2*(2), 167–171.

Zamai, L., Ponti, C., Mirandola, P., Gobbi, G., Papa, S., Galeotti, L., ... Vitale, M. (2007). NK cells and cancer. *The Journal of Immunology*, *178*(7), 4011–4016.

Zhang, G. J., & Adachi, I. (1999). Serum interleukin-6 levels correlate to tumor progression and prognosis in metastatic breast carcinoma. *Anticancer Research*, *19*(2), 1427–1432.

Zhang, L., Conejo-Garcia, J. R., Katsaros, D., Gimotty, P. A., Massobrio, M., Regnani, G., ... Rubin, S. C. (2003). Intratumoral T cells, recurrence, and survival in epithelial ovarian cancer. *New England Journal of Medicine*, *348*(3), 203–213.

Zhang, M., Guo, R., Zhai, Y., & Yang D. (2003). LIGHT sensitizes IFN gamma-mediated apoptosis of MDA-MB-231 breast cancer cells leading to down-regulation of anti-apoptosis Bcl-2 family members. *Cancer Letters, 195*(2), 201–210.

Zhang, N., Wardwell, P. R., & Bader, R. A. (2013). Polysaccharide-based micelles for drug delivery. *Pharmaceutics*, *5*(2), 329–352.

Zhao, T., Mao, G., Mao, R., Zou, Y., Zheng, D., Feng, W., ... Chen, Y. (2013). Antitumor and immunomodulatory activity of a water-soluble low molecular weight polysaccharide from *Schisandra chinensis* (Turcz.) Baill. *Food and Chemical Toxicology*, *55*, 609–616.

Zheng, X., Liu, Z., Li, S., Wang, L., Lv, J., Li, J., ... Qian, F. (2016). Identification and characterization of a cytotoxic polysaccharide from the flower of *Abelmoschus manihot*. *International Journal of Biological Macromolecules*, *82*, 284–290.

Zhao, L., Dong, Y., Chen, G., & Hu, Q. (2010). Extraction, purification, characterization and antitumor activity of polysaccharides from *Ganoderma lucidum*. *Carbohydrate Polymers*, *80*(3), 783–789.

Zhao, X., Ma, S., Liu, N., Liu, J., & Wang, W. (2015). A polysaccharide from *Trametes robiniophila* inhibits human osteosarcoma xenograft tumor growth *in vivo*. *Carbohydrate Polymers*, *124*, 157–163.

Zhou, G., Sun, Y., Xin, H., Zhang, Y., Li, Z., & Xu, Z. (2004). *In vivo* antitumor and immunomodulation activities of different molecular weight lambda-carrageenans from *Chondrus ocellatus*. *Pharmacological Research*, *50*(1), 47–53.

Zhu, J., Liu, W., Yu, J., Zou, S., Wang, J., Yao, W., & Gao, X. (2013). Characterization and hypoglycemic effect of a polysaccharide extracted from the fruit of *Lycium barbarum* L. *Carbohydrate Polymers*, *98*(1), 8–16.

LIST OF PUBLICATIONS AND PAPERS PRESENTED

Publications

1. **Razali, F. N**., Ismail, A., Abidin, N. Z., & Shuib, A. S. (2014). Stimulatory effects of polysaccharide fraction from *Solanum nigrum* on RAW 264.7 murine macrophage cells. *PloS One*, *9*(10), e108988.

2. **Razali, F. N**., Sinniah, S. K., Hussin, H., Abidin, N. Z., & Shuib, A. S. (2016). Tumor suppression effect of *Solanum nigrum* polysaccharide fraction on breast cancer via immunomodulation. *International Journal of Biological Macromolecules*, *92*, 185–193.

Papers presented

1. **Razali, F. N**., Ghazali, M. S., Abidin, N. Z., & Shuib, A. S. (2013). Immunomodulatory effects of *Solanum nigrum* polysaccharide on RAW 264.7 cell line. UniSZA Postgraduate Research Conference 2013, 7–8th September 2013 at University Sultan Zainal Abidin (UniSZA), Terengganu, Malaysia.

2. **Razali, F. N**., Ghazali, M. S., Abidin, N. Z., & Shuib, A. S. (2013). Effects of *Solanum nigrum* polysaccharide fraction SN-ppF3 on RAW 264.7 macrophage cell. The International Postgraduate Conference on Science and Mathematics 2013, 5–6th October 2013 at Convention Hall, E-Learning Building Universiti Pendidikan Sultan Idris (UPSI) Perak, Malaysia

3. **Razali, F. N**., Abidin, N. Z., & Shuib, A. S. (2014). Anti-cancer potential by *Solanum nigrum* polysaccharide fraction through immunomodulation on tumor bearing mice. The Third Thailand-Malaysia Graduate Forum in Life Sciences, Food Sciences and Agriculture 2014, 16–20 December 2014 at Kasetsart University, Bangkok, Thailand.

4. **Razali, F. N**., Hussin, H., Abidin, N. Z., & Shuib, A. S. (2015). Tumor suppression effect by *Solanum nigrum* polysaccharide fraction on animal model through immunomodulation. The 2nd International Conference of Traditional and Complementary Medicine on Health 2015, 24–25 October 2015 at GIS NTU Convention Centre, Taipei, Taiwan.

OPEN ACCESS Freely available online

PLOS ONE

Stimulatory Effects of Polysaccharide Fraction from *Solanum nigrum* on RAW 264.7 Murine Macrophage Cells

Faizan Naeem Razali[1], Amirah Ismail[1], Nurhayati Zainal Abidin[1], Adawiyah Suriza Shuib[1,2]*

1 Institute of Biological Sciences, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia, 2 University of Malaya Centre for Proteomics Research, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia

Abstract

The polysaccharide fraction from *Solanum nigrum* Linne has been shown to have antitumor activity by enhancing the $CD4^+$/$CD8^+$ ratio of the T-lymphocyte subpopulation. In this study, we analyzed a polysaccharide extract of *S. nigrum* to determine its modulating effects on RAW 264.7 murine macrophage cells since macrophages play a key role in inducing both innate and adaptive immune responses. Crude polysaccharide was extracted from the stem of *S. nigrum* and subjected to ion-exchange chromatography to partially purify the extract. Five polysaccharide fractions were then subjected to a cytotoxicity assay and a nitric oxide production assay. To further analyze the ability of the fractionated polysaccharide extract to activate macrophages, the phagocytosis activity and cytokine production were also measured. The polysaccharide fractions were not cytotoxic, but all of the fractions induced nitric oxide in RAW 264.7 cells. Of the five fractions tested, SN-ppF3 was the least toxic and also induced the greatest amount of nitric oxide, which was comparable to the inducible nitric oxide synthase expression detected in the cell lysate. This fraction also significantly induced phagocytosis activity and stimulated the production of tumor necrosis factor-α and interleukin-6. Our study showed that fraction SN-ppF3 could classically activate macrophages. Macrophage induction may be the manner in which polysaccharides from *S. nigrum* are able to prevent tumor growth.

Citation: Razali FN, Ismail A, Abidin NZ, Shuib AS (2014) Stimulatory Effects of Polysaccharide Fraction from *Solanum nigrum* on RAW 264.7 Murine Macrophage Cells. PLoS ONE 9(10): e108988. doi:10.1371/journal.pone.0108988

Editor: Rasheed Ahmad, Dasman Diabetes Institute, Kuwait

Received April 12, 2014; **Accepted** September 2, 2014; **Published** October 9, 2014

Funding: This work was funded by the Ministry of Higher Education, Malaysia (FRGS FP001/2011A) and University Malaya Postgraduate Research Grant (PPP PG057-2012B). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* Email: adawiyah@um.edu.my

Introduction

In recent decades, the engineering and production of increasingly efficient chemotherapies has become possible. Despite this, there continues to be a need for additional targeted therapies with fewer side effects and greater specificity. Among the newer options for treatment is the possibility to modulate the immune response within the patient, making the patient's own immune system more capable of eliminating cancer cells.

Studies have shown that a variety of polysaccharides can act as immunomodulators, helping to stimulate the immune system or and enhanced immune response have been observed in these animals.

The herbal plant *Solanum nigrum* is widely distributed throughout the world, extending from tropical to temperate regions [6]. Because of the folkloric belief that it imparts numerous benefits, including curing cancer, it has been studied extensively [7]. Studies done by several researchers showed that crude extract from *S. nigrum* suppresses free radical–mediated DNA damage [8], induces necrosis in SC-M1 stomach cancer cells [9], inhibits 12-*O*-tetradeca-noylphorbol-13-acetate-induced tumor promotion

International Journal of Biological Macromolecules 92 (2016) 185–193

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac

Tumor suppression effect of *Solanum nigrum* polysaccharide fraction on Breast cancer via immunomodulation

Faizan Naeem Razali [a], Saravana Kumar Sinniah [a], Huzlinda Hussin [b], Nurhayati Zainal Abidin [a], Adawiyah Suriza Shuib [a,c,*]

[a] Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia
[b] Department of Pathology, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, Serdang, Selangor, Malaysia
[c] University of Malaya Centre for Proteomics Research, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia

ARTICLE INFO

Article history:
Received 1 April 2016
Received in revised form 21 June 2016
Accepted 25 June 2016
Available online 27 June 2016

Keywords:
Plant polysaccharide
Solanum nigrum
Immunomodulation

ABSTRACT

A polysaccharide fraction from *Solanum nigrum*, SN-ppF3 was shown previously to have an immunomodulatory activity where it could possibly be used to enhance the host immune response in fighting cancer. The non-toxic SN-ppF3 was fed orally to breast tumor bearing-mice with concentrations of 250 and 500 mg/kg for 10 days. During the treatment period, size of the tumor and weight of the mice were monitored. At the end of the treatment, blood, tumor, spleen and thymus were harvested for physiological and immunological analyses. After the treatment, the tumor volume and tumor weight were significantly inhibited by 65% and 40%, respectively. Based on the histological observation, the treatment of SN-ppF3 resulted in the disruption of tumor cells morphology. The increase in infiltrating T cells, NK cells and macrophages were observed in tumor tissues of the treated mice, which partly explained the higher apoptosis tumor cells observed in the treated mice. Moreover, the level of TNF-α, IFN-γ and IL-4 were elevated, while the level of IL-6 was decreased significantly, in serum of the treated mice. These results suggested that tumor suppression mechanisms observed in SN-ppF3-treated mice were most probably due through enhancing the host immune response.

1. Introduction

In the current decade, cancer has still remained the top listed death-causing disease despite of developments in tools of diagnosis, treatment and prevention. Among these, breast cancer is the most common-death causing cancer. According to Seigel et al. in the year of 2015, it was estimated that breast cancer contributed approximately 29% of all new female cancers in the United State [1]. Although the major modern therapies and techniques have become possible in treating this cancer, the treatments still have some drawbacks such as unavoidable toxic due to nonspecific action towards cancer cells which would affect the quality of patients' lives [2]. Moreover, many evidences have demonstrated that the anti-tumor activities of many chemotherapeutic agents have resulted in the toxicity of normal cells and organ damages as well [3]. Thus the identification of novel drugs with a better effectiveness and lower toxicity is necessary. Numerous studies on breast cancer therapies are still ongoing, and one of the possible strategies to encounter this type of cancer is through the improvement of patient or host immune responses.

Evasion of the immune surveillance of the host by tumor cells by modifying the host's immune response is one of the main reasons for the rapid progression of cancer [4]. One of the approaches in overcoming this problem is to modulate the immune response within the host, making the host's own immune system more capable in eliminating cancer cells [5]. There are many mechanisms to

Abbreviations: SN-ppF3, *Solanum nigrum* polysaccharide fraction number 3; FT-IR, Fourier Transform Infrared; CTX, cyclophosphamide; ELISA, enzyme-linked immunosorbent assay; DMEM, Dulbecco's modified eagle medium; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; IC_{50}, inhibitory

APPENDIX

APPENDIX A: GENERAL MATERIALS

The materials used during this experiment and their respective brands and suppliers are listed. All general chemicals were analytical standard grade and were purchased from Sigma-Aldrich Company, unless stated.

1 Chromatographic materials

1.1 Whatman, Maidstone, England

Diethylaminoethyl (DEAE) pre-swollen ion-exchanger resin

1.2 Sigma-Aldrich, St. Louis, MO, USA

Sepharose CL-6B size exclusion chromatography resin

2 Commercial kits

2.1 Cayman Chemical Company, Ann Arbor, MI, USA

Phagocytosis assay kit (IgG-FITC conjugated)

2.2 Bio-Rad, Hercules, CA, USA

Bradford Protein Quantification kit

2.3 GE Healthcare, Piscataway, NJ, USA

2-D protein extraction kit

2.4 Abcam, Cambridge, UK

TNF-α mouse ELISA kit

IFN-γ mouse ELISA kit

IL-4 mouse ELISA kit

IL-6 mouse ELISA kit

2.5 Sigma-Aldrich, St. Louis, MO, USA

Limulus amebocyte lysate (LAL) E-TOXATE^{TM} kit

2.6 Cell Signalling Technology, Danvers, MA, USA

PathScan Inflammation Multi-Target Sandwich ELISA kit

2.7 Roche, Basel, Switzerland

In situ Cell Death Detection kit (FITC conjugated)

3 Antibodies

3.1 Cayman Chemical, Ann Arbor, MI, USA

Anti-mouse iNOS primary polyclonal antibody

3.2 BioVision Instrument, Milpitas, CA, USA

Anti-mouse β-actin primary polyclonal antibody

3.3 Abnova, Taipei City, Taiwan

Anti-rabbit IgG secondary antibody

3.4 Abcam, Cambridge, UK

Anti-CD69 NK cells (Cy5® conjugated) antibody

Anti-CD8 T cells (Phycoerythrin conjugated) antibody

Anti-F4/80 macrophage (FITC conjugated) antibody

APPENDIX B: PREPARATION OF SOLUTIONS / REAGENTS

1 Standard solutions

1.1 Phosphate-buffered saline, pH 7.8

NaCl	9.36 g
KCl	0.25 g
Na_2HPO_4	0.25 g
KH_2HPO_4	1.42 g

The solution was made up to 1 L with distilled water and pH was adjusted to 7.8 by using hydrochloric acid (HCl) or sodium hydroxide (NaOH). The solution was filtered through filter paper and stored at room temperature.

1.2 50 mM sodium phosphate buffer, pH 7.4

Sodium phosphate monobasic	1.60 g
Sodium phosphate dibasic	10.40 g

The solution was made up to 1 L with distilled water and pH was adjusted to 7.4 by using phosphoric acid (H_3PO_4) or NaOH. The solution was filtered through filter paper and stored at room temperature. The solution was diluted with distilled water by 10× serial dilution for 5 mM of sodium phosphate buffer (pH 7.8) preparation.

1.3 Tris-Buffered Saline

Tris	12.11 g
NaCl	9.00 g
Distilled water	1 L

Tris was dissolved in 900 mL distilled water and the pH of the solution was adjusted to 7.5 by addition of HCl. NaCl was then added and the solution was made up to 1 L with distilled water.

1.4 2 L of 80% ethanol

To prepare the 80% ethanol solution, 400 mL of distilled water were added to 1600 mL of absolute ethanol.

1.5 1 L of 70% ethanol

To prepare the 70% ethanol solution, 300 mL of distilled water were added to 700 mL of absolute ethanol.

1.6 100 mL of 95% ethanol

To prepare the 95% ethanol solution, 5 mL of distilled water were added to 95 mL of absolute ethanol.

1.7 Phenol solution, 5%

To prepare the phenol solution, 5 grams of phenol crystal was dissolved in 90 mL of distilled water. The solution was stirred vigorously until phenol crystal was completely dissolved. The solution was made up to 100 mL with distilled water. The solution was stored at room temperature until further use.

1.8 1 L of 1.5 M NaCl solution

To prepare the 1.5 M of NaCl solution, 87.66 g of NaCl was dissolved in 1 L of distilled water. The solution was filtered through filter paper no. 1 (Whatman)

1.9 Normal saline (NaCl solution, 0.9%)

NaCl	9.00 g
Distilled water	1 L

To prepare the normal saline, NaCl was dissolved in 900 mL of distilled water and stirred vigorously until NaCl was completely dissolved. The solution was made up to 1 L, autoclaved and stored at room temperature until further use.

2 Cell culture

2.1 1 L of DMEM solution, pH 7.4

To prepare the basic DMEM medium, 13.4 g/L of DMEM high glucose powder was dissolved in 900 mL of steriled distilled water before 2 g of sodium hydrogen carbonate and 0.5206 g of HEPES were added. Solution pH was adjusted to 7.4 with 1 M of NaOH or 1 M of HCl solution. The solution was made up to 1 L with steriled distilled water and filtered through sterile Minisart 0.2 μM membrane filter (Sartorius Stedim Biotech, Goettingen, Germany) in sterile condition and stored at 4°C until further use.

2.2 100 mL of DMEM supplemented with 10% FBS

To prepare the DMEM supplemented with 10% FBS, 10 mL FBS, 2 mL of 100× penicillin/streptomycin and 1 mL of 100× amphotericin B were added to 87 mL of DMEM. The solution was filtered through sterile Minisart 0.2 μM membrane filter (Sartorius Stedim Biotech) in sterile condition and stored at 4°C until further use.

3 Cytotoxicity evaluation by [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide] (MTT) cytotoxicity assay

3.1 10 mL of 5 mg/mL MTT solution

To prepare the MTT solution, 50 mg of MTT powder was diluted in 9 mL of PBS. The solution was made up to 10 mL with PBS and filtered through sterile Minisart 0.2 μM membrane filter (Sartorius Stedim Biotech) in sterile condition. The solution was aliquoted into 1 mL each and stored at -20°C until further use.

4 Measurement of nitric oxide production

4.1 Sulphanilamide solution

To prepare the sulphanilamide solution, 1 g of 98% sulphanilamide was dissolved in 90 mL of 5% H_3PO_4. The solution was stirred vigorously until sulphanilamide powder was completely dissolved. The solution was made up to 100 mL with 5% H_3PO_4 and stored at 4°C until further use.

4.2 N-1-napthylethylenediamine dihydrochloride (NED) solution, 0.1%

To prepare the NED solution, NED powder was dissolved in 90 mL of distilled water. The solution was stirred vigorously until NED powder was completely dissolved. The solution was made up to 100 mL with distilled water and stored at 4°C until further use.

4.3 Nitrite standard stock solution, 0.1 M

To prepare the nitrite stock solution, 0.69 g of sodium nitrite was dissolved in 90 mL of distilled water. The solution was stirred vigorously until sodium chloride powder was completely dissolved. The solution was made up to 100 mL with distilled water and stored at 4°C until further use.

4.4 Nitrite standard solution, 100 µM

To prepare the nitrite standard solution, 1 µL of nitrite standard stock solution was diluted in 999 µL of DMEM medium. The solution was freshly prepared prior to use.

5 Detection of inducible nitric oxide synthase by Western blot

5.1 Tris-sucrose buffer, 10 mM, pH 7.0

Tris-base	1.21 g
Sucrose	85.6 g
Distilled water	1 L

Tris-base and sucrose powders were dissolved in 800 mL of distilled water. Solution pH was adjusted to 7.0 by using HCl or NaOH. The solution was made up to 1 L with distilled water and stored at 4°C until further use.

5.2 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

A. Acrylamide, 30% and N,N'-methylenebisacrylamide, 0.8%

Acrylamide	60.0 g
N,N'-methylenebisacrylamide	1.8 g
Distilled water	200 mL

To prepare the solution A, acrylamide and N,N'-methylenebisacrylamide were dissolved in distilled water and the solution was made up until 200 mL. The solution was deionised by using amberlite MB-1, stirred for 1 hour and filtered through filter paper number 1 (Whatman). The solution was stored in a dark bottle at 4°C until further use.

B. Tris-HCl, pH 8.8, 1.5 M

Tris-base	36.23 g
Distilled water	200 mL

To prepare the solution B, Tris-base was dissolved in 150 mL of distilled water. The solution pH was adjusted to 8.8 by using 1 M of HCl or NaOH. The solution was made up to 200 mL with distilled water and stored at 4°C until further use.

C. Sodium dodecyl sulfate (SDS), 10%

SDS	10.00 g
Distilled water	100 mL

To prepare the solution C, SDS powder was dissolved in 90 mL of distilled water. The solution was stirred vigorously until SDS powder was completely dissolved. The solution was made up to 100 mL with distilled water and stored at room temperature until further use.

D. Ammonium persulfate (APS), 10%

APS	0.10 g
Distilled water	1 mL

To prepare the solution D, APS powder was dissolved in 800 µL of distilled water. The solution was stirred vigorously until APS powder was completely dissolved. The solution was made up to 1.0 mL with distilled water. The solution was freshly prepared prior to use.

E. N, N, N'tetramethylethylenediamine (TEMED)

F. Tris-HCl, 0.5 M, pH 6.8

Tris-base	12.11 g
Distilled water	200 mL

To prepare the solution F, Tris-base powder was dissolved in 150 mL of distilled water. The solution was stirred vigorously until Tris-base powder was completely dissolved. The solution pH was adjusted to 6.8 using 1 M of HCl or NaOH. The solution was made up to 200 mL with distilled water and stored at 4°C until further use.

5.3 SDS-PAGE sample buffer, 4×

Glycerol	2.00 mL
Bromophenol blue	20 mg
DTT	200 mg
SDS	400 mg
Solution E	2.50 mL
Distilled water	20.00 mL

To prepare the SDS-PAGE sample buffer, solution E was mixed together with glycerol, DTT, SDS and bromophenol blue. The solution was made up to 20 mL with distilled water. SDS-PAGE sample buffer was aliquoted with the volume of 720 µL into 1 mL microcentrifuge tube and stored at -20°C until further use. Prior the used of sample buffer, 72 µL of β-mercaptoethanol was freshly added to each tube and vortexed to mix.

5.4 Trailing buffer (Cathode buffer), pH 8.3

Tris-base	3.03 g
Glycine	14.41 g
SDS	1.00 g
Distilled water	1 L

To prepare the trailing buffer, Tris-base, glycine and SDS were dissolved in 800 mL of distilled water. The pH of the solution was adjusted to 8.3 using 1 M of HCl or NaOH. The solution was made up to 1 L with distilled water and stored at 4°C until further use.

5.5 Transfer buffer, pH 8.3

Tris-base	3.03 g
Glycine	14.41 g
Methanol, 10%	100 mL
Distilled water	1 L

To prepare the transfer buffer, Tris-base and glycine were dissolved in methanol. The solution was diluted with 800 mL of distilled water and pH of the solution was adjusted to 8.3 using 1 M of HCl or NaOH. The solution was made up to 1 L with distilled water and stored at 4°C until further use.

5.6 Tris-buffered saline (TBS) Tween-20 buffer, pH 7.4

Tris-base	6.05 g
NaCl	8.76 g
Tween-20 solution	1 mL
Distilled water	1 L

To prepare the TBS Tween-20 buffer, Tris-base and NaCl were dissolved in 900 mL of distilled water and 1 mL of Tween-20 solution was added. The solution was stirred vigorously until powders were completely dissolved. The solution was made up to 1 L with distilled water and stored at 4°C until further use.

5.7 Gelatin blocking solution, 3%

Gelatin powder	3.00 g
TBS Tween-20, pH 7.4	100 mL

Gelatin powder was dissolved with 90 mL of TBS Tween-20 and stirred at 50°C until completely dissolved. The solution was made up to 100 mL with TBS Tween-20. The solution was freshly prepared and cooled to room temperature prior to use.

6 Pinocytosis analysis

6.1 Neutral red (NR) solution, 50 µg/mL

NR powder	0.04 g
Sterile distilled water	10 mL

To prepare the NR solution, NR powder was dissolved in 8 mL of sterile distilled water. The solution was stirred vigorously until NR powder was completely dissolved. The solution was made up to 100 mL with steriled distilled water. The solution was centrifuged at 1000 rpm for 10 minutes to precipitate the needle-like crystal and kept in a dark tube. The solution was freshly prepared prior to use.

6.2 NR washing solution

Calcium chloride ($CaCl_2$)	1.00 g
Formaldehyde solution	500 µL
Distilled water	100 mL

To prepare the NR washing solution, $CaCl_2$ was dissolved in 80 mL of distilled water and 500 µL of formaldehyde solution was added. The solution was stirred vigorously until $CaCl_2$ was completely dissolved. The solution was made up to 100 mL with distilled water and stored in a dark bottle at 4°C until further use.

6.3 NR resorb solution

Acetic acid solution	1 mL
Absolute ethanol	50 mL
Distilled water	49 mL

To prepare the NR resorb solution, absolute ethanol was diluted with 49 mL of distilled water and 1 mL of acetic acid solution was added. The solution was stirred and stored in a dark bottle at 4°C until further use.

APPENDIX C: PREPARATION OF STANDARD CURVES

1 D-glucose

The standard curve was constructed based on several glucose concentrations. To prepare 100 mg/mL of glucose solution, 100 mg of D-glucose powder was dissolved in 1 mL of distilled water. Glucose solution at concentration of 50, 25, 12.5, 6.25, 3.125 and 1.56 mg/mL were prepared by serial diluting 100 mg/mL of glucose solution with distilled water at 1:1 dilution factor. Fifty microliter of glucose solution at each concentration was pipetted into a 96-wells microplate. Then, 150 µL of absolute sulphuric acid was added into each well, followed immediately by 30 µL of 5% phenol solution. The plate was heated in a 95°C water bath for 10 minutes and the absorbance at 490 nm was immediately measured by Multiskan Go microplate spectrophotometer (Thermo Scientific).

2 Bovine serum albumin (BSA)

The standard curve was constructed based on several BSA concentrations, provided in the kit. Fifty microliter of BSA at concentration of 2, 1.75, 1.5, 1.25, 1, 0.5, 0.25 and 0 mg/mL, were pipetted into a 96-wells microplate. Then, 200 µL of Bradford reagent was added into each well and incubated in a dark condition for 10 minutes. The absorbance at 595 nm was immediately measured by Multiskan Go microplate spectrophotometer (Thermo Scientific).

3 Nitrite

The standard curve was constructed based on several nitrite concentrations. Nitrite solution at concentration of 50, 25, 12.5, 6.25, 3.125 and 1.56 µM were prepared by serial diluting 100 µM of nitrite solution (Appendix B4.4) with fresh culture medium at 1:1 dilution factor. One-hundred microliter of nitrite solution at each concentration

was pipetted into a 96-wells microplate. Then, 50 µL of sulphanilamide solution was added into each well and the plate was incubated in the dark for 5–10 minutes at room temperature. After that, 50 µL of NED solution (Appendix B4.2) was added to each well and the plate was incubated again in the dark for another 5–10 minutes at room temperature. The absorbance at 520 nm of was immediately measured using Multiskan Go microplate spectrophotometer (Thermo Scientific).

4 Cytokines

The standard curves of TNF-α IFN-γ IL-4 and IL-6 were constructed based on several concentrations of each recombinant cytokine solution provided in the respective ELISA kit. One-hundred microlitre of each recombinant cytokine solution was pipetted into a 96-wells microplate coated with respective anti-mouse TNF-α, IFN-γ, IL-4 or IL-6 and the plate was incubated overnight at 4°C. Then, the recombinant cytokine solutions were discarded, and the wells were washed 4 times with 300 µL washing buffer. After that, 100 µL of biotinylated anti-mouse antibodies for TNF-α, IFN-γ, IL-4 or IL-6 were added, respectively into each well, and the plate was incubated for 1 hour at room temperature with gentle shaking. The solution was discarded, and the wells were washed again before 100 µL of horseradish peroxidase-streptavidin solution was added into each well. The plate was incubated for 45 minutes at room temperature with gentle shaking. After the final wash, 100 µL TMB One-Step Substrate Reagent was added to each well, and the plate was incubated for another 30 minutes in the dark with gentle shaking. Lastly, 50 µL of stop solution was added into each well to stop the colour development, and the absorbance at 450 nm was immediately measured by a Multiskan Go microplate spectrophotometer (Thermo Scientific).