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American Ginseng Modulation of Immune Function and Phytochemical Analysis

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Graduate Program in Pharmacology and Toxicology
A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of
Philosophy
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AMERICAN GINSENG MODULATION OF IMMUNE FUNCTION AND
PHYTOCHEMICAL ANALYSIS

(Thesis format: Integrated Article)

by

Chike G Azike

Graduate Program in Pharmacology and Toxicology

A thesis submitted in partial fulfilment
of the requirements for the degree of
Doctor of Philosophy

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

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Abstract

The relationship between American ginseng immunostimulatory and immunoinhibitory effects and the unique bioactive fractions of its different extracts namely aqueous (AQ) and alcoholic (ALC) extracts was investigated. AQ extract up-regulated the production of nitric oxide (NO), tumour necrosis factor- α (TNF- α) and interleukin-6 (IL-6), while ALC extract did not upregulate macrophage function. ALC extract but not AQ extract suppressed lipopolysaccharide (LPS) induced macrophage NO and TNF- α production. Macrophage-stimulating activity of the AQ extract was inhibited in the presence of ALC extract. Fractionation of AQ extract revealed that its crude polysaccharides (PS) are the only immunostimulatory phytochemical. Fractionation study of ALC extract showed that its macromolecule and ginsenoside fractions contribute to the extract's immunoinhibitory effect. ALC extract which was devoid of PS, was immunoinhibitory whereas the AQ extract which contained PS, was immunostimulatory. These effects may be considered as the paradoxical immunomodulatory actions of ginseng.

Recent studies suggest that ginseng PS also suppress induced proinflammatory responses. Investigation was performed *ex vivo* and *in vivo* to determine whether American ginseng roots polysaccharides (AGRPS) stimulates basal innate immune function and at the same time can suppress LPS proinflammatory response. An *in vitro* mechanistic study was used to identify the fractions responsible for AGRPS immunobioactivities. Orally administered AGRPS exerted immunostimulation and suppressed LPS immune response under basal and LPS proinflammatory conditions *ex vivo* and *in vivo*. Similar AGRPS immunostimulatory and immunosuppressive effects were observed *in vitro*, and these AGRPS immunodulatory effects were mediated primarily by acid PS and its species with molecular weights ≥ 100 kDa

and 50 - 100 kDa.

The intestinal absorption of orally administered immunomodulatory AGRPS is yet to be ascertained. Absence of a method to analyze ginseng PS created the need for a novel method to investigate the intestinal absorption of orally administered unlabeled AGRPS into systemic circulation. Perchloric acid-protein precipitation of plasma and high performance size exclusion chromatography (HPSEC) with right angle light scattering detection was used as novel approach to analyze AGRPS in plasma of rats after oral administration of AGRPS. Outcome of this study indicates that orally administered immunomodulatory AGRPS is absorbed from the gastrointestinal tract into systemic circulation.

Keywords: American ginseng, aqueous extract, alcoholic extract, phytochemical, polysaccharides, macromolecules, immunostimulation, suppression of LPS induced immunologic response, *ex vivo*, *in vivo*, *in vitro*, gastrointestinal tract absorption, plasma analysis, systemic circulation.

CO-AUTHORSHIP

Published Chapters of this thesis have been marked as so on the title page of that chapter.

Experimental work and initial manuscript preparation was performed by Chike G Azike who also received considerable aid from colleagues and supervisors as follows:

CHAPTER 3:

1. Chike G Azike, Paul A Charpentier, Jirui Hou, Hua Pei, Edmund MK Lui (2011). The Yin and Yang actions of North American ginseng root in modulating the immune function of macrophages. *Chinese Medicine*, 6, 1-12.
2. Chike G Azike, Paul A Charpentier and Edmund MK Lui (2014). Novel macromolecules of American ginseng root Alcoholic extract suppresses Lipopolysaccharide induced production of inflammatory mediators *in vitro*. Manuscript in preparation.

The RAW 264.7 (ATCC TIB 67) murine macrophage cell lines were provided by Dr. Jeff Dixon. Hua Pei lyophilized the ginseng extracts and maintained the murine macrophages (Raw 264.7) culture. PolyAnalytik London Ontario, Canada performed the gel permeation chromatography of American ginseng extracts. Dr. Edmund MK Lui and Dr. Paul A Charpentier supervised the project and aided in manuscript preparation. The preparation of American ginseng polysaccharides, fractionation of American ginseng extracts via size exclusion chromatography, ultrafiltration, *in vitro* bioassays and writing of manuscript were done by Chike G Azike.

CHAPTER 4:

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Romeh, Hua Pei, John T Arnason, Paul A Charpentier (2012). Bioactive Polysaccharides

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Phytochemical and Pharmacological Characterization. The Complex World of

Polysaccharides. InTech Publications, Chapter 19, 513-534.

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Suppression of Innate Immune Function by American Ginseng Polysaccharides:

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Dr. Edmund MK Lui, Dr. Paul A Charpentier and Dr. John T Arnason supervised the project and aided in manuscript preparation. Dr. José A Guerrero-Analco, Sherif J Kalda and Chike G Azike performed the preparation, ion exchange chromatography, size exclusion chromatography and ultrafiltration of American ginseng polysaccharides. *Ex vivo*, *in vivo* and *in vitro* bioassays and writing of manuscript were done by Chike G Azike.

CHAPTER 5:

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Plasma by High Performance Size Exclusion Chromatography. Manuscript in preparation.

Dr. William Z. Xu and Ahmad A. Romeh provided guidance in the selection of columns and maintenance of High Performance Size-Exclusion Chromatography (HPSEC) instrument. Dr. Edmund MK Lui and Dr. Paul A Charpentier supervised the project and aided in manuscript preparation. Administration of American ginseng polysaccharides to rats, collection & processing of plasma samples, HPSEC instrumental analysis of American ginseng polysaccharides in plasma and writing of manuscript were done by Chike G Azike.

DEDICATION

This Thesis is dedicated to God Almighty, the LORD Most High, the great King of all the earth (Psalm 47).

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LIST OF ABBREVIATIONS

Abbreviation	Full Name
ALC	Alcoholic
AGRPS	American Ginseng Root Polysaccharides
AP-1	Activator protein 1
AQ	Aqueous
AUC	Area Under the Concentration–time curve
BAL	Broncho Alveolar Lavage
CAM	Complementary or Alternative Medicine
C _{max}	Maximum plasma Concentration
COX-2	Cyclooxygenase-2
Da	Dalton
DEAE	Diethylaminoethyl
DC	Dendritic cells
ELISA	Enzyme-Linked Immunosorbent Assay
ERK1/2	Extracellular signal-regulated kinases 1/2
GAP	Good Agricultural Practice
GFC	Gel Filtration Chromatography
GPC	Gel Permeation Chromatography
GMP	Good Manufacturing Practices

GRPS	Ginseng root polysaccharides
HPLC	High Performance Liquid Chromatography
HPSEC	High Performance Size Exclusion Chromatography
IKK	Inhibitory Kappa Kinase
iNOS	inducible Nitric Oxide Synthase
IR	Infrared
IRAKs	Interleukin-1 receptor-associated kinases
IL-6	Interleukin-6
JNKs	Jun N-terminal kinases
kDa	kilo Dalton
LPS	Lipopolysaccharide
LC	Liquid Chromatography
MAPK	Mitogen-Activated Protein Kinase
MyD88	Myeloid Differentiation factor 88
Mw	Molecular weight
NF- κ B	Nuclear Factor kappa B
NK	Natural Killer
NHPs	Natural Health Products
NMR	Nuclear Magnetic Resonance
NO	Nitric Oxide

OGRIC	Ontario Ginseng Research & Innovation Consortium
PAMPs	Pathogen-Associated Molecular Patterns
PI3K	Phosphoinositide 3-kinase
PRRs	Pattern Recognition Receptors
PBS	Phosphate-Buffered Saline
PGE ₂	Prostaglandin E ₂
PS	Polysaccharides
PPD	Protopanaxadiol
PPT	Protopanaxatriol
RI	Refractive Index
SD	Standard Deviation
SEC	Size Exclusion Chromatography
TAK1	Transforming growth factor-beta Activated Kinase 1
TIR	Toll/IL-1 receptor
TIRAP	TIR domain-containing adaptor protein
TLRs	Toll-like receptors
TLR-4	Toll-like receptor 4
TRAF6	TNF receptor associated factor 6
TRAM	TRIF-related adaptor molecule
TM	Traditional Medicine

T_{\max}	Time to attain C_{\max}
TM	Traditional medicine
TNF- α	Tumor Necrosis Factor-alpha
UV	Ultraviolet
WHO	World Health Organisation

Chapter 1

INTRODUCTION AND LITERATURE REVIEW

1. Introduction

1.1 Medicinal Plants

1.1.1 The Use of Medicinal Plants in Traditional Medicine

Our experience with medicinal plants can be traced back to biblical times as described in the book of Ezekiel chapter 44 verse 12 which states ‘and the leaf thereof for medicine’ [1]. Since ancient times mankind has utilized the properties of plants not just for food and shelter, but also for health and well-being’ [2]. Early empirical observations served as the basis for the use of plants as prophylactic and therapeutic agents in herbal medicine [3]. Traditional medicine can be described as the combination of knowledge and practice used for diagnosing, preventing, or curing disease which relies exclusively on past experience and observation handed from generation to generation, verbally or in writing [4]. Traditional medicine (TM) covers a wide variety of medicinal plants and their concoctions which vary from country to country and region to region. Practice of herbal medicine is often referred to as "complementary" or "alternative" medicine (CAM) in some countries. Since the 1990s its use has surged in many developed and developing countries as Eastern and Western medicines intersect [5].

Traditional Chinese, East Indian Ayurveda, Native American and African medicine are examples of different systems of traditional medicine, with the philosophy and practices of each being influenced by the prevailing diseases, environments for plant growth, and geographic areas within which they first evolved. Generally, in each of these regions, a common philosophy evolved with a holistic approach to life, i.e. equilibrium of the mind, body, and the environment, and an emphasis on health rather than on disease [3-4, 6-9].

The use of medicinal plants is a central part of all these systems of traditional medicine in which a restoration of health, rather than on treating a particular ailment or disease from which the patient is suffering is usually the main goal [8-9].

A medicinal plant (herb) is any plant which, in one or more of its organs or parts (e.g. leaf, flower, fruit, seed, stem, wood, bark, root, rhizome, juice, gum, fixed oil, resin) contains a variety of substances that can be used for therapeutic purposes or which are precursors for the synthesis of useful drugs [4].

Scientists from academic institutions and industrial firms have turned to plants as a source of either new drugs or of compounds from which more efficacious or less toxic drugs can be developed based on their long-standing use in traditional medicine [10]. The World Health Organization (WHO) has estimated that 80% of the world's rural population relies on TM's use of plant extracts or their active principles for their primary health care needs. Despite tremendous progress in synthetic chemistry, some 25% of prescription medicines are still derived either directly or indirectly from plants [2, 11-12].

A 2010 survey shows that 73% of Canadians regularly take natural health products (NHPs), Health Canada defines NHPs as: vitamins and minerals, herbal remedies, homeopathic medicines, traditional medicines such as traditional Chinese medicines, probiotics, or other products like amino acids and essential fatty acids. NHPs must be safe to use as over-the-counter products and do not require a prescription to be sold [13].

1.1.2 The Use of Medicinal Plants in Drug Discovery

Of the 252 drugs considered as basic and essential by the WHO, 11% are exclusively of plant origin and a significant number are synthetic drugs obtained from natural precursors [14]. Examples of important drugs obtained from plants are taxol which is isolated from *Taxus* (*T. brevifolia* and *T. bacata*), artemisinin from *Artemisia annua*, digoxin from *Digitalis* spp., quinine and quinidine from *Cinchona* spp., vincristine and vinblastine from *Catharanthus roseus*, atropine from *Atropa belladonna* and morphine and codeine from *Papaver somniferum* [2]. About 6% of higher plant species (angiosperms and gymnosperms) have been screened for biological activity and 15% of them have been reported to be phytochemically active [14]. Since only 5–15% of the higher plants have been systematically investigated for the presence of bioactive compounds, nature's biodiversity remains largely unexplored [15].

Different approaches to drug discovery using higher plants include: chemical screening, biological assays, biological activity reports (e.g., ecology based), or ethnomedical (traditional medicine) use of plants [10]. A diversified approach to drug discovery involves the observation, description, and experimental investigation of indigenous medicinal plants and their biological activities. It is based on botany, chemistry, biochemistry, pharmacology, and many other disciplines that contribute to the discovery of natural products that are pharmacologically active [16]. Of the more than 120 pure drugs derived from plants in current commercial use, three quarters were discovered through scientific investigations of traditional uses [17]. Phytochemical and biological characterization of plants used in traditional herbal medicine is an important tool in the scientific research of potential medicinal agents which can be used for the prevention, risk reduction and treatment of human diseases [18].

The goals of using plants as sources of therapeutic agents are to: *a*) isolate bioactive compounds for use as drugs; *b*) produce bioactive compounds that can be used for synthesis to produce entities of higher activity and/or lower toxicity, *c*) use agents as pharmacologic tools and *d*) use the whole plant or part of it as a herbal medicine [16].

Interest in drugs of plant origin is due to several reasons, namely, conventional medicine can be inefficient (e.g. side effects and ineffective therapy), incorrect use of synthetic drugs results in side effects and other problems, a large percentage of the world's population does not have access to conventional pharmacological treatment, and NHPs are considered harmless [2]. Phytomedicines exist as extracts which are concentrated preparations of a liquid, powdered, or viscous consistency that are ordinarily made from dried plant parts (the crude drug) by maceration or percolation [19].

1.1.3 Quality, Safety and Efficacy of Herbal Products from Medicinal Plants

As the world's market demand for herbal products from medicinal plants increases, quality control is of critical necessity to ensure that they are of consistent composition (quality), safety, and efficacy. Medicinal plants do not supply its phytochemicals with a consistent, composition, and unlike chemical drugs with a single entity, they contain multiple components. Factors such as soil, climate, species, age, geographical origin, cultivation method, processing (harvesting, storage, and manufacturing techniques), and contamination (pesticide, microbial and heavy metal) can affect the composition, safety, and efficacy of medicinal plants and their NHPs [19-20]. When species are harvested from wild plant populations, collectors may inadvertently confuse desired species with close relatives, or even with unrelated species that are superficially similar in appearance. There may also be deliberate substitution of alternate species that are

difficult to differentiate, especially when the financial incentive is great [21].

The use of good agricultural practice (GAP) and good manufacturing practice (GMP) is encouraged by health regulatory agencies to ensure consistent quality from the point of cultivation and harvesting of medicinal plants until the stage of processing and final delivery of the NHPs [22]. The WHO has published general guidelines for GMP procedures which can be employed for the manufacture of plant derived herbal products involving; the raw material production, botanical taxonomic identification to assure species identification; the processing and manufacturing stage, and analytical procedures similar to those employed for the manufacture of conventional drugs to assure quality and purity by appropriate protocols [23-25]. Ensuring quality can be controlled in part by removing plant material that does not meet strict quality standards and limiting further processing of plants that are not sufficiently consistent in their relevant constituents. Thus, quality control of plant derived NHPs begin with the selection and mixing of the herbal raw materials [19].

A major challenge when using herbal products is being able to consistently formulate a product via the identification of a particular “marker compound” which is believed to be responsible for the physiological effect [26]. A reported study on selected commercial ginseng products prepared from *Panax ginseng* and *Panax quinquefolius* marketed as botanical supplements in North America in the 1995-1998 period showed that the ginsenoside contents of *P. ginseng* and *P. quinquefolius* products ranged from 0.00% to 13.54% and from 0.009% to 8.00%, respectively, and close to 26% of these products did not meet label claims [27-28].

The percentage of a particular marker for a particular plant derived NHPs varies from product to product, and in some cases batch to batch from the same company [26]. Often these marker

compounds are chemicals that are merely characteristic components of the herb in question and may not have been tested for their actions or therapeutic efficacy in pharmacologic test models or in clinical studies. Chemical characterization (extraction, fractionation, purification and structural identification) are only meaningful when the purified and identified marker compounds (fractions) have the same or similar biological activity as the plant or its extract [18]. Proper *in vitro* and *in vivo* assay methods need to be established for each step of the fractionation and purification process [29]. Only those with sufficient activity should be used for further purification.

The presence of multiple active compounds in herbal products can provide either synergistic or antagonistic effects that may not be achievable by any single-component. These complex interactions can present a unique challenge for bioactivity-guided fractionation, because the relative activity of fractions may decrease with greater purity and may even be lost entirely [8]. The activity of the individual active components of botanicals can be assessed by recombining the fractions after separation followed by confirmation of biological activity [29]. Consistency of botanical therapeutic products can only be achieved when the active marker compounds are identified and their biological activities verified.

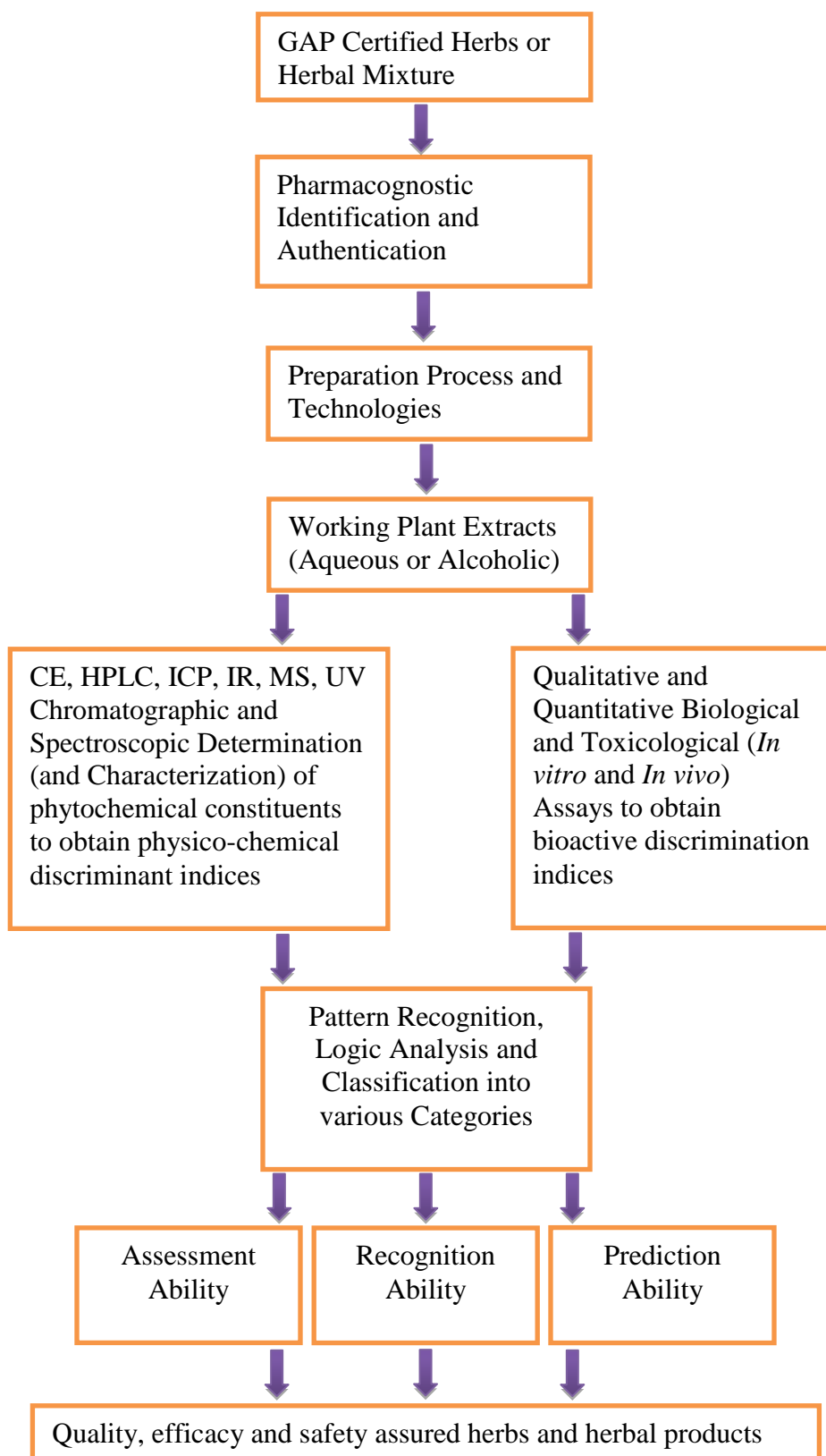


Figure 1.1 - Summarized Scientific Approach of Evaluating Herbs and Herbal products [30].

Quality control of plant derived NHPs involve finger printing of phytochemical markers to ensure consistency in quality, safety and efficacy for consumers. Depending on the technology and solvent used, chromatographic and spectrometric techniques can generate chemical chromatographs and spectra that characterize the multicomponent active principle as uniquely as a fingerprint. Phytochemical fingerprinting of test samples with authentic reference standards is a fundamental quality control step for ensuring consistency. This is particularly true when little is known about the relation of these constituents to actions and efficacies or about the quantitative/qualitative makeup of the remaining components [19].

The initial challenge in the quality control process is isolating and analyzing medicinal plants and their NHPs due to the complexity of their sample matrices and the presence of multiple phytochemical components. Isolation of active constituents from medicinal plants serves as a means by which constituents can be processed into safe medicinal products tailored towards obtaining a quality product that has a consistent, uniform composition [31]. The nature of the solvent and of the extraction and drying processes critically affects the phytochemical composition of the finished product. Polar compounds are soluble in water, while lipophilic constituents are soluble in alcohol. For example an aqueous extract of medicinal plant such as ginseng will have a different spectrum of ingredients than an extract that has been isolated using ethanol [19].

Novel technologies are required for separating and isolating these phytochemicals before they are characterized using physicochemical techniques. A high quality chemical library of reference standards is vital for research in the structure-activity relationship, and investigation of the mechanism of NHPs [14, 18]. Some reference compounds are commercially available, such as ginsenosides, but their high cost and limited supply make many studies challenging [32]. In

addition, insufficient availability of phytochemical reference standards such as ginseng polysaccharides adds another level of difficulty and challenge to evaluate these components from medicinal plants. Chromatographic or spectral phytochemical characterization is used and accepted worldwide for the evaluation of medicinal plants and their NHPs. This fingerprinting enables the detection and quantitation of desired markers present and for the assessment of the pattern of the phytochemicals [19].

There is also a belief that plant derived NHPs are inherently safe without side effects when properly used at normal therapeutic doses. However, these NHPs may also have undesirable side effects and herb–drug or herb–herb interactions are possible [8]. Where safety information is lacking on any medicinal plants being contemplated for NHPs, relevant research must be performed prior to its employment [20]. The WHO has established guidelines for such studies [33]. Adverse events, including drug-herb interaction must also be monitored to promote a safe integration of efficacious herbal medicine into conventional medical practices [20]. Hence there is a need to screen medicinal plants such as ginseng to identify novel bioactives for the promotion of human health.

1.2 Ginseng

The name ginseng comes from the Chinese words “Jen Sheng,” meaning “man-herb,” because of the humanoid shape of the root or rhizome of the plant, which is the part of the plant most commonly used for extraction. The ginseng plant is a deciduous perennial belonging to the Family Araliaceae and genus *Panax*. The genus name of ginseng "*Panax*" is derived from the Greek *pan* (all) *akos* (cure), meaning "cure-all" or “all healing,” which describes the traditional belief that ginseng has properties to heal all aspects of the body [34-37]. There are about thirteen

different species of ginseng which have being identified all over the world. The most commonly used species of ginseng are *P. ginseng* (Asian ginseng) and *P. quinquefolius* (American ginseng). *P. ginseng* has been used in the Orient for thousands of years, while *P. quinquefolius* has been used by Native Americans for at least hundreds of years [9, 34-44].

In 1714 while at St Louise, Canada (near Montreal), Father Lafitau stumbled across ginseng growing at the site of a new house with an appearance that accurately fit the ginseng roots Father Jartoux (French Jesuit missionary) described to him in a letter in 1709 from China [36]. American ginseng was first introduced in the “*New Compilation of Materia Medica*” in 1757. There are three kinds of American ginseng namely; cultivated, simulated wild, and wild. American ginseng is also cultivated in some Asian countries, like China. As a perennial herb, most wild populations of American ginseng thrive in the upland, north and east-facing woods where shade and loamy soils are typical [40]. American ginseng has been harvested in North America primarily for export to Asia since the 1700s and is highly valued for its medicinal and herbal use [41]. The quantity of wild ginseng was not sufficient to meet the demand, so experiments on cultivation were undertaken in 1878 and was achieved in Fabius, New York by George Stanton, a retired tinsmith turned to farmer. Cultivation of *Panax ginseng* in Asia started around 11 B.C. by transplantation of wild ginseng. In 1122 A.D., ginseng cultivation was also attempted through the propagation of transplanted ginseng from seeds [36]. American ginseng is distributed in the eastern temperate forest areas of North America; Ontario and Quebec in Canada, Minnesota, Wisconsin, Oklahoma and Georgia in the United States of America [40].

Ginseng is prepared and used in several ways: as fresh ginseng (sliced and eaten, or brewed in a tea), white ginseng (peeled and dried), red ginseng (peeled, steamed, and dried), extract (tincture or boiled extract), powder, tea, tablet or capsule [35-36]. According to Traditional Chinese

Medicine (TCM), Asian ginseng is believed to have a ‘warm’ or ‘yang’ property, while American ginseng has a ‘cool’ or ‘ying’ characteristic [37]. Ginseng products are often referred to as “tonics,” a term that has been replaced by “adaptogen”. The term “adaptogen” can be defined as an agent that increases resistance to physical, chemical, and biological stress and builds up general vitality, including the physical and mental capacity for work [43]. Ginseng root is used as a medicinal plant in traditional herbal medicine to improve overall health, restore the body to balance, help the body to heal itself, and to reduce stress [37, 44].

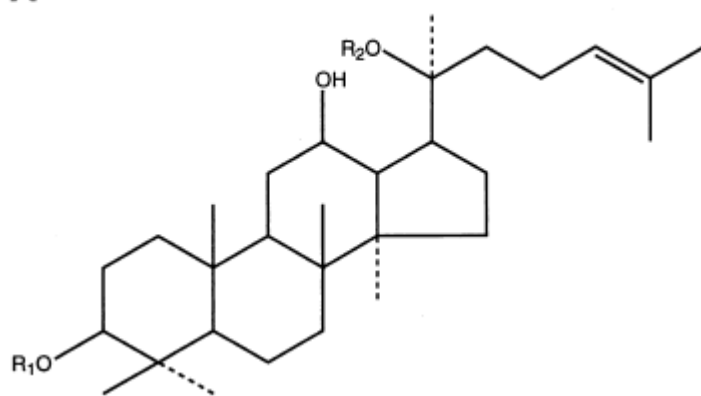
Ginseng’s broad spectrum of biological activity has been attributed to its multiple bioactive components namely ginsenosides, polysaccharides, peptides, polyacetylenic alcohols, and fatty acids. These known active phytochemicals are present in most ginseng species, although there can be slight variation in different species [39, 44]. Scientific studies have shown that ginseng exhibits a wide range of beneficial pharmacological effects including immunomodulation, anti-tumor, anti-oxidation, anti-depression, hypoglycemia, inhibition of gastric lesions, attenuation of leptin-induced cardiac hypertrophy, heart protection against ischemia and reperfusion injury, prevention of glucose-induced oxidative stress, prevention of diabetic nephropathy, retinopathy and cardiomyopathy [38, 41, 45-59].

1.2.1 Ginseng Bioactives

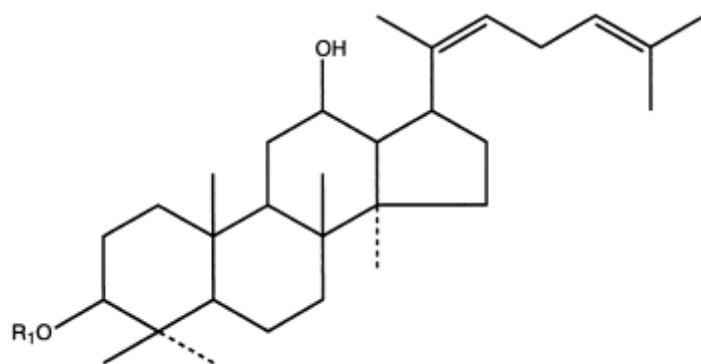
1.2.1.1 Ginsenosides

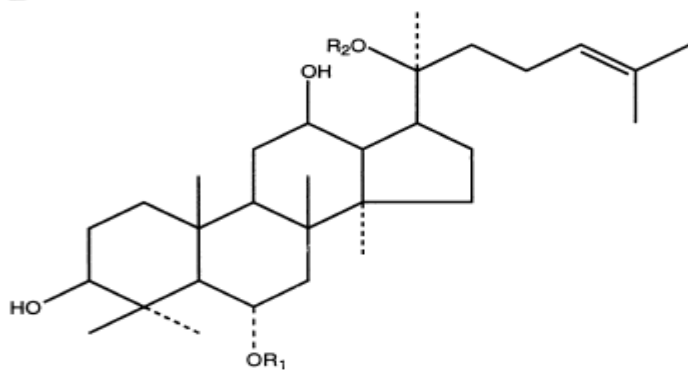
Ginsenosides belong to a family of steroids with a four trans-ring rigid steroid skeleton that shares a unique triterpenoid saponin structure of the dammarane type (Figure 1.2). More than 100 different ginsenosides have been isolated from the roots, leaves, stems, flower buds, and berries of Asian and American ginseng which exhibit considerable structural variation [40].

Ginsenosides differ from one another by the type of sugar moieties, sugar number, and site of sugar attachment at positions C-3, C-6, or C-20. The structural isomerism and stereoisomerism, the number and site of attachment of hydroxyl groups, and available modified side chain at C-20 also increase their diversity. Ginsenosides from ginseng are divided into several groups. Protopanaxadiol (PPD) and protopanaxatriol (PPT) groups are the main constituents; while ocotillol and oleanane groups are minor ones [37, 39-40]. The PPD group has sugar moieties attached to the β -OH at C-3 and/or C-20, and the PPT group has sugar moieties attached to the α -OH at C-6 and/or β -OH at C-20. The ocotillol group has a five-membered epoxy ring at C-20, and the oleanane group has a modified C-20 side chain [40]. Ginsenoside Rb1, Re, Rd, Rc, Rg1, and Rb3 are the six major saponins in American ginseng, and the variability in individual ginsenosides and total ginsenoside amount in different commercial products of American ginseng may be responsible for different or even opposing reported pharmacological activities, which is in part associated with natural variations such as climate, geographical location and cultivation length conditions [40]. Quality control of ginseng products will ensure consistency in quality, safety and efficacy for consumers. Ginseng extracts (aqueous and alcoholic) contain relative variable amount of ginsenoside components [54]. Ginsenosides have been reported to exert numerous pharmacological activities including immunomodulatory, anti-oxidant, anti-inflammatory, anti-cancer, anti-obese and anti-diabetic effects [60-62].

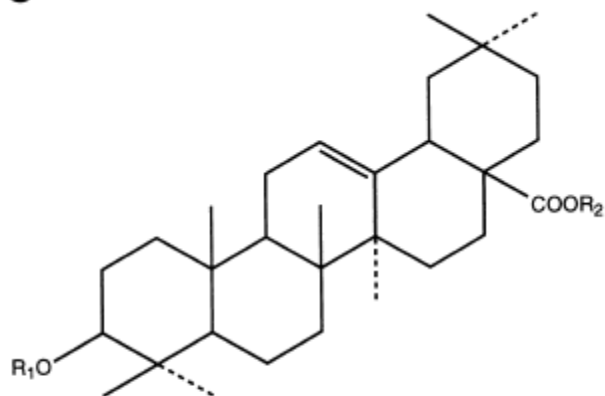
A**Panaxadiols**

Ginsenoside	R ₁	R ₂
Rb ₁	-glc(2-1)glc	-glc(6-1)glc
Rb ₂	-glc(2-1)glc	-glc(6-1)arap
Rc	-glc(2-1)glc	-glc(6-1)araf
Rd	-glc(2-1)glc	-glc
Rg ₃	-glc(2-1)glc	-H
Rh ₂	-glc	-H
Rh ₃	-glc	

**Figure 1.2 - Chemical Structures of Panaxadiol ginsenosides [39].**

B**Panaxatriols**

Ginsenoside	R ₁	R ₂
Re	-glc(2-1)rha	-glc
Rf	-glc(2-1)glc	-H
Rg ₁	-glc	-glc
Rg ₂	-glc(2-1)rha	-H
Rh ₁	-glc	-H

C**Oleanic acid**

Ginsenoside	R ₁	R ₂
Ro	-glcUA(2-1)	-glc

Figure 1.3 - Chemical Structures of Panaxatriol ginsenosides and Oleanic acid (nonsteroidal saponin) [39].

1.2.1.2 Polysaccharides

Plant polysaccharides are biopolymers of various monosaccharides linked together through glycosidic bonds, resulting in complex structures. Plants polysaccharides are usually isolated by hot water extraction of the plant material, after which they are purified and precipitated with alcohol from the water extract [63]. It is noteworthy that, in comparison with other biopolymers such as proteins and nucleic acids, polysaccharides offer the highest capacity for carrying biological information because they have the greatest potential for structural variability [64]. Scientific studies have demonstrated that plant polysaccharides such as water-soluble polysaccharides used in NHPs are non-toxic and exhibit a number of beneficial biological activities, including immunostimulation, anti-tumor, wound-healing, hematopoietic, radioprotective, anti-ulceric and anti-atherosclerotic effects [65-66].

Ginseng polysaccharides are biopolymers formed from a complex chain of monosaccharides rich in L-arabinose, D-galactose, L-rhamnose, D-galacturonic acid, D-glucuronic acid and D-galactosyl residue linked together through glycosidic bonds, resulting in complex macromolecular architectures [46, 63, 67]. Their molecular weights range from 3500 to 2,000,000 Da [47, 67-72] which contribute to their diverse physicochemical properties and biological activities. CVT-E002™ (sold commercially as COLD-FX®) is a herbal product rich in poly-furanosyl-pyranosyl-saccharides extracted by an aqueous method from the root of American ginseng as described in its patent [67-68]. The process of preparing ginseng fraction CVT-E002 comprises: combining American ginseng dried ground root with a first solvent comprising an alcohol in a proportion of about 7 to 9 ml of first solvent per gram of ginseng. The resulting solution is then heated at a temperature of about 80 to 100° C for a time period of about 2 to 4 hours, to produce a first ginseng solution.

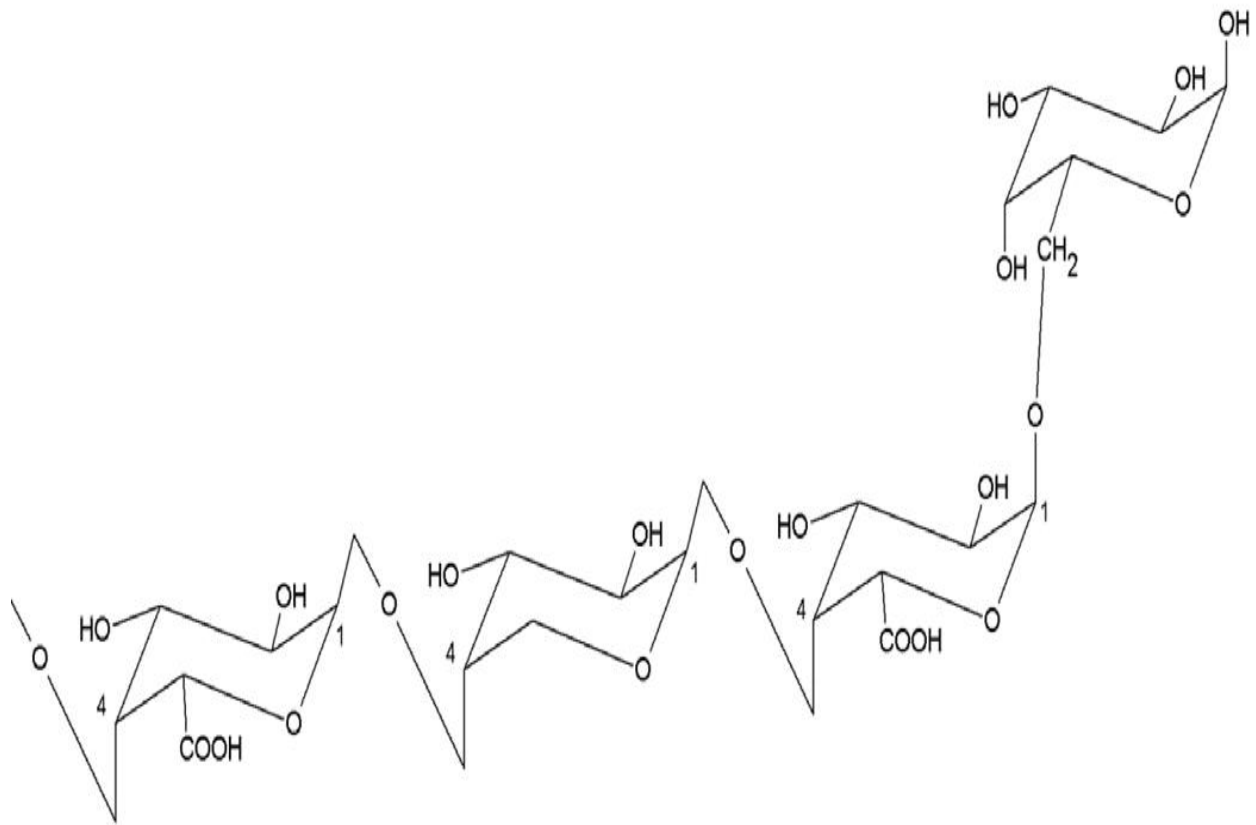


Figure 1.4 - Chemical Structure of Ginseng Polysaccharides [69].

Thereafter, the first ginseng solution is separated to produce an alcohol/ginseng solution and a first ginseng residue. Thereafter, the first ginseng residue is combined with water in a proportion of about 7 to 9 ml of water per gram of ginseng residue. The ginseng residue solution is then heated at a temperature of about 80 to 100° C for a time period of about 2 to 4 hours, to produce a ginseng residue solution. Thereafter, the ginseng residue solution is separated to produce a second ginseng residue and an aqueous extract solution containing a ginseng extract. The aqueous extract solution is then dried to produce ginseng fraction CVT-E002 [68].

1.3 Immunomodulation

The immune response of a host is its key defense and surveillance system capable of eradicating invading infectious microorganisms (bacteria, fungi, virus, and protozoa) and maintains homeostasis required for a normal healthy condition. The immune system is divided into the innate and adaptive (specific) immunity which can distinguish between foreign pathogenic micro-organisms and the body's cells and tissues via surface cell receptors that can recognize toxic surface pathogen-associated molecular patterns (e.g. lipopolysaccharides of Gram negative bacteria) as foreign [73]. Components of the innate immunity are macrophages, natural killer cells (NK-cells), dendritic cells (DC), granulocytes (neutrophils, basophils, eosinophils), proinflammatory mediators e.g. cytokines (tumor necrosis factor, interleukins), reactive oxygen /nitrogen species (e.g. nitric oxide) and prostanoids (e.g. prostaglandins). While that of the adaptive (acquired or specific) immunity include T lymphocytes, B lymphocytes and antibodies [74].

Compounds that are capable of interacting with the immune system to up-regulate or down-regulate specific aspects of the host immune response can be classified as immunomodulators or

biologic response modifiers [75]. A broad spectrum of such compounds are still being investigated and characterized. Biopolymers like plant polysaccharides which have long been believed to have benign biologic properties, have recently been shown to act as biological response modifiers. They can either up-regulate or down-regulate specific aspects of biological responses of the host [65-66]. A current significant problem is the rise of microbial infections and their resistance to synthetic antimicrobial agents and naturally derived antibiotics. Unlike the use of antibiotics and antimicrobials to kill pathogens, a key immunotherapeutic strategy to address this challenge will be to identify bioactive agents which can interact with the host immune response defense to enhance its ability to fight diseases and infections or neutralize immunotoxic response.

1.3.1 Macrophage-mediated Innate Immunity

Macrophages, first described by Metchnikoff in the 1880s, are large mononuclear phagocytic cells, which are derived from monocytes which originate from haematopoietic stem cells in the bone marrow. Monocytes differentiate further into macrophages as they leave the blood and enter the tissue. In tissues, a small number of macrophages differentiate under the influence of cytokines and, depending on the tissue type, they may become osteoclasts (bone), kupffer cells (liver), microglia (brain), alveolar macrophages (lung) and peritoneal macrophages (peritoneum) [76]. Fast acting macrophage-mediated innate immunity is the first line of defense in identifying, neutralizing, destroying and removing microbial pathogens and influencing the subsequent slow adaptive immune response. Macrophages act by means of a number of different mechanisms: (a) directly via phagocytosis by destroying bacteria, parasites, viruses and tumor cells; (b) indirectly via the secretion of proinflammatory mediators (e.g. tumor necrosis factor-alpha [TNF- α]) which

can activate other cells; (c) as accessory cells, by processing antigen and presenting digested peptides to T lymphocytes; and (d) by repairing tissue damage [65, 77].

The pathogen-associated molecular patterns (PAMPs) of pathogens (bacteria to fungi, protozoa, and viruses) are the targets of innate immune recognition with the help of their pattern recognition receptors (PRRs) which can distinguish between self (host) and pathogens via their PAMPs [78]. During activation of macrophage function by plant polysaccharides or lipopolysaccharide (LPS) endotoxin (a PAMP) from microbial infection caused by Gram negative bacteria, plant polysaccharides or LPS ligand binds to the transmembrane PRRs such as Toll-like receptors (TLRs) of innate macrophage cells which induce down-stream intracellular events [79]. As seen in Figure 1.5, the recognition and binding of plant polysaccharides or LPS ligand by Toll-like receptor 4 (TLR-4) leads to the recruitment of various cytoplasmic TIR (Toll/IL-1 receptor) domain-containing adaptors such as MyD88 (myeloid differentiation factor 88), TIRAP (TIR domain-containing adaptor protein) and TRAM (TRIF-related adaptor molecule) [80]. MyD88 recruits IRAKs (interleukin-1 receptor-associated kinases) which then activates TRAF6 (TNF receptor associated factor 6), leading to the activation of TAK1 (transforming growth factor-beta activated kinase 1). At the point of TAK1 activation, the signaling pathway bifurcates. One limb of the pathway leads to TAK1 activating the IKK (inhibitory kappa kinase) complex which phosphorylates and degrades I κ B, resulting in the release and translocation of NF- κ B (nuclear factor kappa B) transcription factor from the cytoplasm into the nucleus. Activated NF- κ B binds to the promoters of diverse proinflammatory mediators including TNF- α , interleukin-6 (IL-6), and enzymes such as iNOS (inducible nitric oxide synthase) and COX-2 (cyclooxygenase-2), thereby activating the transcription and expression of their genes [78, 81]. The other limb of the pathway activates one or more members

of the MAPK (mitogen-activated protein kinase) kinases such as p38, JNKs (Jun n-terminal kinases) and ERK1/2 (extracellular signal-regulated kinases 1/2), which leads to the activation of AP-1 (activator protein 1) transcription factor that induces the transcription of proinflammatory mediators [79, 80]. The expression of TNF- α , IL-6, iNOS and COX-2 induced by activated NF- κ B transcription factor results in the production of TNF- α , IL-6, nitric oxide (NO) and prostaglandin E2 (PGE₂) respectively. NF- κ B is highly activated in inflammatory disease conditions such as endotoxemia [82]. LPS induced macrophage overproduction of TNF- α , IL-6, NO and PGE₂ have been shown to play critical roles in the pathological process of many inflammatory diseases, including endotoxemia and septic shock [83-90]. The presence of a large amount of LPS endotoxin in the bloodstream, as observed during severe gram-negative bacterial infections or as caused by translocation of enterobacteria from the gut, induces excessive macrophage stimulation with uncontrolled production of proinflammatory mediators and cytokines. This endotoxin is harmful and leads to endotoxemia with dramatic pathophysiological reactions such as fever, leukopenia, tachycardia, tachypnea (acute respiratory failure), hypotension, disseminated intravascular coagulation, myocardial dysfunction and multi-organ failure which ultimately results in endotoxic shock syndrome [91-98].

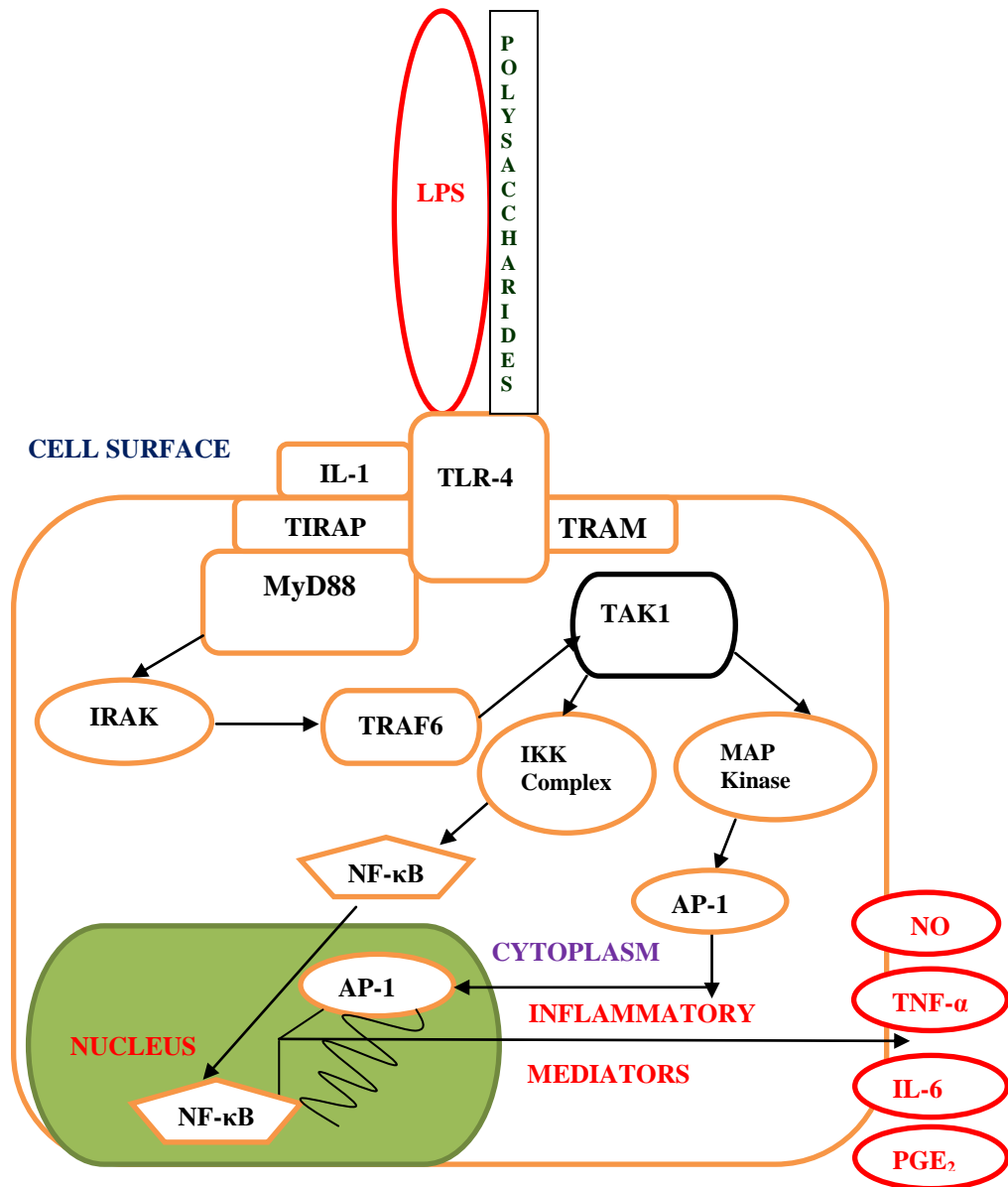


Figure 1.5 – LPS and Plant polysaccharides initiation of Innate Immune response via TLR-4 Signaling pathway [78-81].

LPS is a glycolipid that constitutes the major portion of the outermost membrane of Gram-negative bacteria and is essential for bacterial growth and survival [97]. It is a complex, negatively charged molecule composed of a polysaccharide chain called the O-specific chain and a lipid moiety referred to as lipid A. The latter is the actual toxic moiety of the LPS molecule and contains phosphate groups shown to be essential for its immunostimulatory activity [98]. The interaction of lipid A moiety of LPS with macrophages is important and subsequent cellular activation results in the release of systemically active proinflammatory molecules, which in turn mediates systemic toxicity. LPS is an extremely potent toxin: macrophages can be activated at concentrations of LPS as low as 1 pg/mL [97]. LPS endotoxin exerts its profound immunotoxic effects by stimulating host cells (mainly monocytes/ macrophages, but also endothelial cells, smooth muscle cells, and neutrophils) to produce and release proinflammatory mediators and cytokines (NO, IL-6 and TNF- α). The presence of high amounts of LPS leads to the release of these proinflammatory mediators and cytokines in large quantities, resulting in the described pathophysiological reactions [91]. The suppression of LPS immunotoxic (anti-inflammatory) effects of various immunomodulation compounds have been evaluated using both *in vitro* and *in vivo* models [99-101]. The ability of an agent to inhibit LPS-induced macrophage overproduction of proinflammatory mediators is called an immunosuppressive (anti-inflammatory) effect, while the ability of an agent to stimulate or enhance macrophage production of proinflammatory mediators is called an immunostimulatory effect. Antibiotics capable of killing Gram negative bacteria are currently used in the management of endotoxin mediated inflammatory diseases, although the continued rise in antibiotic resistance and mortality from infections caused by these organisms has led to investigation of beneficial therapies aimed at inhibiting or neutralizing the toxic action of endotoxins including LPS. Polymyxin B, a molecule that binds tightly to LPS

endotoxins, has been investigated and found to neutralize many endotoxic activities (including lethality), and was more protective than antibodies to core LPS. Unfortunately, the neurotoxicity and nephrotoxicity of polymyxin B limits its potential as a therapeutic agent [102]. Hence a less toxic anti-LPS agent would be highly desirable as a therapeutic (prophylactic or curative) agent. Therapies directed at the neutralization of proinflammatory mediators or LPS that are promising in experimental models have been largely ineffective in clinical trials. Therefore, the development of new therapies is of major interest [103-105].

1.3.2 Modulation of Macrophage Function as a Target for Immunotherapy

The innate immunity (e.g. macrophage function) of a host which is responsive to LPS is also a known target for plant polysaccharides which are biological response modifiers. Plant polysaccharides can up-regulate macrophages production of proinflammatory mediators to fight infection, they can also neutralize or suppress immunotoxic response by down-regulating macrophage production of proinflammatory mediators [65, 75]. Bioactive compounds which can inhibit LPS endotoxin from triggering excess macrophage production of NO, TNF- α and IL-6 proinflammatory mediators will be very useful in the prevention and treatment of inflammatory diseases such as endotoxemia. New approaches to the prophylaxis of diseases like endotoxemia can be based on comprehensive blockade of the LPS signaling pathways in macrophages. The immune response to LPS can take a number of different forms (immunostimulation and immunosuppression). Low doses of endotoxin have been known since the 1940s to induce a state of tolerance (desensitization), in which the immune response to subsequent LPS challenge is altered. This alteration is characterized by suppression of proinflammatory mediators and upregulation of anti-inflammatory mediators [106]. Mouse macrophages as well as human monocytes exposed to LPS have been reported to show an inability to respond to further LPS

challenge. The key readout for tolerance in these cells was the drastic reduction of TNF α production as compared to the cells exposed to LPS only once [107].

Interestingly, bioactive polysaccharides have been shown to exhibit both macrophage-mediated immunostimulatory and suppression of induced proinflammatory effects [46, 65, and 108-109]. A possible mechanism by which plant polysaccharides suppress LPS-induced macrophage stimulatory effect may be through their ability to desensitize immune cells (e.g. macrophages) from LPS toxic stimulation, similar to the tolerance ability of LPS pre-exposure to desensitize subsequent LPS challenge. Bioactive polysaccharides can serve as useful prophylactic and therapeutic agents for immune and inflammatory diseases. The ability to neutralize LPS immunotoxicity is a desired requirement for future immunobioactive compounds that can be used as a prophylactic against endotoxemia. Ginseng polysaccharides may provide such immunobioactive compounds. Evaluation of an herb like ginseng provides an opportunity for the discovery of novel agents that can combat disease conditions mediated by LPS such as endotoxemia.

1.3.3 Ginseng Modulation of Immune Function

Different immunomodulatory effects of ginseng have been reported, including both immunostimulatory and immunosuppressive effects [110-119]. Aqueous (AQ) and alcoholic (ALC) ginseng extracts have been reported to exert immunostimulatory [53, 110-113] and immunoinhibitory [45, 114-118] effects respectively. On the contrary, ginseng AQ extract has also been reported to possess immunoinhibitory effects [119]. The basis for the apparent paradoxical immunomodulatory effects is unclear but may be attributed to different experimental conditions, e.g. choice of extraction solvents. There is a need to delineate the paradoxical

immunomodulatory effect of ginseng and provide a basis for explaining the apparently contradictory reporting in the literature. The anti-inflammatory (immunosuppressive) effect of ginseng ALC extract has been attributed to the combined effects of its ginsenosides constituent [45], with molecular weights less than 3KDa [120-122]. The immunoinhibitory macromolecules of ginseng ALC extract are not well known and are overlooked by many investigators who focus mainly on ginsenosides biological activities. There is paucity of data concerning the immunomodulatory effects of ginseng ALC extract macromolecular components.

Ginseng polysaccharides are generally known for their immunostimulatory effects [53, 111, 123]. CVT-E002™ a natural health product of American ginseng polysaccharides is used for the prevention and treatment of the common cold and has been reported to exhibit immunostimulatory effects namely; enhances IL-2 and interferon gamma (INF- γ) productions in murine spleen cells [67], increased proliferation of spleen cells (B lymphocytes), increased plasma level of immunoglobulin G, enhanced macrophage production of NO, TNF- α , IL-6 [124], elevated the number of spleen, bone marrow and natural killer cells [125]. CVT-E002 has been shown to be effective for preventing acute respiratory illness due to influenza and respiratory syncytial virus [126]. Recent studies suggest that ginseng polysaccharides also suppress induced proinflammatory responses, e.g. one study reported that ginseng polysaccharides inhibited immunological response associated with collagen-induced arthritis [127]. Intravenous treatment of mice with ginseng polysaccharides was reported to exert a protective effect against *Staphylococcus aureus*-infected septic mice by suppressing early acute inflammation [128-129]. Another study revealed that intranasal and intravenous administered ginseng polysaccharides showed a protective effect on influenza viral infection by lowering levels of inflammatory cytokine (IL-6) and lung viral titers [69]. Studies also suggest that CVT-E002 suppresses

induced immunoinflammatory responses which include reduction in the activation of neutrophils [130], inhibition in the development of allergic airway inflammation and airway hyper-responsiveness [131], decrease in LPS-induced spleen production of IL-2 and IFN- γ production [46].

There have been different reports of ginseng acidic and neutral polysaccharides (PS) immunomodulatory effects in the literature [72]. Kim et al. (1990) reported that acidic PS and neutral PS of *P. ginseng* (Asian ginseng) may stimulate B cells and macrophages [48], Fan et al. (2010) reported that neutral PS of *P. ginseng* stimulated the proliferation of lymphocytes; increased natural killer cell cytotoxicity; enhanced the phagocytosis and NO production by macrophages and increased the level of TNF- α in serum [132]. Zhou et al. (2009) reported that both neutral PS and acidic PS of *P. ginseng* were potent B and T cell stimulators [71], Tomoda et al. (1993) reported that two acidic PS of *P. ginseng* enhance the phagocytic activity of macrophages [133]. Shin et al. (2004) reported that acidic PS of *P. ginseng* shows immunomodulatory activities via macrophage NO production [134], while the study by Kim et al. (1998) showed that ginsan an acidic PS from of *P. ginseng* induces Th1 cell and macrophage cytokines [135]. Sonoda et al. (1998) found that one acidic PS of *P. ginseng* was a potent inducer of IL-8 production by human monocytes and THP-1 cells [136]. Lemmon et al. (2012) reported that the immunostimulatory effects of ginseng PS (*P. quinquefolius*) are mediated by PS with a molecular weight higher than 100 kDa [137].

1.4 Phytochemical Analysis of Ginseng Polysaccharides

For the separation, purification and physicochemical characterization of biopolymers such as ginseng polysaccharides (PS), size exclusion chromatography (SEC), also known as gel

permeation chromatography (GPC) or gel filtration chromatography (GFC) is a widely accepted analytical method [54]. The determination of molecular weights of polymers is one of the primary uses of SEC/GPC, and currently most SEC/GPC analyses are performed by comparing the molecular weight of a sample against standards of known molecular weight. This method is often described as classical SEC/GPC. A newer method is becoming increasingly common, which uses multiple detectors to provide absolute molecular weight information [138] which is referred to as high performance SEC or HPSEC in this thesis.

Size exclusion chromatography utilizes a non-interactive mode of separation with a stationary phase composed of a macromolecular gel containing a porous network. As the PS sample passes through the column containing the gel, the components of the sample are separated. Elution order is based on molecular size, with molecules with a hydrodynamic (retention) volume larger than the largest pores of the stationary phase not being able to penetrate the pores of the gel. These large molecules then pass through the space between gel particles un-retarded, while molecules with smaller hydrodynamic volumes enter the pores and the open network of the gel, and are separated depending on their size and shape [138]. Analytical SEC combined with multiple detectors such as refractive index (RI), ultraviolet (UV), and multi-angle light scattering is a rapid method to separate, characterize the structure, average molecular weight, and chain conformation of PS [139]. Light scattering detectors measure the light scattered inelastically (i.e., Rayleigh scattering) and, with the use of the Zimm relationship, the PS weight-average molecular weight (M_w) can be obtained directly [140]. Analytical SEC with a light scattering detector directly measures the PS molecular mass and potentially molecular size (root-mean square radius), and the conformation or shape of the polymers can be derived from their correlation.

The intensity from a light scattering detector is proportional to three important variables: molecular mass, concentration, and the specific refractive index increment. The RI detector is used to measure polymer concentrations across the chromatographic peaks [138, 140]. The viscosity detector measures the pressure drop and, in combination with a concentration detector, allows calculation of the intrinsic viscosity (inverse molecular density). This structural information can be used to probe such important features of the polymer system as its shape and branching characteristics [138]. The ability of SEC to determine the molecular weight averages and molecular weight distribution of naturally occurring polymers makes it a chromatographic method of choice in the separation and analysis of PS.

1.5 Absorption of Orally Administered Ginseng Polysaccharides

The oral route is the most convenient way to administer drugs and bioactives (e.g. ginseng PS), with absorption across the intestinal barrier into systemic circulation being prerequisite for these biochemical compounds to exert their pharmacological effects. Therefore it has been of great interest to study intestinal absorption of biochemical compounds even if the possibilities are limited for obvious reasons such as their physicochemical properties (e.g. lipophilicity/hydrophilicity and molecular weight) [141]. The natural function of the gastrointestinal tract (GIT) is to digest and absorb nutrients which sustain the living system. After oral administration, biochemical compounds travel across the gastrointestinal barrier into systemic circulation [142-143]. Orally administered ginsenosides have been reported to demonstrate low bioavailability due to their low membrane permeability across the intestinal mucosa, active biliary excretion, decomposition in the stomach, metabolism in the large intestine and elimination in the liver. Of these factors, low membrane permeability and active biliary

excretion has been attributed to be the two major factors that limits the absorption of orally administered ginsenosides from the gastrointestinal tract into systemic circulation [144-147].

PS of medicinal plants such as *Radix ophiopogonis* (Mw = 4.8 kDa) have been reported to have low bioavailability of about 1.7 % in rats after oral administration, which was attributed to their large molecular size and hydrophilic characteristics [148]. Orally administered ginseng PS have been reported to exert numerous pharmacological activities including immunomodulation [46, 124- 125, 130-131], anti-depressant [50] and anti-ulcer effects [149], but their absorption from the GIT into systemic circulation is yet to be ascertained.

Despite PS such as ginseng PS being soluble in water, their weak chromophore group and the complexity of the plasma matrix are barriers to overcome in their analysis in plasma. Chromatographic analytical methods have been used to analyze plant PS tagged with a fluorescent label or subjected to a post column fluorescence derivatization method with fluorometric detection in biological matrices (e.g. plasma) after *in vivo* treatments [148, 150-156]. Pending the establishment of the safe use of fluorescently-labeled PS for human studies, the development of a chromatographic method to analyze unlabeled PS in plasma will serve as a useful tool in human studies. The knowledge of bioactive PS concentrations in plasma will enhance their prophylactic and therapeutic use in herbal medicine.

However, there is currently no available method to analyze ginseng PS in plasma. Such a method will be a useful tool in determining whether orally administered ginseng PS is absorbed from the GIT into systemic circulation. Perchloric acid-protein precipitation of plasma and high performance size exclusion chromatography with right angle light scattering detection is a new approach to analyze orally administered unlabeled ginseng PS in rat plasma. This new approach

is a key that opens the door to investigating the absorption of ginseng PS from gastrointestinal tract (GIT) into systemic circulation after oral administration of ginseng PS.

A possible mechanism by which polysaccharides is absorbed via the GIT may be endocytosis, whereby molecules are engulfed by the membrane and move through the intestinal cell in vesicles, which would be subsequently released on the membrane's other side [147]. Orally administered PS (GFPBW1, β -1,3-linked glucan, extracted from *Grifola frondosa*, WGE, α -1,4-linked glucan, extracted from *Gastrodia elata* and Lentinan extracted from *Lentinula edodes*) have been reported to be detected in rat serum by high performance liquid chromatography analysis. These PS were found to be internalized in human intestine mucosa epithelial cells and the underlying mechanism of their intestinal absorption was found via clathrin-mediated endocytosis [157].

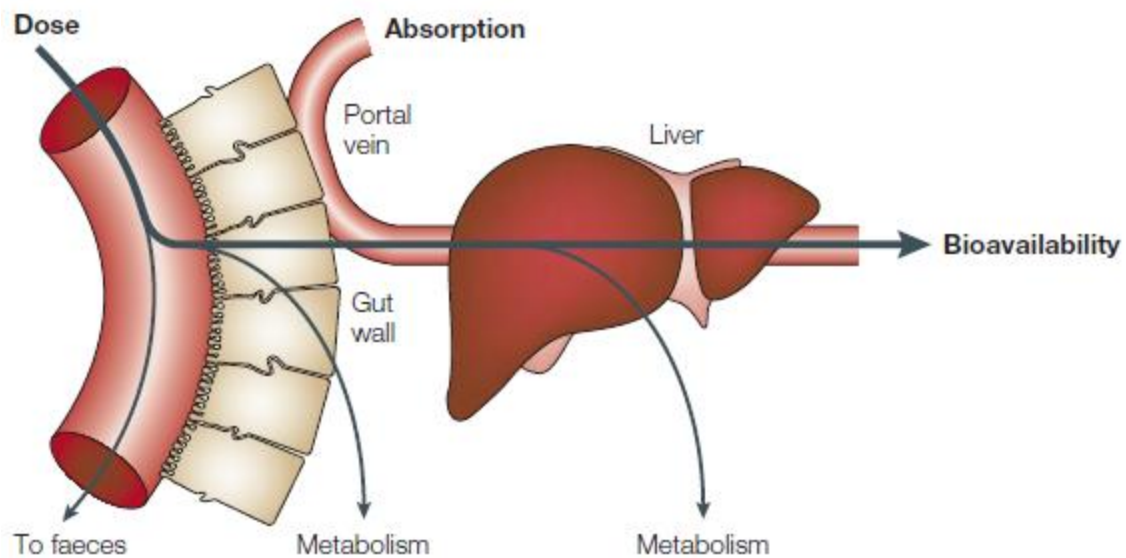


Figure 1.6 – Gastrointestinal absorption of orally administered biochemical compounds into systemic circulation [142].

1.6 Summary and Knowledge Gaps to be addressed

Medicinal plants (e.g. American ginseng) contain multiple bioactive compounds with long-standing use in traditional medicine; hence they are a rich source of potential prophylactics and biotherapeutics for the improvement of human health including the immune function. *In vitro* and *in vivo* bioassay screen of medicinal plants and their bioactives help to fill in the gap where efficacy and safety data is lacking. The extract solvent matrix (aqueous or alcoholic) and their bioactive components play a crucial role in the biological effects of medicinal plants and their natural products. This is especially true of immunotherapeutics, where their up-regulatory and down-regulatory interaction with innate component of the immune system (e.g macrophages) determines their observed immunopharmacological effects. Identification of the bioactive(s) and extract of medicinal plants and natural products associated with immunomodulatory effect(s) is vital to provide appropriate prophylaxis or therapy. The use of chromatography technology is a good tool for the isolation, fractionation and phytochemical analysis of medicinal plants and their bioactives in extract and plasma matrices.

The relationship between American ginseng immunostimulatory and immunoinhibitory effects and the unique bioactive fractions of its different extracts namely AQ and ALC extracts will be investigated as the first knowledge gap to be addressed in chapter three of this thesis. This first knowledge gap will be addressed by examining the immunomodulatory effects of AQ extract, ALC extract and their fractions on murine macrophage (RAW 264.7) production of NO and cytokine. The suppression of LPS-stimulation by AQ extract, ALC extract and their fractions will also be examined *in vitro*. The second knowledge gap that will be addressed in chapter three of this thesis is the identity of immunoinhibitory fractions of ginseng ALC extract. To address

this second knowledge gap, the suppression of LPS-stimulation by the ginsenoside and macromolecule fractions of ginseng ALC extract will be examined *in vitro*.

In view of the reported paradoxical immunomodulatory effects of ginseng polysaccharides, the first knowledge gap that will be addressed in chapter four of this thesis is; whether American ginseng roots polysaccharides (AGRPS) stimulates basal innate immune function and at the same time can suppress LPS induced proinflammatory response *ex vivo* and *in vivo*. To address this first knowledge gap, the *ex vivo* and *in vivo* immunomodulatory effects after oral administration of AGRPS extract will be studied in adult rats by measuring cultured alveolar macrophage production of NO and changes of plasma cytokine level, modification of LPS immunological effects by AGRPS extract will also be examined followed with a lung histochemical study. In view of the different reports of ginseng acidic PS and neutral PS immunomodulatory effects, the second knowledge gaps that will be addressed in chapter four of this thesis are; (i) the identity of AGRPS bioactive fractions. (ii) the relationship between AGRPS bioactive fractions physicochemical properties (ionic charge and molecular weight) and their influence on AGRPS up-regulation and down-regulation of macrophage function under basal and LPS endotoxic conditions. To address these second knowledge gaps an *in vitro* mechanistic study will be used in chapter four to identify the underlying immunobioactive fractions of AGRPS extract by examining; (i) the immunobioactivities of AGRPS extract and its fractions (isolated by ion exchange chromatography and ultrafiltration) using murine macrophages (RAW 264.7) and rat alveolar macrophages stimulation of NO and cytokine. (ii) the suppression of LPS-stimulation by AGRPS extract and its fractions pretreatments *in vitro*.

There is currently no available method to analyze ginseng PS in plasma. Such a method will be a useful tool in determining whether orally administered ginseng PS is absorbed from the GIT into

systemic circulation. Perchloric acid-protein precipitation of plasma and high performance size exclusion chromatography with right angle light scattering detection will be used in chapter five of this thesis as a new approach to analyze orally administered unlabeled AGRPS in rat plasma. This new approach will address the GIT absorption of orally administered AGRPS into systemic circulation gap in knowledge.

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Chapter 2

SPECIFIC AIMS AND HYPOTHESES

2.1 SPECIFIC AIMS 1

1. To evaluate the immunostimulatory and immunoinhibitory effects of ginseng aqueous and alcoholic extracts.
2. To identify what bioactive fractions in these extracts cause the immunomodulatory effects.

Choice of solvents influences the bioactive components in plant extracts including American ginseng aqueous (AQ) and alcoholic (ALC) extracts. This factor is often overlooked by many investigators who focus mainly on the pharmacological effects of ginseng extracts. Inconsistent immunomodulatory effects of ginseng have been reported, including both immunostimulatory [1-5] and immunosuppressive effects [6-12]. The basis for the apparent paradoxical immunomodulatory effects is unclear but may be attributed to different experimental conditions, *e.g.* choice of extraction solvents.

Chapter three of this thesis investigates whether the paradoxical immunomodulatory effect is related to unique bioactive fractions of different American ginseng extracts, namely AQ and ALC extracts. AQ and ALC ginseng extracts have been reported to exert immunostimulatory [2-3] and immunoinhibitory [6, 9-10] effects respectively. On the contrary, ginseng aqueous extract have also been reported to possess immunoinhibitory effect [12]. There is need to delineate the paradoxical immunomodulatory effect of ginseng and provide a basis for explaining the apparently contradictory reporting in the literature. The relationship between American ginseng immunostimulatory and immunoinhibitory effects and the unique bioactive fractions of its different extracts namely aqueous (AQ) and alcoholic (ALC) extracts was investigated.

HYPOTHESIS:

Ginseng AQ extract is immunostimulatory while its ALC extract is immunoinhibitory.

To address the hypothesis, AQ and ALC extracts were prepared and their immunostimulatory effects were studied *in vitro* in murine macrophages (Raw 264.7) by measuring the direct production of proinflammatory mediators and cytokines. The suppression of lipopolysaccharide (LPS) induced production of proinflammatory mediators and cytokines by the two extracts was evaluated as readout for their immunoinhibitory effects. Gel permeation chromatography was used to separate and isolate fractions of ginseng AQ and ALC extracts, which were then characterized *in vitro* for immunostimulatory and immunoinhibitory effects.

2.2 SPECIFIC AIMS 2

1. Investigate the effects of American ginseng root polysaccharide (AGRPS) extract on basal immune function *ex vivo* and *in vivo*, and determine whether or not this treatment would also suppress the proinflammatory response of LPS endotoxin.

2. Identify the bioactive fractions responsible for AGRPS extract immunobioactivities; separate AGRPS extract by ionic charge and molecular weight to examine their immunobioactivities.

Polysaccharides of ginseng root are known to stimulate the immune system [13-15] but have recently been shown to suppress response to proinflammatory challenge [16-18]. Chapter four of this thesis aims to determine whether AGRPS stimulates basal innate immune

function and at the same time can suppress LPS proinflammatory challenge. The bioactive fractions responsible for AGRPS extract immunobioactivities was identified by investigating the influence of variations in ionic charge and molecular weight on AGRPS immunomodulatory effects.

HYPOTHESES:

- 1. AGRPS extract possess both immunostimulatory and immunosuppressive effects under basal and LPS endotoxic conditions respectively.**
- 2. The ionic charge and molecular weight variations contribute to AGRPS immunomodulatory activities.**

To address these hypotheses, the *ex vivo* and *in vivo* immunomodulatory effects after oral administration of AGRPS extract were studied in adult rats by measuring cultured alveolar macrophage production of NO and changes of plasma cytokine level. The modification of LPS immunological effects by AGRPS extract was examined followed by a lung histochemical study. To identify the bioactive fractions responsible for AGRPS extract immunobioactivities, fractions of AGRPS extract isolated by ion exchange chromatography and ultrafiltration were used for *in vitro* mechanistic study. For this study, murine macrophages (RAW 264.7) and rat alveolar macrophages were used to evaluate the stimulation of NO and cytokine by AGRPS extract and its fractions. The suppression of LPS-stimulation by AGRPS extract and its fractions was also examined *in vitro*.

2.3 SPECIFIC AIMS 3

1. Develop and apply a method for the determination of AGRPS in plasma.
2. Ascertain that orally administered AGRPS can be absorbed from the gastrointestinal tract into systemic circulation.

A recent study with rat shows that orally administered AGRPS exerts *ex vivo* and *in vivo* immunomodulatory effects [19]. *Ophiopogon japonicas*, *Radix ophiopogonis*, chitosan ester, dermatan sulfate and marine sulfated polysaccharides of high molecular weights have been analyzed in biological matrices (e.g. plasma) of animals after polysaccharide treatments using various chromatographic and spectroscopic analytical methods [20-26]. The weak chromophore group of AGRPS and complex nature of plasma matrix are challenging barriers to overcome to analyze AGRPS in plasma. Chromatographic methods involving fluorescent labeling (derivatization) coupled with fluorometric detection used for plasma analysis of polysaccharides shows that; high molecular weight polysaccharides such as *Radix Ophiopogonis* polysaccharides are absorbed from the gastrointestinal tract (GIT) into systemic circulation after oral administration [25]. There is no method available to detect and quantify AGRPS in plasma and show that orally administered AGRPS is absorbed from the GIT into systemic circulation. In chapter five of this thesis, perchloric acid-protein precipitation of plasma and high performance size exclusion chromatography (HPSEC) with right angle light scattering detection is used as a new approach to analyze orally administered unlabeled ginseng polysaccharides in rat plasma.

HYPOTHESIS:

Orally administered AGRPS is absorbed from the GIT into systemic circulation.

To address the hypothesis, AGRPS was isolated from plasma of rats orally treated with AGRPS, using protein precipitation with perchloric acid. The AGRPS analyte was then chromatographed on a HPSEC system and monitored using right angle light scattering detector system. To ascertain the intestinal absorption of orally administered AGRPS, enzyme hydrolytic method accompanied with HPSEC analysis was used to verify the identity of the AGRPS chromatographic peak in plasma of orally AGRPS treated rats. The time-dependent change in plasma concentration after oral administration of AGRPS in rats was evaluated to provide additional evidence of the intestinal absorption of orally administered AGRPS into systemic circulation.

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Chapter 3

IN VITRO IMMUNOSTIMULATORY AND ANTI- INFLAMMATORY EFFECTS OF AMERICAN GINSENG AQUEOUS AND ALCOHOLIC EXTRACTS

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3.1 Introduction

Ginseng is a perennial herb of the Araliaceae family. Asian ginseng (*Panax ginseng* C.A. Meyer, *Ren Shen*) and American ginseng (*Panax quinquefolius* L., *Xiyangshen*) are the most commonly used ginseng species. While Asian ginseng has been used for thousands of years as a tonic to improve overall health, restore the body to balance, help the body to heal itself and reduce stress [1], the medicinal use of American ginseng traces back about 400 years ago. Canada is currently the largest producer of American ginseng [1-3]. Recognized by the Canadian regulatory agency as a natural health product for use as an adaptogen (biological response modifier) [4], American ginseng is a multi-action herb with a wide range of pharmacological effects on the central nervous system, cardiovascular system and endocrine secretion, reproductive and immune function [5].

Ginseng has influences on both innate and adaptive immunity. Macrophage-mediated innate immunity is the first line of defense against microbial pathogens and influences the subsequent adaptive immune response. Macrophages kill pathogens and cancer cells directly *via* phagocytosis and indirectly *via* the production of various pro-inflammatory mediators (e.g. NO) and cytokines (e.g. TNF- α) [6]. However, over production of pro-inflammatory mediators [7] may result in inflammatory diseases and/or tissue injury which are then managed by immune-suppressive agents. Modulation of macrophage function, e.g. up-regulation of inflammatory mediator production *in vitro* or suppression of its stimulation by LPS has been used as an experimental tool to evaluate immunostimulatory and anti-inflammatory potency of herbal products respectively [8].

Ginseng contains bioactive compounds such as ginsenosides, which are steroidal saponins

containing different sugar moieties and possessing different lipid-solubility [5] and polysaccharides (PS) consisting of complex chain of monosaccharides rich in L-arabinose, D-galactose, L-rhamnose, D-galacturonic acid, D-glucuronic acid and D-galactosyl residue [9-10]. There is paucity of data concerning the immunomodulatory effects of ginseng alcoholic (ALC) extract macromolecular components. Choice of solvents influences the bioactive components in the extracts. This factor is often overlooked by many investigators who focus mainly on biological activities.

Inconsistent immunomodulatory effects of ginseng have been reported, including both immunostimulatory and immunosuppressive effects [11-22]. Aqueous (AQ) and ALC ginseng extracts have been reported to exert immunostimulatory [11-15] and immunoinhibitory [16-21] effects respectively. On the contrary, ginseng AQ extract has also been reported to possess immunoinhibitory effects [22]. The basis for the apparent paradoxical immunomodulatory effects is unclear but may be attributed to different experimental conditions, *e.g.* choice of extraction solvents. There is a need to delineate the paradoxical immunomodulatory effect of ginseng and provided a basis for explaining the apparently contradictory reporting in the literature.

In this study, the apparent paradoxical effects of ginseng were characterized by examining the immunomodulatory effect of AQ and ALC extracts prepared from 4-year-old Ontario grown American ginseng roots in RAW 264.7 murine macrophage cell line. Characteristics of the immunobioactive fractions of ginseng were also explored.

3.2 Materials

RAW 264.7 (ATCC TIB 67) murine macrophage cell lines were provided by Dr. Jeff Dixon (Department of Physiology and Pharmacology, University of Western Ontario, Canada). Sephadex G75 was purchased from GE healthcare bio-sciences AB (Sweden). Cell culture medium and reagents were purchased from Gibco laboratories (USA). BD OptEIA ELISA kits tumour necrosis factor- α and interleukin-6 (BD Biosciences, USA). LPS from *Escherichia coli* and Griess reagent were purchased from Sigma-Aldrich (USA), Amicon ultra-15 centrifugal filter units with molecular weight cut-off pore size of 100 KDa, 50 KDa, 30 KDa and 10 KDa were purchased from Millipore (USA).

3.2.1 Ginseng and its extracts

Four-year-old American ginseng roots collected in 2007 from five different farms in Ontario, Canada were provided by the Ontario Ginseng Growers Association. Ginseng extracts from each farm were prepared individually and combined to produce composite extracts which were used for phytochemical and pharmacological studies.

3.3 Methods

3.3.1 Preparation of the AQ, ALC and Crude PS ginseng extracts

Dried ginseng root samples were shipped to Naturex (USA) for extraction. Samples were ground between $\frac{1}{4}$ and $\frac{1}{2}$ inch and used to produce the AQ or ALC extract. Briefly, 4kg ground ginseng roots were soaked three times during five hours in 16 L of water or ethanol/water (75/25, v/v) solution at 40°C. After extraction, the solution was filtered at room temperature. The excess solvent was then removed by a rotary evaporator under vacuum at 45°C. The three pools were

combined and concentrated again until the total solids on a dry basis were around 60%. The Central Laboratory of Ontario Ginseng Research & Innovation Consortium (OGRIC) lyophilized these concentrates with a freeze dryer (Labconco, USA) at -50°C under reduced pressure to produce AQ or ALC ginseng extract in powder form. Yield of the powder extracts from the concentrates was about 66%. The yields of the final extract (mean \pm standard deviation of % extractive) from the initial ground root were 41.74 ± 4.92 and 35.30 ± 5.01 for the AQ and ALC extracts respectively. A solution of AQ extract in distilled water (1 g/10mL) was prepared, and the crude PS was precipitated by the addition of four volumes of 95% ethanol. The PS fraction was collected by centrifugation at $350\times g$ (Beckman Model TJ-6, USA) for 10 minutes and lyophilized to produce the crude PS extract.

3.3.2 Chromatography of ginseng extracts

3.3.2.1 Sephadex G-75 Chromatography

Five hundred milligrams of AQ or ALC ginseng extract was dissolved in 5 mL distilled water and then fractionated by loading to a calibrated Sephadex G-75 column (47×2.5 cm) equilibrated and eluted with distilled water mobile phase at 4°C with a flow rate of 1 mL/min. Absorbance of the eluates was monitored at 230 nm, which is the wave length of maximum absorption for the extracts determined from a UV scan. 5 mL fractions were collected and four major fractions (I-IV) were collected and lyophilized to produce four sub-fractions (I-IV) (Figure 5) for the study of bioactivity distribution.

3.3.2.2 Size Exclusion Chromatography for PS analysis

Size exclusion chromatography of AQ, ALC and PS ginseng extract was carried out at 40°C with an AquaGel PAA-200 Series column (8×300 mm, PolyAnalytik, USA) connected to a Viscotek (Varian Instruments, USA) gel permeation chromatography system with OmniseC software (version 4.5, Viscotek, USA) for data acquisition. Solutions of AQ, ALC and PS extract (5 mg/mL) were filtered with 0.2 µm nylon filter and used for analysis. Each sample (100 µl) was injected and eluted with 0.05 M sodium nitrate (NaNO₃) mobile phase at a flow rate of 1 mL/min and monitored using a multiple detectors system for light scattering, refractive index and viscosity. Pullulan polysaccharide reference standard was analyzed as a positive control.

3.3.2.3 Fractionation of the ALC extract by Molecular Weight

Powdered ALC extract (800 mg) was dissolved in 40 mL of distilled water and 15 mL was placed in a 100 kDa amicon ultra-15 centrifugal filter unit (Millipore, USA) and centrifuged at 10000rpm for 30 minutes. Fractions of the extract with molecular weight of 100 kDa and above (≥ 100 kDa) were retained on the filter unit, while fractions of less than 100 kDa in the filtrate obtained from this process was then placed in a 50 kDa amicon ultra-15 centrifugal filter unit and centrifuged as indicated above. Fractions of the extract with molecular weight of less than 100 and above 50 kDa (100 - 50 kDa) were retained on the filter unit, while fractions of less than 50 kDa in the filtrate obtained from this process was then placed in a 30 kDa amicon ultra-15 centrifugal filter unit and centrifuged as indicated above. Fractions of the extract with molecular weight of less than 50 and above 30 kDa (50 - 30 kDa) were retained on the filter unit, while components of less than 30 kDa in the filtrate was then placed in a 10 kDa amicon ultra-15 centrifugal filter unit and centrifuged as indicated above. Fractions of the extract with molecular

weight of less than 30 and above 10 kDa (30 - 10 kDa) were retained on the filter unit, while fractions of less than 10 kDa in the filtrate was then placed in a 3 kDa amicon ultra-15 centrifugal filter unit and centrifuged as indicated above. Fractions of the extract with molecular weight of less than 10 and above 3 kDa (10 - 3 kDa) were retained on the filter unit, while components of less than 3 kDa in the final filtrate was termed the ginsenoside fraction since ginsenosides have molecular weights less than 3kDa [23-25]. Ten mL of distilled water was used to wash and remove the fractions of ALC extract of different molecular weights ≥ 100 kDa, 100 - 50 kDa, 50 - 30 kDa, 30 - 10 kDa, 10 - 3 kDa, < 3 kDa (ginsenoside fraction) retained on the filter units, after which they were lyophilized at -50°C under reduced pressure (Labconco, USA).

3.3.3 Cell culture

Mouse macrophage cell line RAW 264.7 was grown and subjected to no more than 20 cell passages in Dulbeccos Modified Eagle's Medium supplemented with 10% Fetal Bovine Serum, 25 mM HEPES, 2mM Glutamine, 100 IU/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin. Cells in RPMI-1640 medium supplemented with 10% Fetal Bovine Serum, 25 mM HEPES, 2 mM Glutamine, 100 IU/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin were seeded in 96-well tissue culture plates at a density of 1.5×10^5 cells per well and maintained at 37°C in a humidified incubator with 5% CO_2 and used for experiments at 60-80% confluency.

3.3.4 Cell treatment

3.3.4.1 Immunostimulatory effect

Experiments to evaluate the dose-related stimulation of inflammatory mediators profile *in vitro* was carried out by treating and incubating macrophages (1.5×10^5 cells / well) with 0, 20, 50 and 200 $\mu\text{g/ml}$ of ginseng extracts, their fractions or 1 $\mu\text{g/mL}$ of LPS (positive control) for 24 hr. The end-points were the 24 hour-production of NO, TNF- α and IL-6 inflammatory mediators.

3.3.4.2 Immunosuppression of LPS-induced effect

To examine the direct inhibitory effect of ginseng extracts or their fractions on LPS-stimulated immune function, macrophages were pretreated with 0, 10, 20 50, 100 or 200 $\mu\text{g/ml}$ of ginseng extracts two hours prior to the addition of 1 $\mu\text{g/mL}$ of LPS. The 24-hour cytokine production induced by LPS was determined by measuring NO, TNF- α and IL-6 levels in the culture medium.

3.3.4.3 Suppression of AQ extract-induced Macrophage NO stimulation by ALC extract

Production of NO by 1.5×10^5 macrophages / well in a 96 well-plate induced by 0, 50 and 200 $\mu\text{g/ml}$ of AQ ginseng extract was determined 24 hr after the presence and absence of 200 $\mu\text{g/ml}$ ALC ginseng extract.

3.3.4.4 Quantification of NO, TNF- α and IL-6

TNF- α and IL-6 concentrations in supernatants from cultured cells were analyzed with Enzyme Linked Immunosorbent Assay (ELISA). Samples were evaluated with mouse cytokine-specific BD OptEIA ELISA kits (BD Biosciences, USA) according to the manufacturer's protocol. NO

production was analyzed as accumulation of nitrite in the culture medium. Nitrite in culture supernatants was determined with Griess reagent (Sigma-Aldrich, USA). Briefly, 50 μ L of culture supernatant from each sample was transferred to wells of a 96-well U-bottom microtiter plate, 50 μ L Griess reagent (containing 0.5% sulfanilic acid, 0,002% N-1-naphtyl-ethylenediamine dihydrochloride and 14% glacial acetic acid) was then added. The ultraviolet (UV) absorbance at 550nm wavelength was measured using Multiskan Spectrum microplate reader (Thermo Fisher Scientific, Finland) with SkanIt software (version 2.4.2, Thermo Fisher Scientific, Finland). Sample nitrite concentrations were estimated from a sodium nitrite standard calibration curve.

3.4 Statistical analysis

Each cell culture experiment was performed in triplicate on three separate occasions. All statistical analyses were performed with GraphPad prism 4.0a Software (GraphPad Software Inc., USA). Data were presented as the mean \pm standard deviation (SD) of triplicates from three independent experiments. Data sets were evaluated by one-way analysis of variance (ANOVA) with Dunnett's *post-hoc* test. $P < 0.001$ was considered to be statistically significant.

3.5 Results

3.5.1 Immunostimulatory effect of the AQ and ALC ginseng extracts in macrophages

in vitro

Evaluation of the immunostimulatory effect of the ginseng extracts on RAW 264.7 murine macrophages revealed that exposure to 20-200 $\mu\text{g/mL}$ of AQ extract significantly up-regulated macrophage production of NO, TNF- α and IL-6 compared to an untreated control in a concentration-dependent manner (Figures 3.1a and 3.1b). The responses to 200 $\mu\text{g/mL}$ of AQ extract in NO and TNF- α production were similar to the maximum stimulatory response induced by 1 $\mu\text{g/mL}$ of LPS. Moreover, the magnitude of maximum stimulatory response pertaining to NO and TNF- α (as a % of the positive control) was much greater than that of IL-6. By contrast, the ALC extract had no apparent immunostimulatory effect (Figure 3.1).

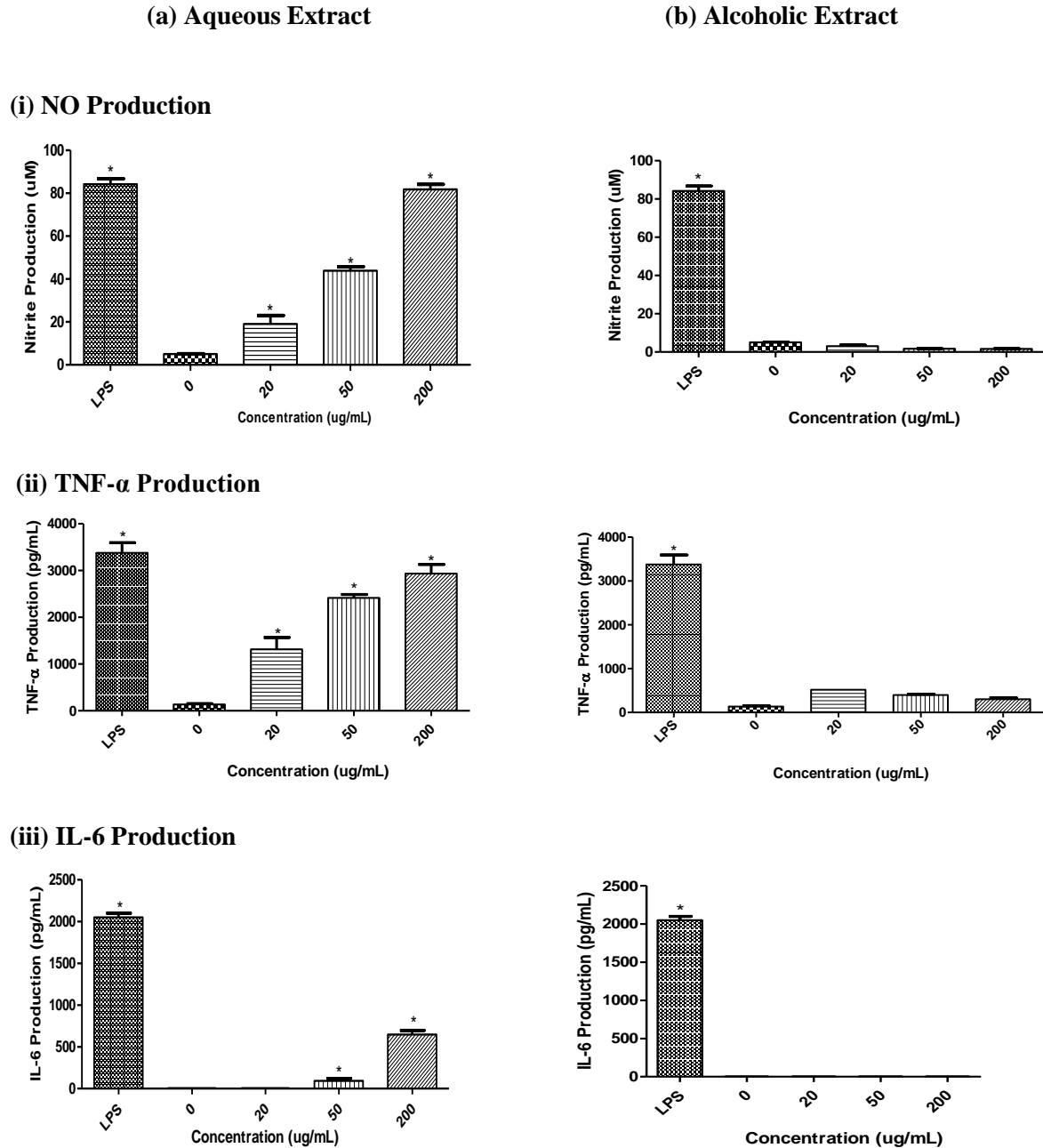


Figure 3.1 - Immunostimulatory effects of the (a) AQ and (b) ALC ginseng extracts on 24 hr macrophage production of (i) NO, (ii) TNF- α and (iii) IL-6. Murine macrophages (RAW 264.7 cells 1.5×10^5 / well) were treated with or without AQ and ALC ginseng extracts (20, 50, 200 $\mu\text{g}/\text{mL}$), LPS (1 $\mu\text{g}/\text{mL}$) for 24 hr and the culture supernatants were analyzed for NO and TNF- α /IL-6 by Griess reaction assay and ELISA respectively. Three independent experiments ($n = 3$) were performed and the data were shown as mean \pm SD. Datasets were evaluated by ANOVA. * Values $P < 0.001$ compared to the untreated (vehicle) control were statistically significant.

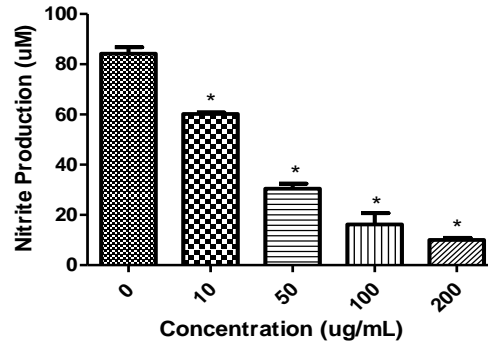
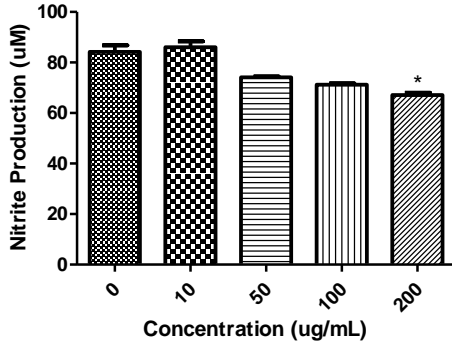
3.5.2 Effect of the AQ and ALC ginseng extracts on LPS-stimulated production of NO and TNF- α in macrophages *in vitro*

Figure 3.2 shows the influence of ginseng extract treatment on LPS-stimulated NO and TNF- α production in macrophages. LPS stimulated 24-hour production of NO markedly, which was significantly suppressed in the presence of 20-200 $\mu\text{g/ml}$ of the ALC extract in a dose-dependent manner (Figure 3.2b). This inhibitory effect appeared to be extract-specific as the AQ extract was marginally effective and only at high concentrations (Figure 3.2a). Figure 3.2 also showed that the influence of ginseng was cytokine-specific, *i.e.* the magnitude of inhibition by ALC extract was much smaller with respect to TNF- α production. Moreover, the AQ extract had either no inhibitory effect at high concentration or additive effect at low concentration.

(a) Aqueous Extract

(b) Alcoholic Extract

(i) NO Production



(ii) TNF- α Production

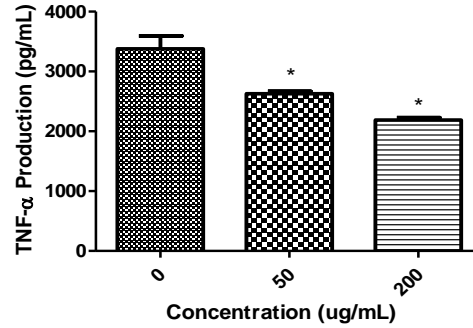
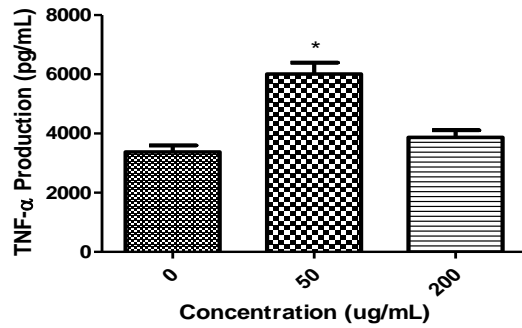


Figure 3.2 -Effect of the (a) AQ and (b) ALC ginseng extracts on LPS-stimulated 24 hr macrophage production of (i) NO and (ii) TNF- α . Murine macrophages (RAW 264.7 cells 1.5×10^5 / well) were pretreated with or without the AQ and ALC ginseng extracts (50, 200 $\mu\text{g/ml}$), for two hours after which LPS 1 $\mu\text{g/ml}$ was added; and 24 hr later the NO and TNF- α contents of the culture supernatants were determined by Griess reaction assay and ELISA, respectively. Three independent experiments ($n = 3$) were performed and the data were shown as mean \pm SD. Datasets were evaluated by ANOVA. * Values $P < 0.001$ compared to the LPS positive control were statistically significant.

3.5.3 Suppression of the AQ ginseng extract-induced immunostimulation by the ALC ginseng extract

To further study the apparent extract-specific paradoxical immunomodulatory effects of ginseng, investigation was done to determine whether the immunostimulation induced by the AQ extract could be suppressed by subsequent treatment with the ALC extract. The dose-related up-regulation of NO production in macrophages by the AQ extract was reduced by 50-65% with exposure to equivalent concentrations of the ALC extract (Figure 3.3).

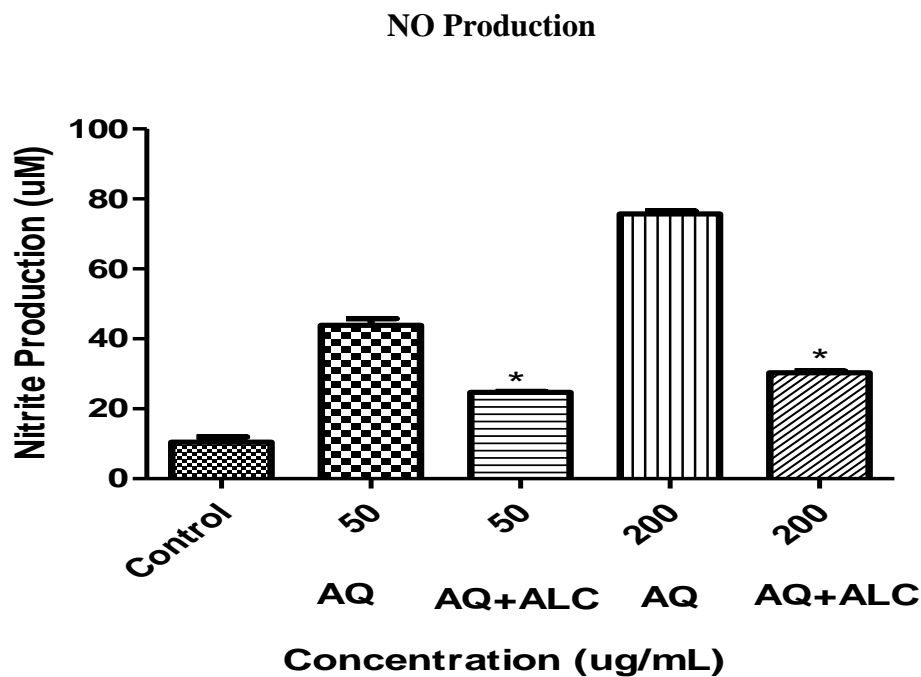


Figure 3.3 - ALC ginseng extract suppressed up-regulation of macrophage NO production by the AQ ginseng extract. Murine macrophages (RAW 264.7 cells 1.5×10^5 / well) were pretreated with or without the 200 $\mu\text{g/ml}$ ALC ginseng extract (50, 200 $\mu\text{g/ml}$), for two hours after which the AQ ginseng extract (50, 200 $\mu\text{g/ml}$) was added, then NO contents of the culture supernatants were determined by Griess reaction assay 24 hr later. Three independent experiments were performed ($n = 3$) and the data were shown as mean \pm SD. Datasets were evaluated by ANOVA. * Values $P < 0.001$ were statistically significant.

3.5.4 Sephadex G-75 Chromatography of the AQ and ALC ginseng extracts

To further study the apparent extract-specific paradoxical immunomodulatory effects of ginseng, the extract-specific bioactive fractions that mediated these effects was examined. Gel filtration of the AQ extract on a calibrated Sephadex G-75 column resulted in the appearance of two major peaks (Fractions I and III) based on UV absorbance at 230 nm (Figure 3.4a). The estimated average molecular weights (Mw) of Fractions I and III were about 73 kDa and 37 kDa respectively; and their yield accounted for 28% and 40% by dry weight of the AQ extract respectively. Since PS of ginseng possesses an immunostimulatory effect [1, 9, 10, 26], the crude PS fraction was isolated from the AQ extract by alcohol (40%) precipitation (with a yield of 10% by weight) and was subjected to a similar chromatographic procedure for comparison. As shown in Figures 3.4a and 3.4c, the major PS peak had a similar elution volume as Fraction I of the AQ extract.

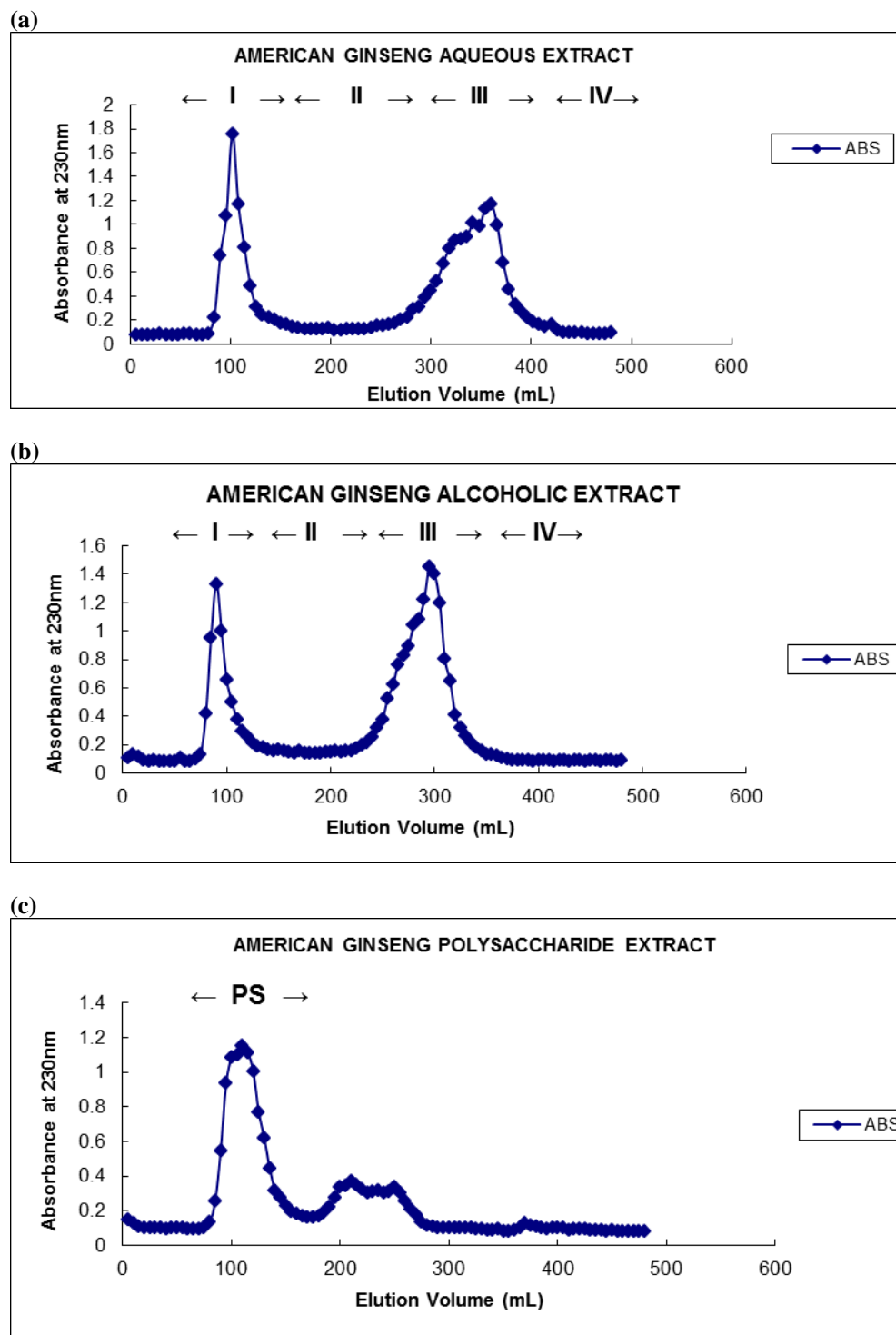


Figure 3.4 - Sephadex G-75 (47×2.5 cm) chromatographic fractionation of the (a) AQ, (b) ALC and (c) PS extracts of ginseng. Column was loaded with 500mg of extract, and then eluted with distilled water at flow rate of 1 mL/min. The y-axis is the UV absorbance at 230 nm while the x-axis represents the elution volume (mL).

3.5.5 Immunobioactive Fraction(s) of the AQ ginseng extract

Figure 3.5 shows the data concerning the stimulation of cytokine production in macrophages by Fractions I and III of the AQ extract. The immunostimulatory activity with respect to NO and TNF- α production was associated only with macromolecules of Fraction I (Mw = ~ 73 kDa) but not Fraction III (Mw = ~ 37 kDa). Fraction I was less active than the PS extract in terms of IL-6 production. Since PS and Fraction I corresponded to 10% and 28% of the AQ extract by weight, it appeared that these isolated fractions could only account for part of the observed immunostimulatory activities of the AQ extract on the basis of the difference in their immunostimulatory potency.

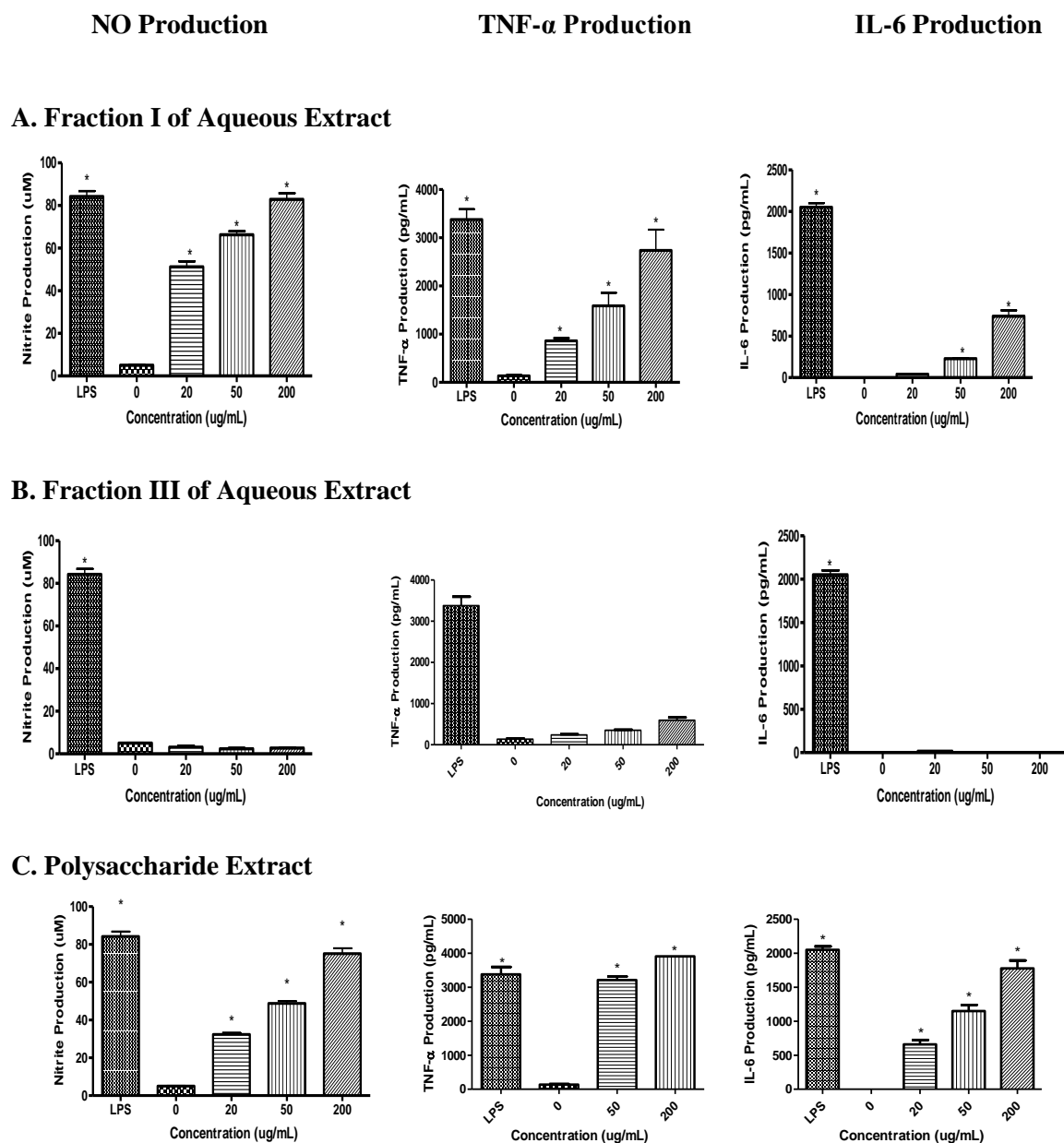


Figure 3.5 - Immunostimulatory effect of Fraction I ($M_w = \sim 73$ kDa) and III ($M_w = \sim 37$ kDa) of the AQ, PS extracts of ginseng. Murine macrophages (RAW 264.7 cells 1.5×10^5 / well) were treated with Fraction I and III of AQ ginseng extract, PS extract of ginseng. (0, 20, 50, 200 $\mu\text{g/ml}$), LPS (1 $\mu\text{g/ml}$) for 24 hr and the NO, TNF- α and IL-6 contents of the culture supernatants were determined. Three independent experiments were performed ($n = 3$) and the data were shown as mean \pm SD. Datasets were evaluated by ANOVA. *Values $P < 0.001$ compared to the untreated (vehicle) control were statistically significant.

3.5.6 Immunobioactive Fraction(s) of the ALC ginseng extract

Fractions I (10%) and III (64%) obtained from the Sephadex G-75 chromatographic profile of the ALC extract contained no immunostimulatory activity (data not shown). Fraction I was not affected by treatment with 40% ethanol (data not shown). This observation was consistent with the lack of PS in the ALC extract. Figure 3.6 indicates that Fraction I ($M_w = \sim 73$ kDa) of ALC extract was particularly more active than Fraction III ($M_w = \sim 37$ kDa): causing significant and dose-dependent reduction in 24-hour NO production by macrophages induced by 1 $\mu\text{g/mL}$ LPS.

NO Production

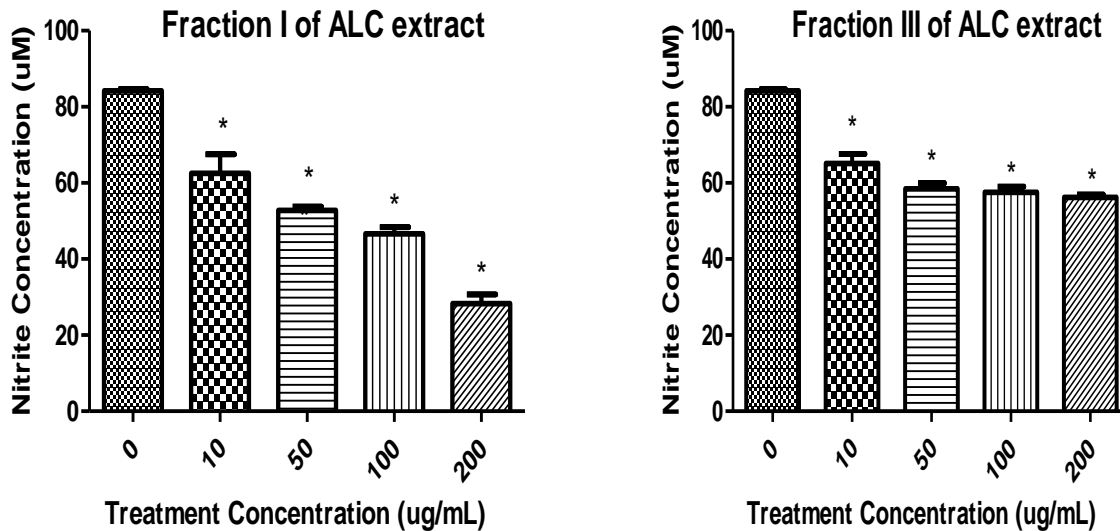


Figure 3.6 - Effect of Fractions I ($M_w = \sim 73$ kDa) and III ($M_w = \sim 37$ kDa) of the ALC extract on LPS-stimulated 24 hr macrophage production of NO. Murine macrophages (RAW 264.7 cells 1.5×10^5 / well) were pretreated with or without the AQ and ALC extracts (10, 50, 100, 200 $\mu\text{g/ml}$) for two hours after which LPS (1 $\mu\text{g/ml}$) was added, and the NO content of the culture supernatants were determined by Griess reaction assay 24 hr later. Three independent experiments were performed ($n = 3$) and the data were shown as mean \pm SD. Datasets were evaluated by ANOVA. * Values $P < 0.001$ compared to the LPS positive control were statistically significant.

3.5.7 Effect of the ginseng ALC extract, its macromolecule and ginsenoside fractions on

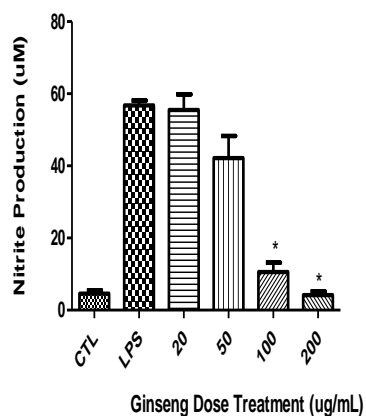
LPS stimulated macrophage production of NO, TNF- α and IL-6

In this study the immunobioactive fractions that contribute to the ginseng ALC extract immunoinhibitory effect were investigated. The extract was fractionated into macromolecule fractions (≥ 100 kDa, 50-100 kDa, 30-50 kDa, 10-30 kDa, 3-10 kDa) and ginsenoside fraction (< 3 kDa). Ginsenosides have been reported to possess molecular weights less than 3 kDa [23-25]. The suppression of LPS induced production of NO, TNF- α and IL-6 by ALC, its macromolecule and ginsenoside fractions was studied in murine macrophages (RAW 264.7 cell line) *in vitro*. LPS stimulated 24-hour production of NO, TNF- α and IL-6 markedly, which was suppressed in the presence of ALC extract, its macromolecule and ginsenoside fractions. The ≥ 100 kDa macromolecule suppressed NO more than the ALC extract at 20-50 $\mu\text{g/ml}$, while at 200 $\mu\text{g/mL}$ the ≥ 100 kDa macromolecule markedly suppressed LPS induced production of TNF- α and IL-6 with a magnitude similar to that of the ALC extract. The 50 - 100 kDa and 30 - 50 kDa macromolecule fractions were quite immunoinhibitory when compared with the ALC extract, they were better than 10 - 30 kDa, 3 - 10 kDa macromolecule fractions and the ginsenoside fraction (< 3 kDa). Data generated from this study suggests that all the macromolecule and ginsenoside fractions exerted immunoinhibitory effect.

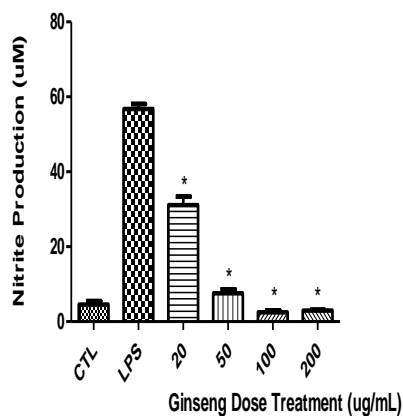
Figure 3.7 - Effect of the ALC ginseng extract, its macromolecules and ginsenoside fraction on LPS-stimulated 24 hr macrophage production of NO

Suppression of LPS-induced Production of NO

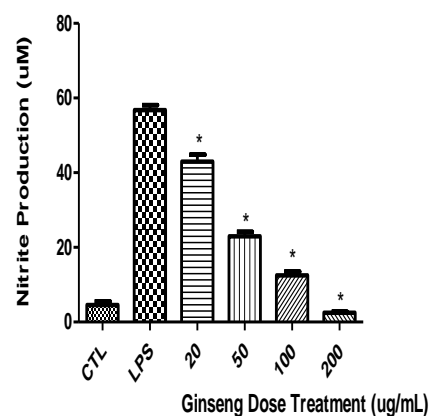
ALC Extract



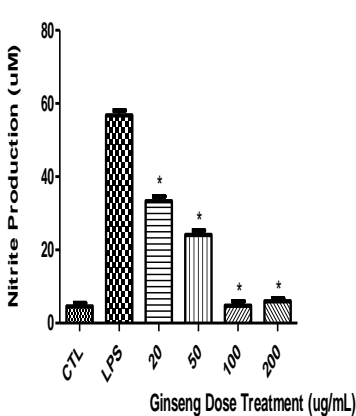
≥100 kDa Macromolecule



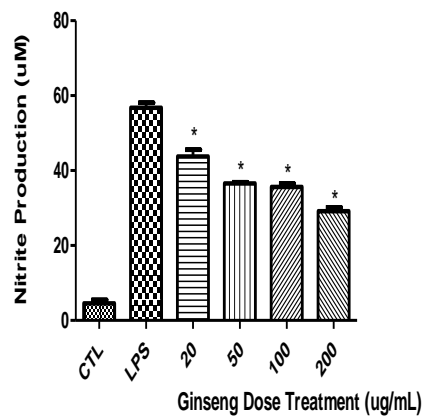
50 - 100 kDa Macromolecule



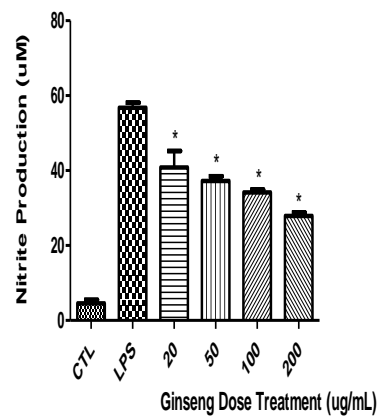
30 - 50 kDa Macromolecule



10 - 30 kDa Macromolecule



3 - 10 kDa Macromolecule



Ginsenoside Fraction (<3 kDa)

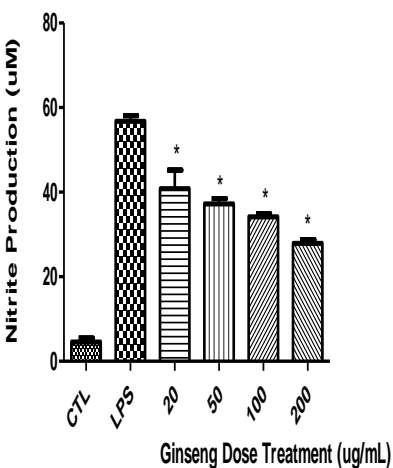
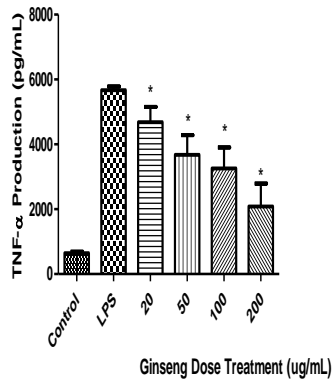


Figure 3.7 - Effect of the ALC ginseng extract, its macromolecules and ginsenoside fraction on LPS-stimulated 24 hr macrophage production of NO. Murine macrophages (RAW 264.7 cells 2.0×10^5 / well) were pretreated with and without the ALC ginseng extract, its macromolecules and ginsenoside fraction (20, 50, 100 and 200 $\mu\text{g/ml}$), for two hours after which LPS 1 $\mu\text{g/ml}$ was added; and 24 hr later the NO contents of the culture supernatants were determined by Griess reaction assay. Three independent experiments ($n = 3$) were performed and the data were shown as mean \pm SD. Datasets were evaluated by ANOVA. *Values $P < 0.001$ compared to the LPS positive control (immunosuppressive effect) were statistically significant.

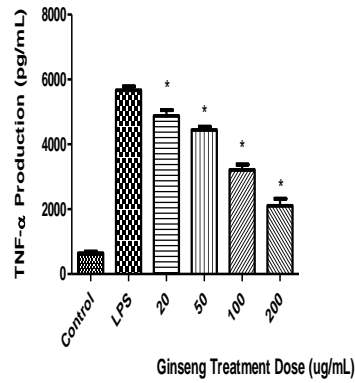
Figure 3.8 - Effect of the ALC ginseng extract, its macromolecules and ginsenoside fraction on LPS-stimulated 24 hr macrophage production of TNF- α

Suppression of LPS-induced Production of TNF- α

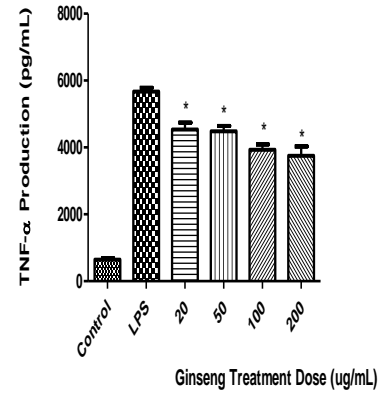
ALC Extract



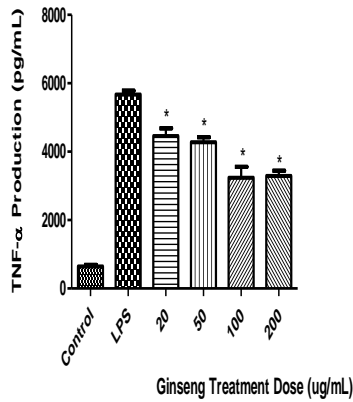
≥ 100 kDa Macromolecule



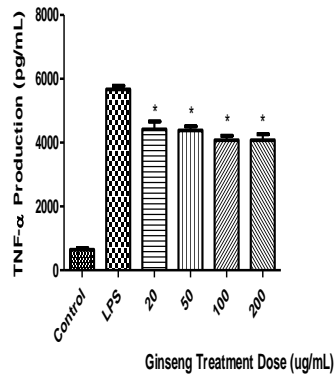
50 - 100 kDa Macromolecule



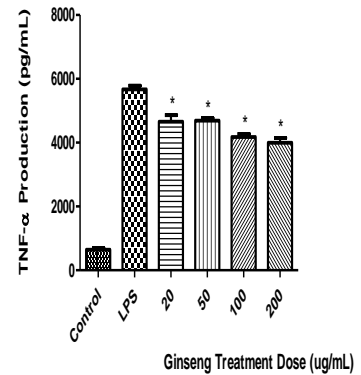
30 - 50 kDa Macromolecule



10 - 30 kDa Macromolecule



3 - 10 kDa Macromolecule



Ginsenoside Fraction (<3 kDa)

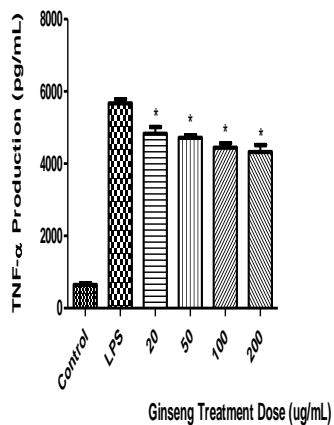
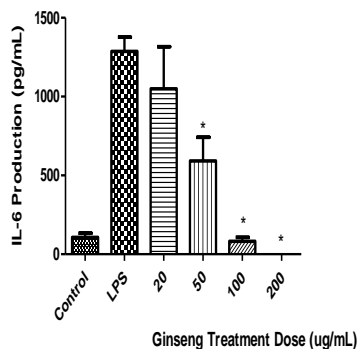


Figure 3.8 - Effect of the ALC ginseng extract, its macromolecules and ginsenoside fraction on LPS-stimulated 24 hr macrophage production of TNF- α . Murine macrophages (RAW 264.7 cells 2.0×10^5 / well) were pretreated with without the ALC ginseng extract, its macromolecules and ginsenoside fraction (20, 50, 100 and 200 $\mu\text{g/ml}$), for two hours after which LPS 1 $\mu\text{g/ml}$ was added; and 24 hr later the TNF- α contents of the culture supernatants were determined by ELISA. Three independent experiments were performed ($n = 3$) and the data were shown as mean \pm SD. Datasets were evaluated by ANOVA. *Values $P < 0.001$ compared to the LPS positive control (immunosuppressive effect) were statistically significant.

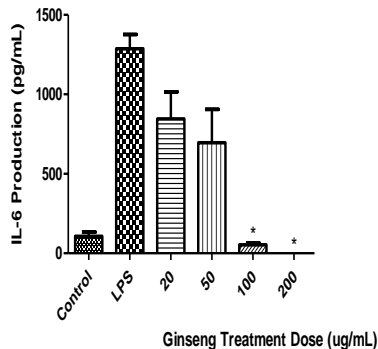
Figure 3.9 - Effect of the ALC ginseng extract, its macromolecules and ginsenoside fraction on LPS-stimulated 24 hr macrophage production of IL-6

Suppression of LPS-induced Production of IL-6

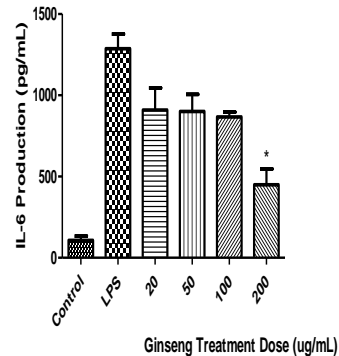
ALC Extract



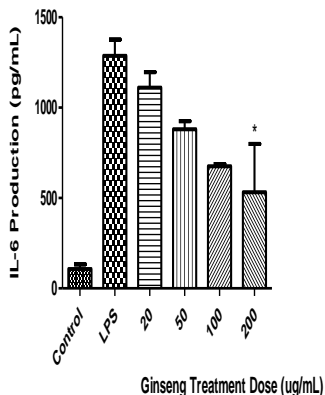
≥100 kDa Macromolecule



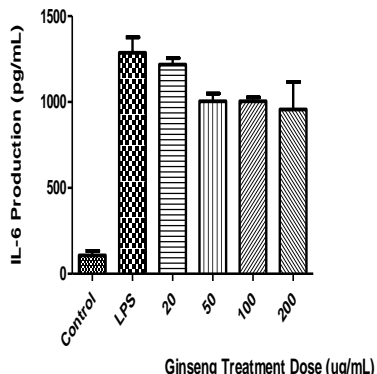
50 - 100 kDa Macromolecule



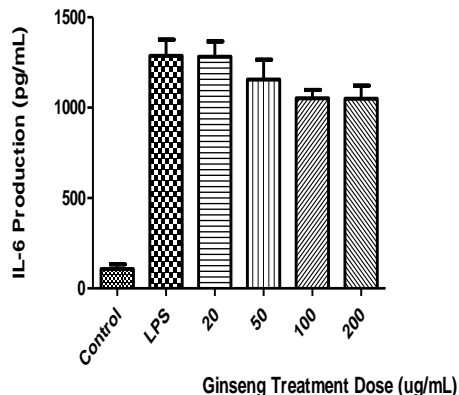
30 - 50 kDa Macromolecule



10 - 30 kDa Macromolecule



3 - 10 kDa Macromolecule



Ginsenoside Fraction (<3 kDa)

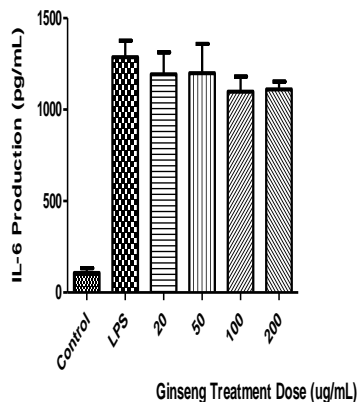


Figure 3.9 - Effect of the ALC ginseng extract, its macromolecules and ginsenoside fraction on LPS-stimulated 24 hr macrophage production of IL-6. Murine macrophages (RAW 264.7 cells 2.0×10^5 / well) were pretreated with without the ALC ginseng extract, its macromolecules and ginsenoside fraction (20, 50, 100 and 200 $\mu\text{g/ml}$), for two hours after which LPS 1 $\mu\text{g/ml}$ was added; and 24 hours later the IL-6 contents of the culture supernatants were determined by ELISA. Three independent experiments were performed ($n = 3$) and the data were shown as mean \pm SD. Datasets were evaluated by ANOVA. *Values $P < 0.001$ compared to the LPS positive control (immuno-suppressive effect) were statistically significant.

3.5.8 Size Exclusion Chromatographic Analysis of AQ and ALC ginseng extracts and their fractions

To further characterize the AQ and ALC extracts in view of the appearance of two peaks identified in their Sephadex G-75 chromatographic profiles at 230nm UV absorbance, size exclusion chromatography with multiple detectors (refractive index, right angle light scattering, lower angle light scattering and viscometer detectors) was used to differentiate the macromolecular constituents from the two extracts. Chromatographic analysis with light scattering detection gave the best qualitative and specific detection in identifying PS and differentiating the extracts. Light scattering data in Figures 3.10 and 3.11 shows the presence of PS in the AQ extract and Fraction I ($M_w = \sim 73$ kDa) of AQ extract on the basis of their similarity to the chromatographic profile of pullulan polysaccharide reference standard ($M_w = 112$ kDa) and the crude PS fraction isolated from ginseng. In contrast, the ALC extract did not contain any detectable PS. Hence crude PS fraction is present in AQ extract and Fraction I of AQ extract but absent in ALC extract. Outcome of this chromatographic analysis showed the significant difference in crude PS fraction composition between the AQ and ALC extracts of ginseng.

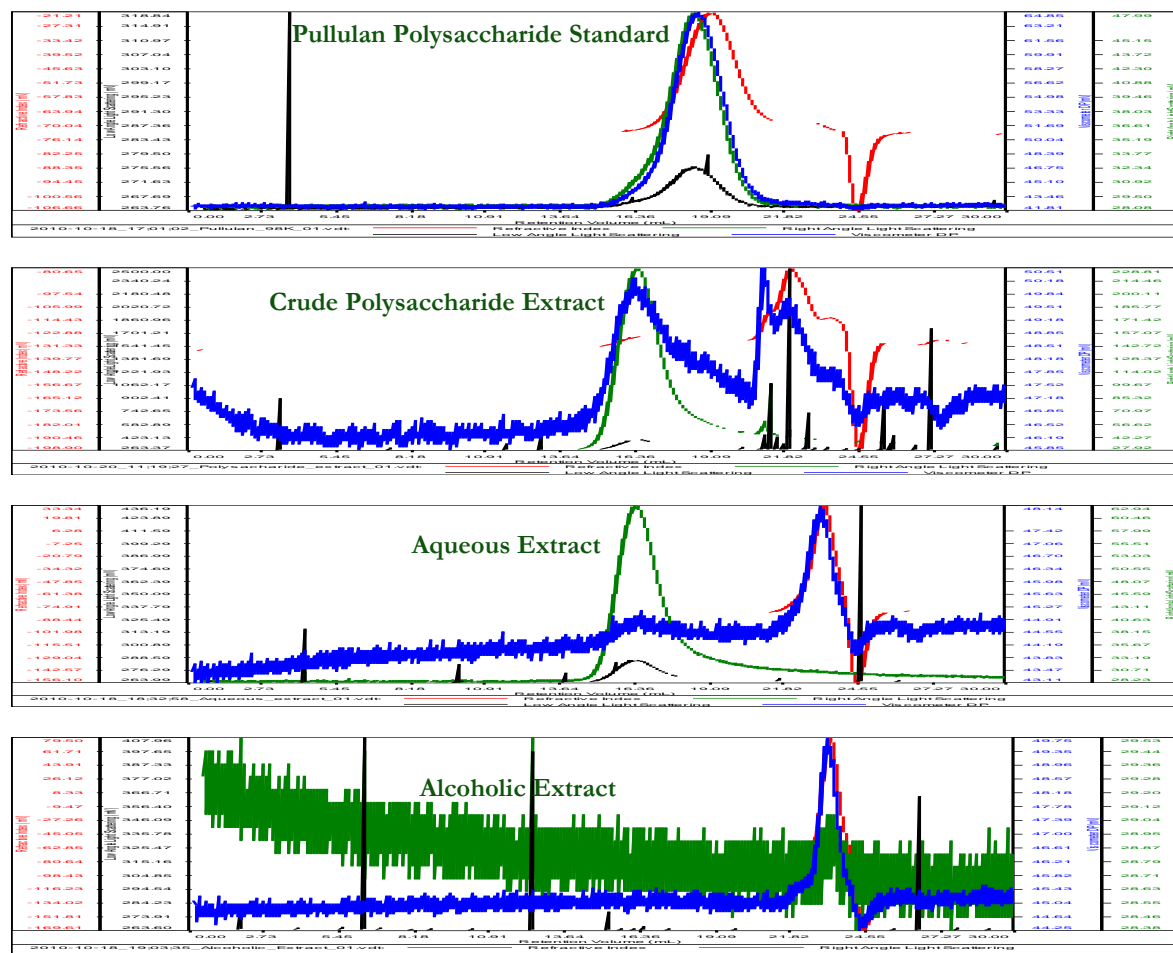


Figure 3.10 – Visual comparison of the chromatograms obtained from size exclusion chromatography of crude PS, AQ and ALC extracts of ginseng. Each sample was injected and eluted with 0.05M NaNO₃ mobile phase, this was monitored with refractive index, right angle light scattering, lower angle light scattering and viscometer detectors at 1mL/min flow rate to differentiate the macromolecular constituents from the extracts. The right angle light scattering detection gave a better Gaussian peak distribution in identifying PS and differentiating the extracts. Pullulan polysaccharide (Mw = 112 kDa) was used as comparable reference standard. The y-axis is the detector response (mV) while the x-axis represents the retention (hydrodynamic) volume (mL). The right angle light scattering detector identified the presence of polysaccharide in AQ and crude PS extracts but not in ALC extract, suggesting the absence of polysaccharide in the ALC extract.

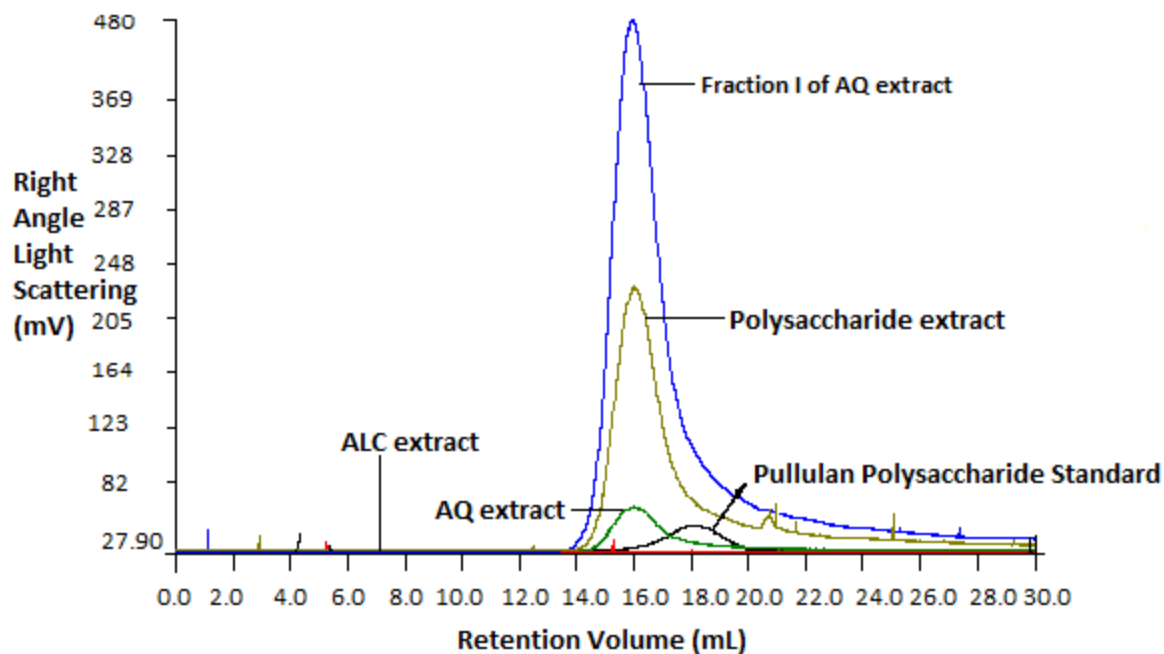


Figure 3.11 – Summarized identification of polysaccharides in Fraction I ($M_w = \sim 73$ kDa) of the AQ extract, PS extract, AQ extract and ALC extract of ginseng by size exclusion chromatography with right angle light scattering detection.

Each sample was injected and eluted with 0.05 M NaNO_3 mobile phase, and this was monitored with right angle light scattering detector at 1 mL/min flow rate. Pullulan polysaccharide was used as a reference standard. The y-axis is the detector response (mV) while the x-axis represents the retention (hydrodynamic) volume (mL). Right angle light scattering detector identified the presence of polysaccharide in Fraction I ($M_w = \sim 73$ kDa) of AQ extract, PS extract and AQ extract but not in ALC extract of ginseng, suggesting the absence of polysaccharide in ALC extract.

3.6 Discussion

The present study delineated the paradoxical immunomodulatory effect of ginseng and provided a basis for explaining the apparently contradictory reporting in the literature. The observed extract-specific immunostimulatory and immunosuppressive effects were described independently by a number of investigators who examined the activity of the aqueous [11-15] or alcoholic [16-21] extracts of ginseng. Moreover, there was a pattern of association of immunostimulation and immunosuppressive activities with aqueous [11-15] and alcoholic [16-21] extracts respectively, with the exception of a study showing an aqueous extract to possess immunosuppressive effect [22]. In light of the observed paradoxical effects and similarity in the yield (% extractive of 41.4 and 35.3) and potency of the AQ and ALC extracts (Figure 1 and 2), the extract-specific inhibitory and stimulatory effect on macrophage function reported in the present study is considered as the paradoxical immunomodulatory actions of ginseng. This concept was considered as an extension of the paradoxical actions of ginseng proposed by other investigators on angiogenesis [27] and cancer cell proliferation [28-29].

Findings on the macrophage-stimulating effect of American ginseng (Figure 3.1) have provided new information on the immunostimulatory property of ginseng in term of its cytokine specificity and dose dependency, which also reflected on the specific pharmacological basis of its biological activity. One study has also demonstrated that the same AQ extract stimulated inflammatory cytokines (IL-1 β , IL-6, TNF- α) as well as IL-10 response by human peripheral blood mononuclear cells (PBMC) [30]. Moreover, the changes reported were not due to LPS contamination of the extracts as documented by Limulus test and direct LPS assay.

While ginseng is generally considered as an immuno-booster or adaptogen [1], a recent study

reported that Rb₁ ginsenoside purified from an alcoholic ginseng extract induced an anti-arthritis effect in an animal model [31]. The present study also showed that the inhibitory effect of the ALC extract could be extended to the stimulation induced by the AQ extract, suggesting that both immunostimulatory and immunosuppressive components were present in ginseng root. Use of certain solvent systems may lead to an inactive extract. Although the magnitude of the inhibition on LPS-induced NO and IL-6 response by the ALC extract was quite significant, the suppressive effect was highly specific to the cytokine involved since TNF- α response was the least affected. Further studies are required to address the signaling pathways specificity of the ALC extract.

Many medicinal plants possess immunostimulatory activity and polysaccharides have been recognized as the primary bioactives giving this activity [26]. In this study, the relative abundance of PS (Fraction I) in the AQ extract (Figure 3.4) was well correlated with its immunostimulatory activity (Figure 3.5). This result supports the immunostimulatory effect that has been reported for CVT-E002TM (sold commercially as COLD-fX®), a natural health product of American ginseng polysaccharides [9-10, 32-36]. Plant bioactive polysaccharides were reported to have molecular weights ranging from 10 kDa to 150 kDa [9-10]. The estimated molecular weight of the ginseng immunostimulatory PS reported in this study was within the stated range. Figures 3.1 and 3.5 indicated that the total macrophage-stimulating activity of the AQ extract was not solely due to Fraction I and/or the PS fraction since the immunostimulatory effects of the AQ extract were more potent than those of the PS or Fraction I. It is possible that some of the bioactive material was lost during isolation or fractionation procedures. Figures 3.10 and 3.11 indicated that Fraction I (Mw = ~73 kDa) macromolecular component of ALC extract was not PS.

It has been suggested that ginsenosides may be involved in immunosuppression [16, 19-20], which is consistent with the immunoinhibitory effect of the ginsenoside fraction (<3 kDa) observed in Figures 3.7-3.9. However, this study showed in Figures 3.6, 3.7, 3.8 and 3.9 that the macromolecule and ginsenoside fractions of ALC extract possess immunoinhibitory effects. Rhule et al. (2006) reported that a combination of ginsenosides did not attenuate TNF- α production to the extent that was observed on exposure to the whole ginseng ALC extract [20], a similar outcome was obtained in this study. This suggests the presence of additional immunoinhibitory bioactives in the extract which was identified in this study to be the macromolecule fractions. They are not well known and are overlooked by many investigators who focus mainly on ginsenosides pharmacological effects. There is a paucity of data concerning the immunomodulatory effects of ginseng ALC extract macromolecule fractions. Results from this finding should provide new directions for researchers exploring anti-inflammatory agents in ginseng. The iNOS (inducible nitric oxide synthase), MAPK (mitogen-activated protein kinase) kinases such as p38, ERK1/2 (extracellular signal-regulated kinases 1/2), PI3K (phosphoinositide 3-kinase), and NF- κ B (nuclear factor kappa B) signaling pathways may mediate the stimulation and suppression of LPS induced NO, TNF- α and IL-6 production effects observed in ginseng AQ and ALC extracts respectively as studies using ginseng AQ [12, 37] and ALC [17] extracts has demonstrated such.

ALC extract suppressed AQ extract up-regulation of macrophage NO production, suggesting that consumption of both AQ and ALC extracts will lead to the loss of AQ extract immunostimulatory health benefit. Findings on the extract-specific immunomodulatory effect have significant implications in the safety, manufacturing, production, development and regulation of products based on ginseng extracts. It is unknown whether the use of organic

solvents or the extraction protocol may influence the potency and characteristics of the extracts of other ginseng species. It is imperative to carry out a systematic analysis of the physiochemical characteristics of various ginseng extracts to determine how these parameters may influence their immunomodulatory properties. The present study provides a lead for identifying immunobioactive constituents of ginseng.

3.7 Conclusion

ALC extract of American ginseng, its novel macromolecule (devoid of PS) and ginsenoside fractions are immunoinhibitory whereas the AQ extract and its PS phytochemical are immunostimulatory. These extract-related anti-inflammatory and pro-inflammatory effects may be considered as the paradoxical immunomodulatory actions of ginseng.

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Chapter 4

STIMULATION AND SUPPRESSION OF INNATE IMMUNE FUNCTION BY AMERICAN GINSENG POLYSACCHARIDES: BIOLOGICAL RELEVANCE AND IDENTIFICATION OF BIOACTIVES

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4.1 Introduction

The immune system consists of both innate and adaptive immunity components. Fast acting macrophage-mediated innate immunity is the first step in the recognition, destruction and removal of microbial pathogens in a host. Macrophages kill pathogens and cancer cells directly via phagocytosis and indirectly via the production of various proinflammatory mediators (e.g. nitric oxide [NO]) and cytokines (e.g. tumor necrosis factor-alpha [TNF- α]) [1]. During microbial infection caused by Gram negative bacteria, lipopolysaccharide (LPS) endotoxin stimulates innate macrophage cells by binding to their Toll-like receptor 4 (TLR-4), and this induces down-stream intracellular events, activates kinases and transcription factors leading to production of proinflammatory mediators [2].

However, excessive macrophage stimulation by LPS during Gram negative bacteria infection generates uncontrolled production of proinflammatory mediators evoking harmful inflammatory response such as endotoxemia. Endotoxemia is characterized by fever, hypotension, myocardial dysfunction, acute respiratory failure, and multiple organ failure. Currently, antibiotics are used in the prophylaxis and therapy of endotoxemia [2-6]. The ability to detoxify or neutralize LPS immunotoxicity is a desired requirement for future bioactive compounds against endotoxemia [7, 8]. Considering the problematic rise of microbial infections and their resistance to synthetic antimicrobial agents and naturally derived antibiotics, a key immunotherapeutic strategy to address this challenge will be to identify immunomodulatory agents which can interact with host immune response defense in such a way as to neutralize or inhibit LPS from triggering excess macrophage production of proinflammatory mediators [9]. The innate immunity (e.g. macrophage function) of a host which is responsive to LPS is also a known target for plant polysaccharides which are biological response modifiers that can up-regulate or down-regulate

macrophage production of proinflammatory mediators [9-10]. Macrophage activation by plant polysaccharides is mediated via their binding to macrophage TLR-4 during initial phase of the immune response. Activation of this receptor leads to intracellular signaling cascades, resulting in transcriptional factor activation and the production of proinflammatory mediators which kills pathogenic microorganisms [9]. Interestingly, polysaccharides from medicinal plants such as *Tripterygium wilfordii* have been reported to suppress LPS-induced macrophage stimulation [11]. The use of plant polysaccharides as a new approach for the prophylaxis of LPS mediated diseases like endotoxemia may be dependent on their ability to inhibit LPS signaling pathways, which will lead to the down-regulation of macrophage production of proinflammatory mediators.

Ginseng root is a multi-action herb, with ginsenosides and polysaccharides being the main bioactive components [12-13]. Previously, ginsenosides were considered to be responsible for most of ginseng's pharmacological effects; however, recent studies indicate that ginseng polysaccharides (PS) also possess a wide range of biological activities. These include immunomodulation, anti-tumor, anti-oxidation, anti-depression, and hypoglycemia [14-18]. Water soluble PS that can be extracted from ginseng root constitute about 10% of its dry weight. These hydrophilic macromolecules are formed from complex chains of monosaccharides, such as L-arabinose, D-galactose, L-rhamnose, D-galacturonic acid, D-glucuronic acid and D-galactosyl residues linked together through glycosidic bonds [19-21]. The monosaccharide composition of American ginseng root polysaccharides (AGRPS) used in this study have been reported to contain glucose, galactose, arabinose, rhamnose and galacturonic acid [22], which is similar to that described previously [20, 23, 24]. Ginseng PS are known to exist as either acidic or neutral forms, and are believed to be heterogeneous in nature, with molecular weights ranging from 3.5 to 2000 kDa [15, 20-21, 23, 25-27]. Ginseng root polysaccharides (GRPS) are generally

known for their immunostimulatory effects [28, 24, 29-31], although recent studies suggests that they also suppress induced proinflammatory responses. Zhao et al. (2011) reported GRPS inhibited immunological response associated with collagen-induced arthritis [32]. Intravenous treatment of mice with GRPS was reported to exert a protective effect against *Staphylococcus aureus*-infected septic mice by suppressing early acute inflammation [33- 34]. One recent study has also revealed that intranasal and intravenous administered GRPS showed a protective effect on influenza viral infection by lowering levels of inflammatory cytokine (IL-6) and lung viral titers [35]. The examination of GRPS immunopharmacological effect under LPS endotoxic condition will shed light if GRPS can exhibit a beneficial prophylactic effect against LPS mediated immune and inflammatory disease conditions like endotoxemia. It's possible that GRPS may inactivate signaling molecules which may account for its ability to suppress an induced proinflammatory immune response.

In view of the reported paradoxical immunomodulatory effects of ginseng polysaccharides, investigation was performed to determine the effects of AGRPS extract on basal immune function, and whether or not this treatment will also suppress LPS immunologic response *ex vivo* and *in vivo*. Physicochemical properties such as water solubility, ionic charge and molecular weight, are known to affect the biological activity of polysaccharides, including immunomodulation [36-38]. An *in vitro* mechanistic study was used to identify the bioactive fractions responsible for AGRPS immunobioactivities. For the *in vitro* mechanistic study, AGRPS extract was separated into ionic charge (acidic and neutral) and molecular weight sub-fractions using ion exchange chromatography and ultrafiltration respectively. The different fractions of AGRPS extract was subsequently examined for their immunobioactivities.

4.2 Materials

4.2.1 Ginseng test materials. Four-year-old American ginseng (*Panax quinquefolius*) roots collected in 2007 from five different farms in Ontario, Canada were provided by the Ontario Ginseng Growers Association [31].

4.2.2 Chemicals and biologicals

Superdex G-200 was purchased from GE Healthcare Bio-Sciences AB (Sweden). Amicon ultra-15 centrifugal filter units with molecular weight cut-off pore size of 100 KDa, 50 KDa, 30 KDa and 10 KDa were purchased from Millipore (USA). Cellu Sep H1® dialysis bags with a molecular weight cut-off pore size of 2 KDa were purchased from Membrane Filtration Products, Inc. (USA). The diethylaminoethyl (DEAE)-Cellulose was purchased from Sigma (Oakville, Ontario). All other chemicals were of analytical grade and used as received. Cell culture medium and reagents were purchased from Gibco laboratories (USA). BD OptEIA ELISA kits tumour necrosis factor- α and interleukin-6 (BD Biosciences, USA). Purified LPS from *Escherichia coli* serotype 0111:B4 and Griess reagent were purchased from Sigma-Aldrich (USA). The RAW 264.7 (ATCC TIB 67) murine macrophage cell lines were provided by Dr. Jeff Dixon (Department of Physiology and Pharmacology, University of Western Ontario, Canada).

4.3 Animals

Adult male Sprague-Dawley rats weighing 200–250g (purchased from Charles River, St. Constant, QC, Canada) were used. The Animal Ethics Review Committee of the University of Western Ontario approved the study (Protocol No: 2009-070).

4.4 Methods

4.4.1 Preparation of Aqueous and AGRPS Extracts

Dried ginseng root samples were shipped to Naturex (USA) for extraction. Samples were ground between ¼ and ½ inch and used to produce the aqueous (AQ) extract. Briefly, 4kg ground ginseng roots were soaked three times during five hours in 16 L of water solution at 40°C. After extraction, the solution was filtered at room temperature. The excess solvent was then removed by a rotary evaporator under vacuum at 45°C. The three pools were combined and concentrated again until the total solids on a dry basis were around 60%. The Central Laboratory of Ontario Ginseng Research & Innovation Consortium (OGRIC) lyophilized these concentrates with a freeze dryer (Labconco, USA) at -50°C under reduced pressure to produce AQ ginseng extract in powder form. Yield of the powder extract from the concentrates was about 66%. The yield of the final extract (mean ± standard deviation of % extractive) from the initial ground root was 41.74±4.92 for the AQ extract.

A solution of AQ extract in distilled water (1g/10mL) was prepared, and the crude AGRPS was precipitated by the addition of four volumes of 95% ethanol. The mixture was left for 1 hr at room temperature for precipitation to occur. The crude AGRPS was collected by centrifugation at 350×g (Beckman Model TJ-6, USA) for 10 minutes and lyophilized to produce crude AGRPS fraction with a yield of about 10 %. The crude AGRPS (10 g) was re-dissolved in 300 mL of water and partitioned five times with Sevag reagent (1:4 n-butanol:chloroform, v/v, 100 mL each) to remove proteins and produce AGRPS [39]. AGRPS was precipitated again by ethanol and lyophilized to produce a light brownish AGRPS extract which was used for the pharmacological and phytochemical studies.

4.4.2 *Ex vivo* and *In vivo* Pharmacological Evaluation

4.4.2.1 *Ex vivo* study to evaluate immunostimulation and suppression of LPS response

Adult male rats (n = 5 per group) were randomly divided into both a sham control and treatment groups. The treatment group received 125 mg/kg of AGRPS or AQ extract dissolved in saline by oral gavage once daily for 3 or 6 consecutive days. The sham control group received saline orally. Animals were anesthetized with intramuscular injection of ketamine 80 mg/kg and xylazine 5 mg/kg and the trachea was cannulated for lung BAL with Dulbecco's phosphate-buffered saline (PBS) at 37°C to collect alveolar macrophages which were then cultured without or with LPS for 24hr and maintained at 37°C in a humidified incubator with 5% CO₂. The *ex vivo* alveolar macrophage NO production from each animal was determined in triplicates. All experiments were done only once.

4.4.2.2 *In vivo* study to evaluate immunostimulation and suppression of LPS response

The rats (n = 5 per group) were given a single daily dose of 125 mg/kg AGRPS or AQ extract in saline by oral gavage for 6 consecutive days. The sham control group (n = 5) received saline orally. Animals were anesthetized with intramuscular injection of ketamine 80 mg/kg and xylazine 5 mg/kg and blood was collected into heparinized tubes by intracardiac puncture, samples were then immediately centrifuged at 3500×g (Beckman Model TJ-6, USA) for 10 minutes and the plasma was separated, aliquoted and stored at -20 °C until use. Plasma TNF- α cytokine levels were measured as a marker for immunostimulation. To evaluate suppression of LPS induced immunological response, rats (10 treated with ginseng and 5 which received saline) were challenged with 5 mg/kg LPS intraperitoneal (IP) injection 24 hr after the last dose of ginseng or saline. Animals were examined 2hr later based on studies revealing that systemic

TNF- α cytokine production peaks 2hr after a LPS challenge [40-42]. The plasma TNF- α level in each animal was determined in triplicate. All experiments were done only once. This immunosuppression model was validated in past studies by other investigators to evaluate the immunomodulatory effects of orally administered pharmacological agents [43, 44].

4.4.2.3 Quantification of NO and TNF- α

TNF- α in culture medium and plasma was measured by ELISA using rat cytokine-specific BD OptEIA enzyme-linked immunosorbent assay (ELISA) kits (BD Biosciences, USA) according to the manufacturer's protocol. Nitrite accumulation in the culture media was measured as an estimate of NO production using Griess reagent (Sigma-Aldrich, USA) as previously described in chapter three.

4.4.2.4 Lung Histochemical Study

Immediately after the collection of blood samples from the anesthetized rats used in the *in vivo* study, the lung samples of the right and left lobes from each rat were removed following animal sacrifice by thoracotomy. The lung samples (four from both lobes) were fixed with 10% formaldehyde solution at room temperature. The lung tissues were embedded in paraffin and cut into 5 mm sections and then stained with hematoxylin & eosin (H&E) by the London Laboratory Services Group of London Health Sciences Centre. Images of histopathologic changes in the lung tissues were observed under a light microscope.

4.4.3 Ion exchange Chromatography of AGRPS

AGRPS extract (3 g) was dissolved in water (200 mL) and loaded on a DEAE-Cellulose column (2.5 X 40 cm) pre-equilibrated with distilled water. The column was eluted first with 1.0 L of

distilled water at a flow rate of 1 mL/min to obtain the unbound or neutral fraction and then with 1.0 L of 0.5 M sodium chloride (NaCl) to obtain the bound or acidic fraction. Absorbance of the eluates was monitored at 230 nm (wave length of maximum absorption for AGRPS determined from a UV scan) and 5 mL fractions were collected. The fractions were concentrated, dialyzed with Cellu Sep H1® dialysis bags (cut off pore size of 2 KDa) against water and freeze dried to give 0.9 g (30 % yield) of the neutral PS fraction and 0.36 g (12% yield) of the acidic PS fraction.

4.4.4 Size Exclusion Chromatography via Superdex G-200 Fractionation of Acidic PS

Five hundred milligrams of acid PS was dissolved in 5 mL distilled water and then fractionated by loading onto a Superdex G-200 column (40×2.5 cm) equilibrated and eluted with distilled water mobile phase at 4°C with a flow rate of 1 mL/min. Absorbance of the eluates was monitored at 230 nm and 5 mL fractions were collected.

4.4.5 Fractionation of Acidic PS via Ultrafiltration

Acidic PS (20 mg) was dissolved in 20 mL of water and 15 mL was placed in a 100 kDa amicon ultra-15 centrifugal filter unit (15mL capacity) and centrifuged at 3500×g (Beckman Model TJ-6, USA) for 30 minutes. The fraction with molecular weight of 100 kDa and above (≥ 100 kDa) were retained on the filter unit, while the fraction of less than 100 kDa in the filtrate obtained from this process was then placed in a 50 kDa amicon ultra-15 centrifugal filter unit and centrifuged as indicated above. The fraction with molecular weight of less than 100 and above 50 kDa (50 - 100 kDa) were retained on the filter unit, while the fraction of less than 50 kDa in the filtrate obtained from this process was then placed in a 30 kDa amicon ultra-15 centrifugal filter unit and centrifuged as indicated above. The fraction with molecular weight of less than 50 and

above 30 kDa (30 - 50 kDa) were retained on the filter unit, while the fraction of less than 30 kDa in the filtrate obtained from the above mentioned process was then placed in a 10 kDa amicon ultra-15 centrifugal filter unit and centrifuged as indicated above. The fraction with molecular weight of less than 30 and above 10 kDa (10 - 30 kDa) was retained on the filter unit. Ten mL of distilled water was used to wash and remove the acidic PS fractions with different molecular weights ≥ 100 kDa, 50 - 100 kDa, 30 - 50 kDa, 10 - 30 kDa retained on the filter units after which they were lyophilized at -50°C under reduced pressure (Labconco, USA).

4.4.6 *In vitro* Pharmacological Evaluation

4.4.6.1 Cell culture

Mouse macrophage cell line RAW 264.7 (grown to 60-80% confluence, and subjected to no more than 20 cell passages from Dr. Jeff Dixon) was cultured in RPMI-1640 medium supplemented with 10% Fetal Bovine Serum, 25 mM HEPES, 2 mM Glutamine, 100 IU/ml penicillin and 100 $\mu\text{g/ml}$ streptomycin. Cells were seeded in 96-well tissue culture plates at a density of 2×10^5 cells per well and maintained at 37°C in a humidified incubator with 5% CO_2 . To collect rat alveolar macrophages animals were anesthetized with intramuscular injection of ketamine 80 mg/kg and xylazine 5 mg/kg and the trachea was cannulated for lung bronchoalveolar lavage (BAL) using 10-mL syringe with three 10ml washes of PBS at 37°C . Fluid recovered from BAL was then centrifuged at 1000 rpm for 5 minutes. Cells were immediately cultured in RPMI-1640 medium supplemented with 10% Fetal Bovine Serum (FBS), 25 mM HEPES, 2 mM Glutamine, 100 IU/mL penicillin and 100 $\mu\text{g/ml}$ streptomycin in 96-well tissue culture plates, at a density of 2×10^5 cells per well at 37°C maintained in a humidified incubator with 5% CO_2 .

4.4.6.2 Cell treatment

4.4.6.2.1 Immunostimulatory effect

Macrophages were treated with 0, 5, 10, 20, 50, and 100 µg/mL (RAW 264.7 cell line) or 0, 50, 100 or 200 µg/mL (rat alveolar macrophages) of AGRPS and AQ extracts, acidic and neutral PS fractions for 24 hr, maintained at 37°C in a humidified incubator with 5% CO₂. Aliquots of culture medium were collected and frozen at -20°C until ready for NO and TNF-α analysis. The experiments were performed in triplicate on three independent occasions.

4.4.6.2.2 Indirect Suppression of LPS immunological response

To examine the indirect inhibitory effect on LPS stimulation *in vitro*, macrophages were pretreated with 0, 5, 10, 20, 50 and 100 µg/mL (RAW 264.7 cell line) or 0, 50, 100 or 200 µg/mL (rat alveolar macrophages) of AGRPS and AQ extracts, acidic and neutral PS fractions for 24hr and maintained at 37°C in a humidified incubator with 5% CO₂. Thereafter macrophages were washed twice with culture medium and then treated with 1µg/mL of LPS to determine the 24 hour cytokine response. The experiments were performed in triplicates on three independent occasions.

4.5 Statistical analysis

All statistical analyses were performed with GraphPad prism 4.0a Software (GraphPad Software Inc., USA). Data were presented as the mean ± standard deviation (SD) for all experiments. Data sets were evaluated by one-way analysis of variance (ANOVA) with Dunnett's post-hoc test. P<0.05 was considered to be statistically significant.

4.6 Results

4.6.1 *Ex vivo* Immunomodulatory Effects of AGRPS and AQ extracts

Oral treatment of rats with AGRPS and AQ extracts for 3 and 6 days produced stimulation of 24 hr *ex vivo* cultured alveolar macrophages (Figures 4.1A and 4.2A) which are higher than sham control as determined by increased production of nitrite, a marker for NO. Only the 6 days AGRPS extract treatment production of NO reached 50% of the LPS positive control (Figure 4.1A), while similar magnitude of response was observed in both the 3 and 6 days AQ extract treatments (Figure 4.2A). The 6 days treatment of AGRPS extract gave two times higher NO production compared to that of the 3 days AGRPS extract treatment. Hence AGRPS extract immunostimulatory response is time-dependent while that of AQ extract is not.

The responsiveness of macrophages collected from the 6 days AGRPS and AQ extract treated animals to LPS stimulation *ex vivo* showed about 50% reduction in NO production as compared to the non-ginseng treated controls (Figures 4.1B and 4.2B). The 3 days AGRPS and AQ extract treatments did not suppress LPS immunological response. Hence AGRPS and AQ extracts suppression of LPS induced immunological response is time-dependent.

Macrophage stimulation is not a requirement to induce suppression of LPS since the 3 days AGRPS and AQ extract treatments was found to exert immunostimulation but did not suppress the LPS response as observed in the 6 days treatment. The 6 days AGRPS and AQ extract treatments exerted both immunostimulation and loss of LPS responsiveness. These data show that orally administered AGRPS and AQ extract has both immunostimulatory and suppression of LPS induced immunological effects.

AGRPS extract

(A) *Ex vivo* Immunostimulatory Effect (B) *Ex vivo* Suppression of LPS immunological Effect

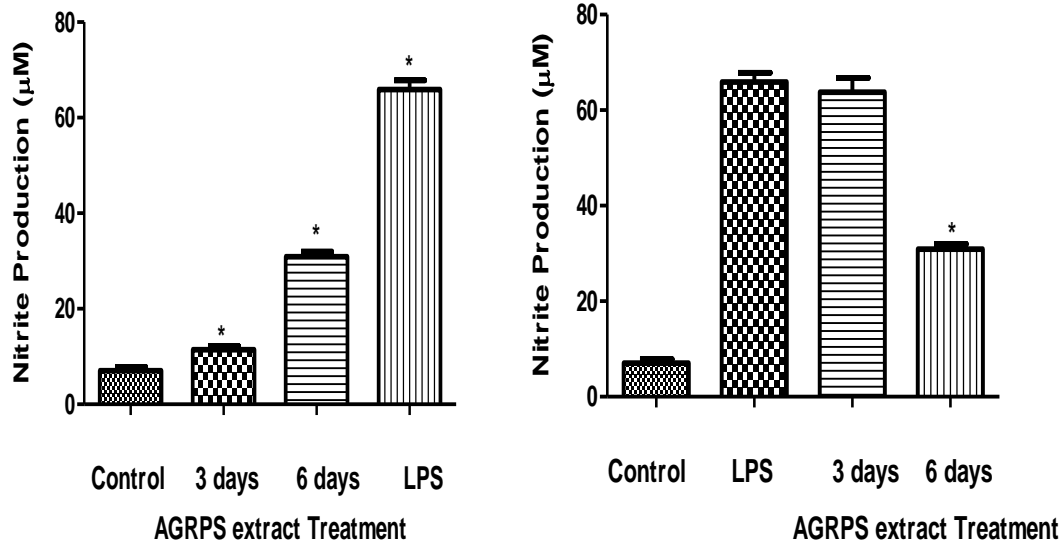


Figure 4.1 - Orally administered AGRPS extract: (A) elevated NO production and (B) reduced LPS-stimulated NO production *ex vivo* in cultured alveolar macrophages. Alveolar macrophages of rats ($n = 5$ per group) treated orally with saline (sham control) or 125 mg/kg AGRPS extract for 3 and 6 days were cultured for 24 hr to measure production of NO (quantified as nitrite production). To determine responsiveness to LPS stimulation, ginseng treated macrophages were exposed to 1ug/ml LPS in culture to determine changes in 24 hr NO production. The *ex vivo* alveolar macrophage NO production from each animal was determined in triplicate and the data were shown as mean \pm SD. Datasets were evaluated by ANOVA. * Values $P < 0.05$ compared to the untreated control were statistically significant.

AQ extract

(A) *Ex vivo* Immunostimulatory Effect

(B) *Ex vivo* Suppression of LPS immunological Effect

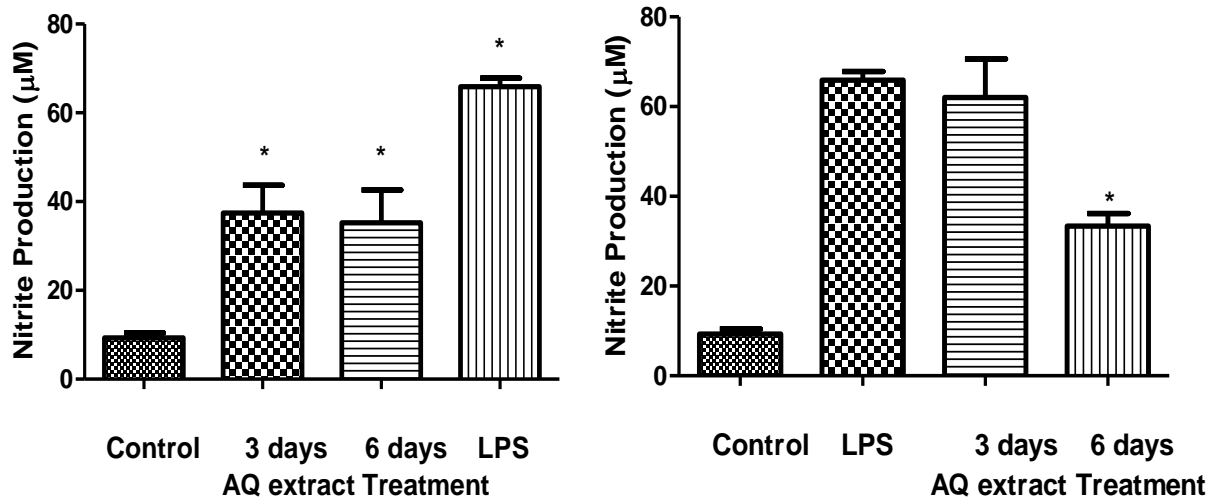


Figure 4.2 - Orally administered AQ extract: (A) elevated NO production and (B) reduced LPS-stimulated NO production *ex vivo* in cultured alveolar macrophages. Alveolar macrophages of rats ($n = 5$ per group) treated orally with saline (sham control) or 125 mg/kg AQ extract for 3 and 6 days were cultured for 24 hr to measure production of NO (quantified as nitrite production). To determine responsiveness to LPS stimulation, ginseng treated macrophages were exposed to 1 $\mu\text{g/ml}$ LPS in culture to determine changes in 24 hr NO production. The *ex vivo* alveolar macrophage NO production from each animal was determined in triplicate and the data were shown as mean \pm SD. Datasets were evaluated by ANOVA. * Values $P < 0.05$ compared to the untreated control were statistically significant.

4.6.2 *In vivo* Immunomodulatory Effect of AGRPS and AQ extracts

In light of the observed AGRPS and AQ extracts *ex vivo* effects on stimulation and suppression of LPS induced stimulation of alveolar macrophage immune function, additional studies were carried out *in vivo* to determine the pharmacological relevance in whole animal response. The 6 days treatment with AGRPS and AQ extracts elevated plasma TNF- α levels compared to the untreated control (Figure 4.3A), suggesting AGRPS extract was more effective. The observed *in vivo* immunostimulatory effects of AGRPS and AQ extracts were very small when compared with that of 5mg/kg LPS. The LPS treatment resulted in marked elevation of plasma TNF, while pretreatment with AGRPS and AQ extracts suppressed LPS induced plasma TNF- α level by 78% and 38% respectively (Figure 4.3B).

To determine whether the suppression of LPS-induced plasma TNF- α level by ginseng extracts has any impact on organ dysfunction in LPS-treated animals, lung histology was evaluated. As shown in Figure 4.4, 2hrs LPS challenge (5mg/kg) did not cause lung morphological and histopathological damages as demonstrated by the absence of neutrophils infiltration from the interalveolar septa into the alveolar space and the absence of alveolar hemorrhage. The 6 days oral AGRPS and AQ extract pretreatments prior to LPS exposure have similar morphology as the LPS challenge and sham control (Figure 4.5). There is dissociation between the changes of plasma TNF- α cytokine production and the lung response.

(A) *In vivo* Immunostimulatory Effect (B) *In vivo* Suppression of LPS immunological Effect

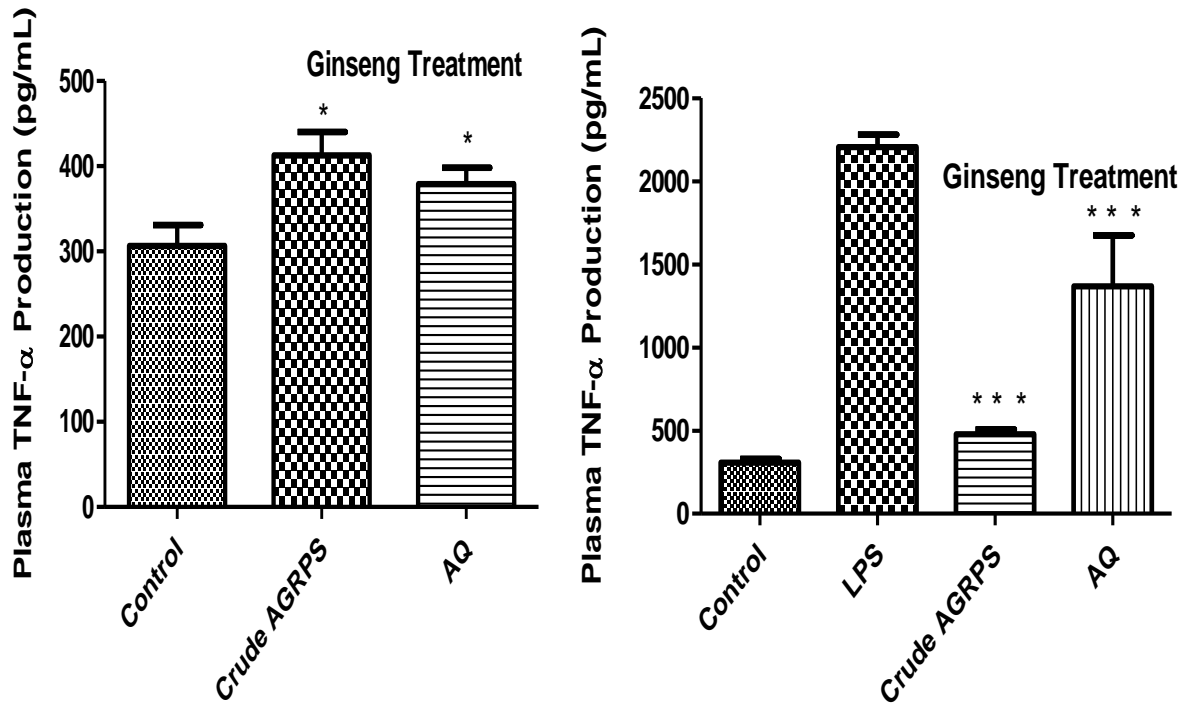
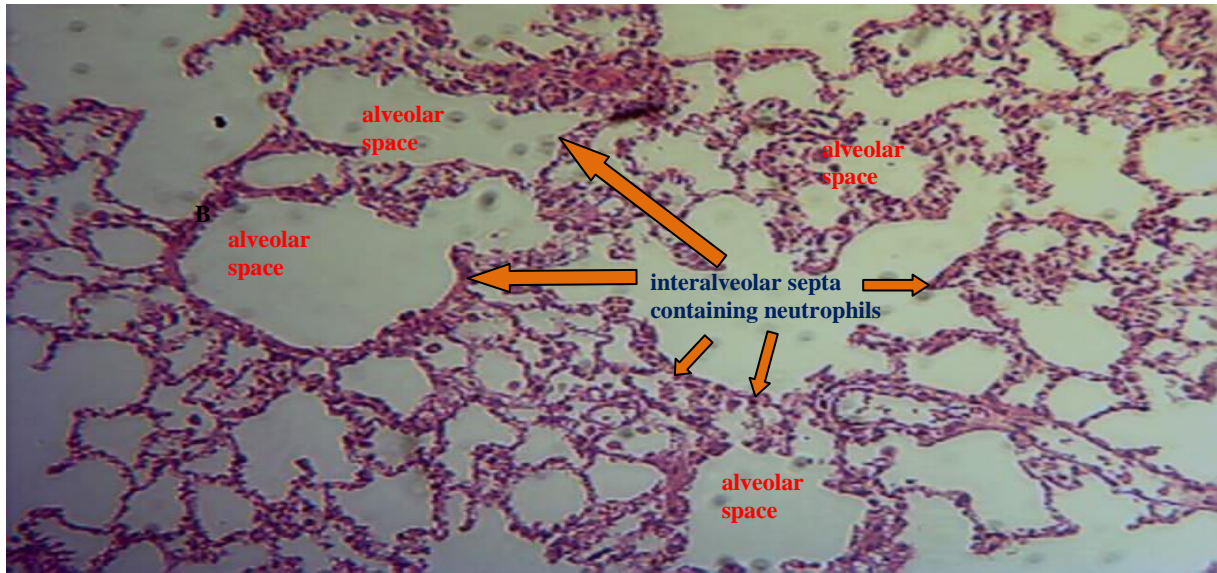


Figure 4.3 - Immunostimulatory and suppression of LPS induced immunological effects of AGRPS or AQ extract treatment *in vivo*. To evaluate immunostimulation, the rats ($n = 5$ per group) were treated orally with saline (sham control) or 125mg/kg AGRPS extract or 125mg/kg AQ extract for 6 days. To evaluate suppression of LPS, the 6 days AGRPS and AQ extract treated rats were challenged for 2hr with 5mg/kg LPS (IP) injection 24 hr after the last dose of saline or AGRPS extract or AQ extract. Plasma TNF- α cytokine concentrations were determined by ELISA. The plasma TNF- α level in each animal was determined in triplicate and the data were shown as mean \pm SD. Datasets were evaluated by ANOVA. * Values $P < 0.05$ compared to the untreated control (immunostimulatory effect) were statistically significant. *** Values $P < 0.05$ compared to the LPS positive control.

Control Group



LPS Group

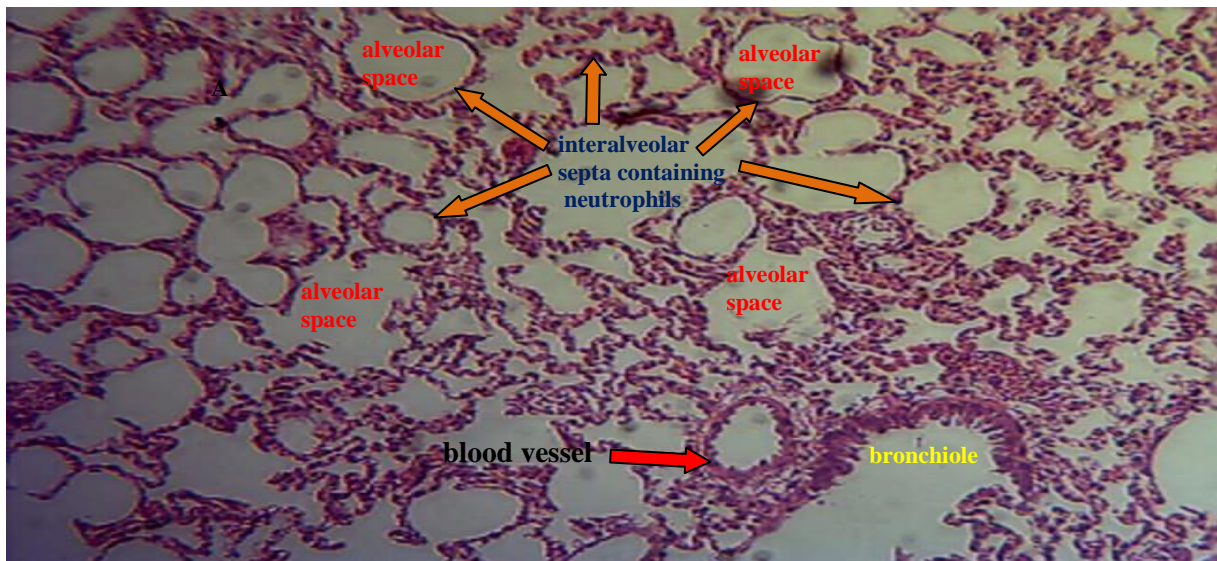
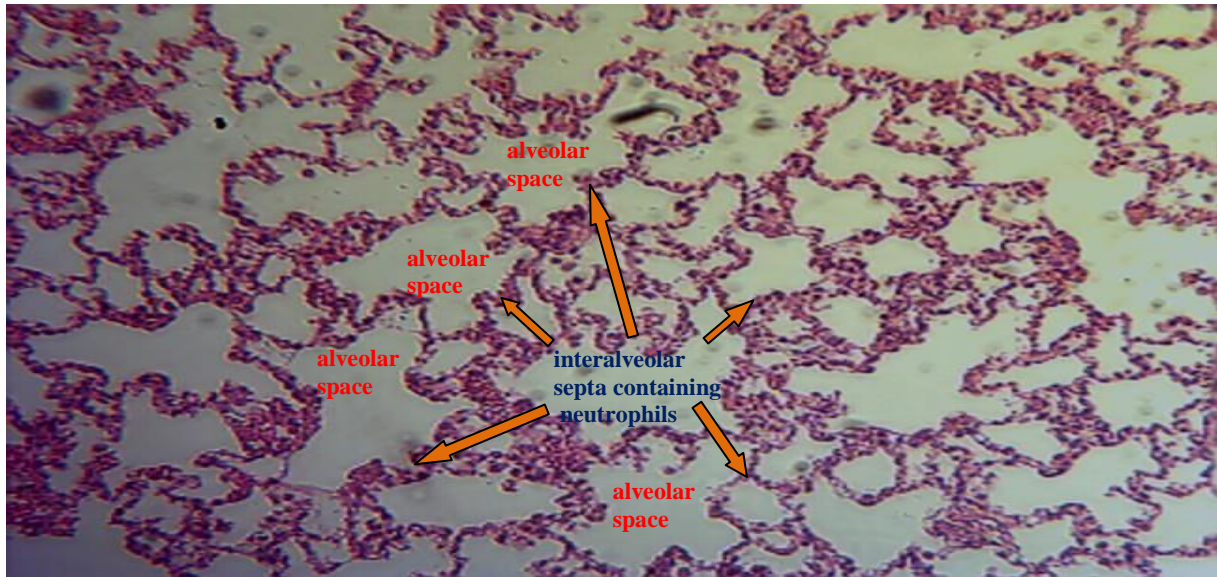


Figure 4.4 – Lung histochemical study of Saline (Control) and LPS treated rats (magnification x40). The rats (n = 5 per group) received saline orally or 5mg/kg LPS (IP) injection. Lungs were processed for histochemical study at the end of 2hrs right after LPS challenge. The diagrams show the alveolar space surrounded by the interalveolar septa containing neutrophils are indicated by the arrows.

LPS + AGRPS Extract Group



LPS + AQ Extract Group

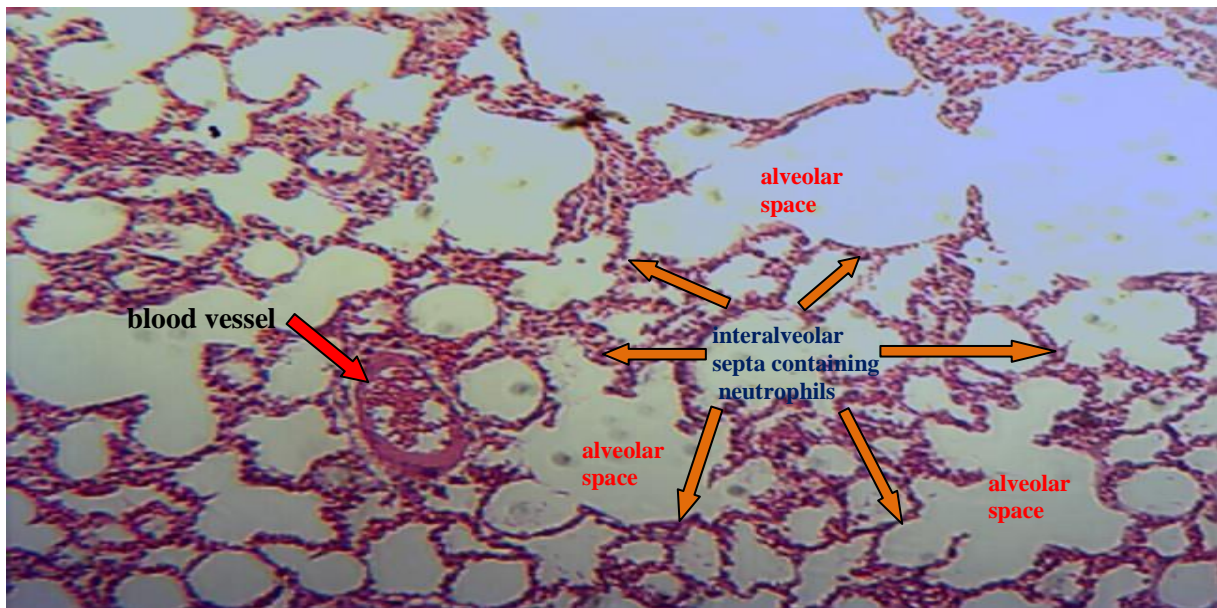


Figure 4.5 - Lung histochemical study of AGRPS and AQ extract treated rats after LPS Challenge (magnification x40). The rats (n = 5 per group) were orally administered with AGRPS or AQ extracts (125mg/kg) prior to 5mg/kg LPS (IP) injection. Lungs were processed for histochemical study at the end of 2hrs right after LPS challenge. The diagrams show the alveolar space surrounded by the interalveolar septa containing neutrophils are indicated by the arrows.

4.6.3 Mechanistic Identification of immunobioactive components of AGRPS

To identify the immunobioactive fractions of AGRPS extract, the extract was fractionated into acidic and neutral species by ion exchange chromatography, which was then fractionated further according to molecular size by size exclusion chromatography via Superdex G-200 fractionation and ultrafiltration fractionation.

4.6.3.1 Ion Exchange Chromatography of AGRPS

Ion exchange chromatography was used to separate AGRPS extract into both acidic (12.0%) and neutral fractions (30.0%). The chromatogram from the ion exchange chromatographic procedure showed the presence of two peaks. The first peak which eluted with the distilled water mobile phase corresponds to the neutral fraction, while the second peak which eluted with the 0.5M NaCl mobile phase corresponds to the acidic fraction. After lyophilization, the resulting neutral fraction was a white cottony substance while the acidic fraction was a light brownish substance similar to AGRPS extract. About 58.0% of the AGRPS materials were not recovered from the chromatographic procedure.

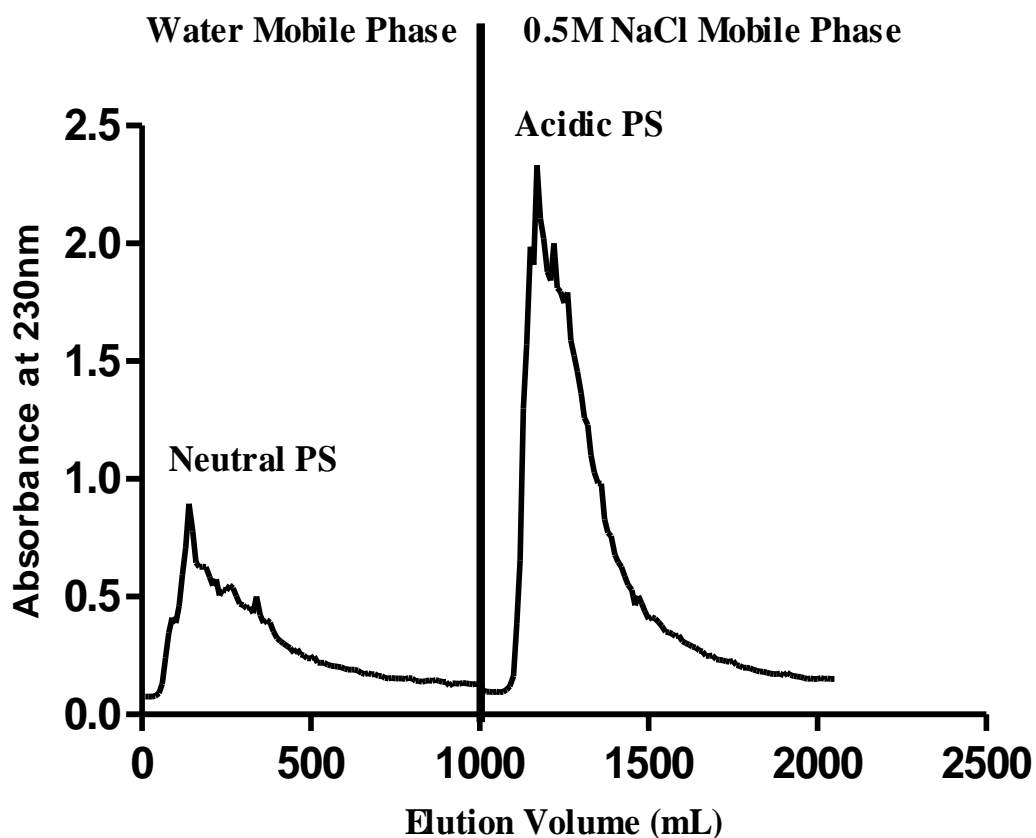


Figure 4.6 – DEAE-cellulose Ion exchange Column (40 × 2.5 cm) chromatographic fractionation of the AGRPS into neutral PS fraction and acidic PS fraction. The column was loaded with 3g of AGRPS extract, and then eluted sequentially with 1000mL distilled water, followed by 1000mL 0.5M NaCl at flow rate of 1mL/min to obtain the neutral and acidic PS fractions respectively. The y-axis is the absorbance at 230 nm while the x-axis represents the elution volume (mL).

4.6.3.2 Size Exclusion Chromatography of Acidic PS

To perform activity guided fractionation of acidic PS, size exclusion chromatography with the aid of a Superdex G-200 column was then used as an analytical technique to examine in more detail the acid PS component. As shown in Figure 4.7, the acidic PS constitutes phytochemicals of different molecular weights with peaks which were poorly separated by the Superdex G-200 column chromatographic technique. Ultrafiltration with molecular weight cut-off of 100 kDa, 50 kDa, 30 kDa and 10 kDa was used as an alternative tool to separate the acid PS into 4 major fractions of different molecular weights (≥ 100 kDa, 50 - 100 kDa, 30 - 50 kDa, 10 - 30 kDa). The immunobioactivities of these fractions were then evaluated.

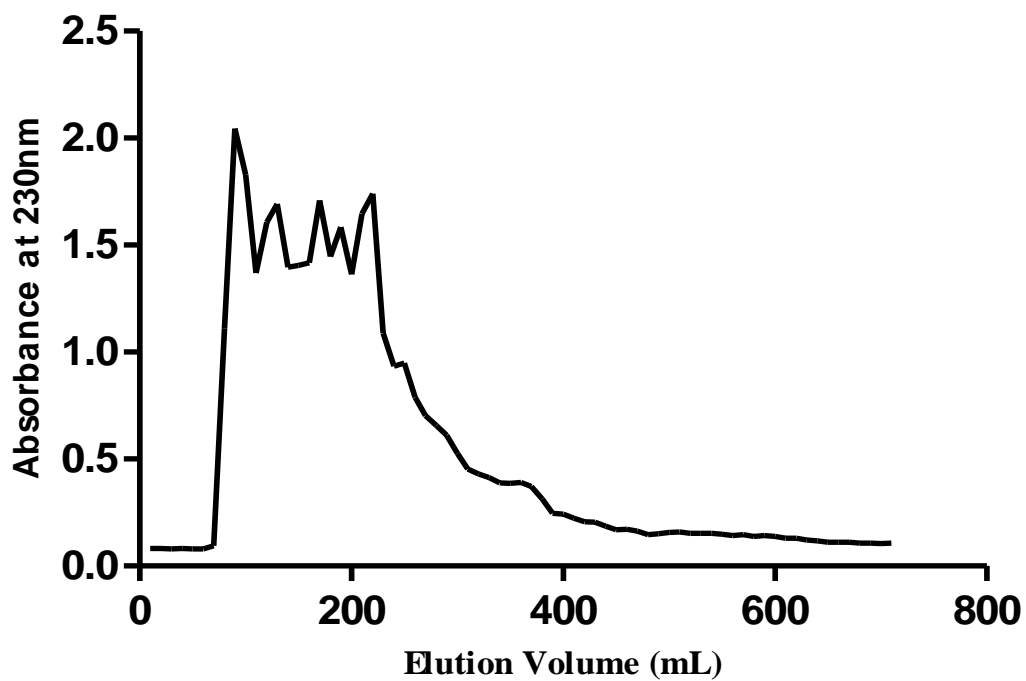


Figure 4.7 – Superdex G-200 size exclusion column (40 × 2.5 cm) chromatographic fractionation of acid PS. The column was loaded with 500mg of acid PS, and then eluted with 700mL distilled water at flow rate of 1mL/min. The y-axis is the absorbance at 230 nm while the x-axis represents the elution volume (mL).

4.6.3.3 *In vitro* immunostimulatory effect of AGRPS and AQ extracts, Acidic and Neutral fractions isolated from AGRPS

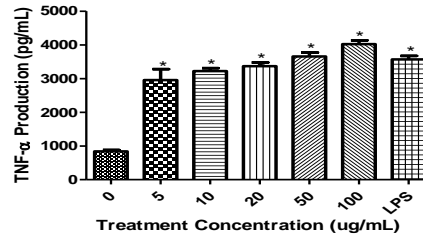
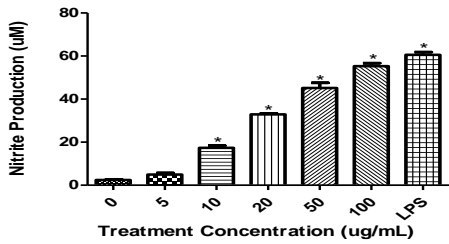
The *in vitro* mechanistic study revealed that AGRPS extract, AQ extract and acidic PS significantly up-regulated murine cell line and rat alveolar macrophage production of NO (nitrite) and TNF- α compared to the untreated control in a dose-dependent manner. Figures 4.8 and 4.9 showed the influence of ionic charge (acidic PS and neutral PS fractions) on AGRPS modulation of macrophage function *in vitro*. The immunostimulatory effects of AGRPS extract, AQ extract and acidic PS show a biphasic dose-response relationship, exhibited by a marked increase in effect at the low dose range. The mechanistic study also indicated that neutral PS did not stimulate production of NO, but showed some significant stimulation of TNF- α production. Figure 4.10 shows the interaction between acidic PS and neutral PS using the composite mixture that simulates the 12% acidic PS fraction and 30% neutral PS yield (ratio 1:2.5 of acidic PS: neutral PS) obtained from the ion exchange chromatographic procedure. This investigation revealed that neutral PS enhances the immunostimulatory effect of acidic PS.

Figure 4.8 - Immunostimulatory effects of AGRPS and AQ extracts, acidic and neutral PS fractions on 24 hours murine macrophage production of (A) NO and (B) TNF- α *in vitro*

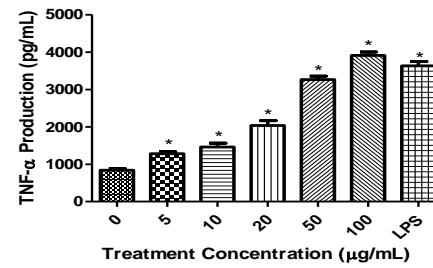
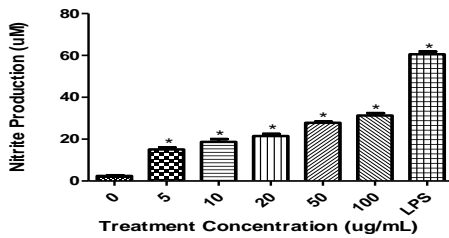
(A) NO Production

(B) TNF- α Production

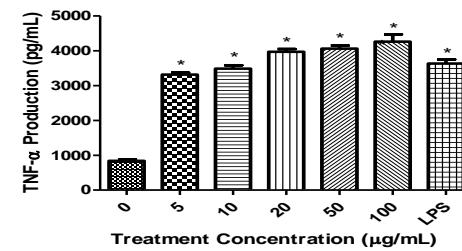
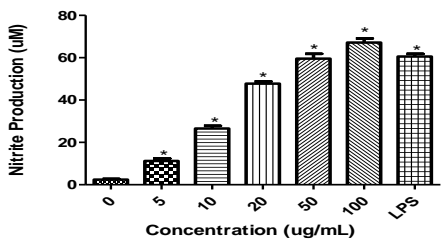
(i) AGRPS extract



(ii) AQ extract



(iii) Acidic PS fraction



(iv) Neutral PS fraction

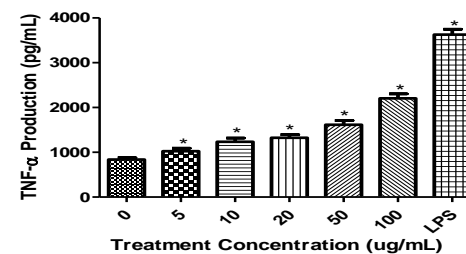
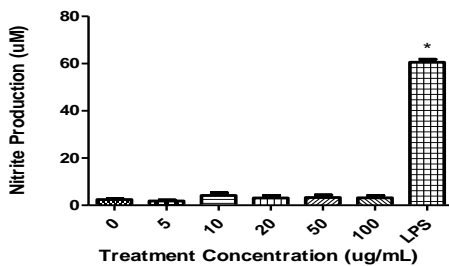


Figure 4.8 - Immunostimulatory effects of AGRPS and AQ extracts, acidic and neutral PS fractions on 24 hours murine macrophage production of (A) NO and (B) TNF- α *in vitro*. Murine macrophages (RAW 264.7 cell line) were treated with 0, 5, 10, 20, 50, 100 $\mu\text{g}/\text{mL}$ of AGRPS and AQ extracts, acidic and neutral PS fractions for 24 hours. The culture supernatants were analyzed for NO and TNF- α by Griess reaction assay and ELISA, respectively. Cells treated with LPS (1 $\mu\text{g}/\text{mL}$) were used as positive controls. The experiments were performed in triplicate at three independent times and the data were shown as mean \pm SD. Datasets were evaluated by ANOVA. * Values $P < 0.05$ compared to the untreated (vehicle) control were statistically significant.

Figure 4.9 - Immunostimulatory effects of AGRPS and AQ extracts, acidic and neutral PS fractions on 24 hours rat alveolar macrophage production of (A) NO (nitrite) and (B) TNF- α *in vitro*

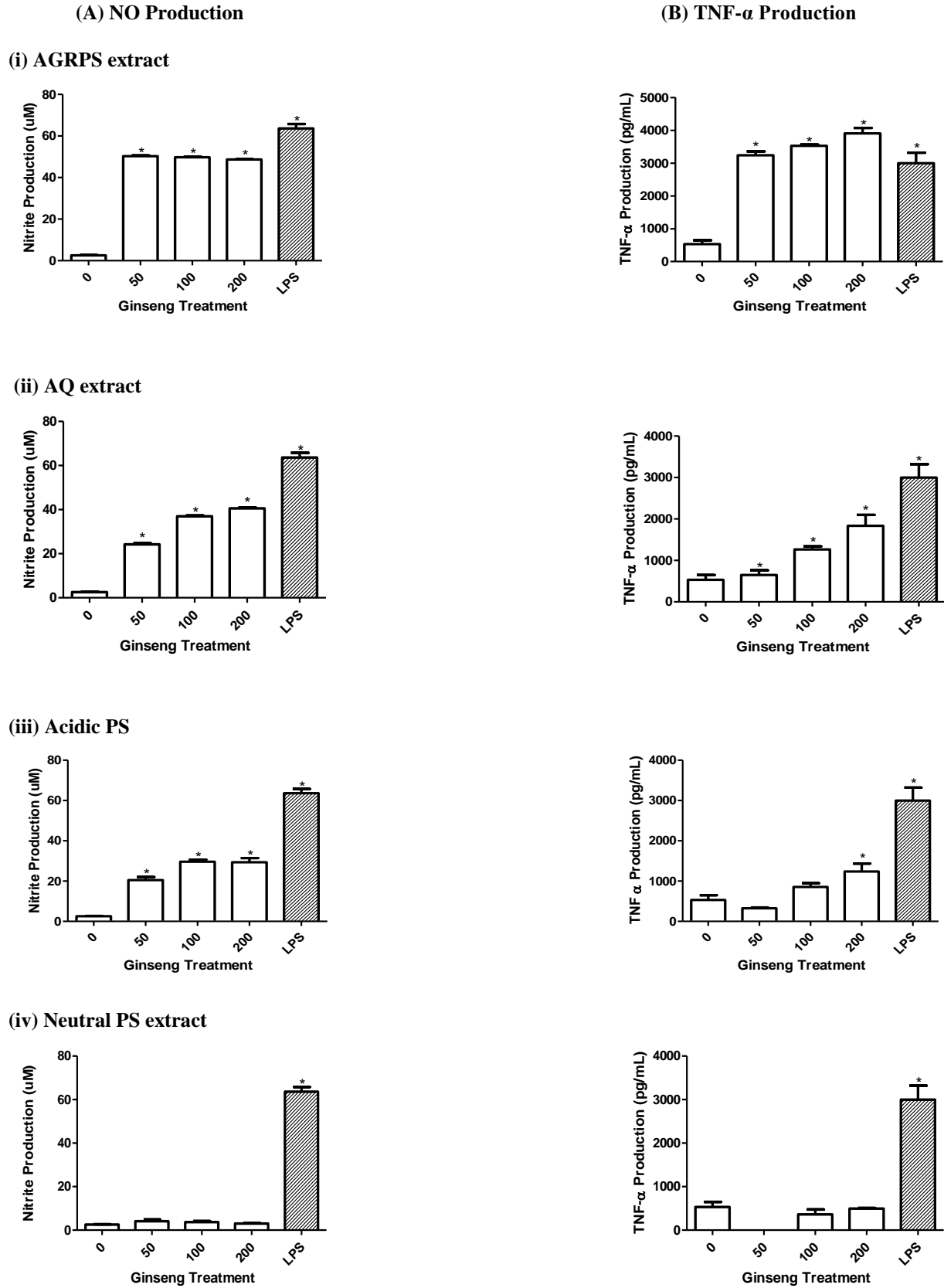
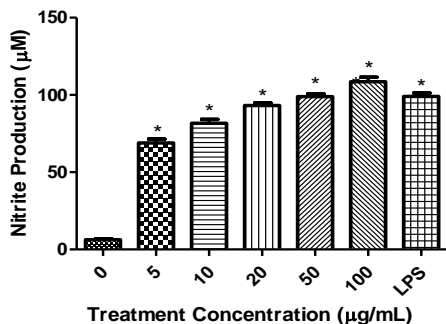


Figure 4.9 - Immunostimulatory effects of AGRPS and AQ extracts, acidic and neutral PS fractions on 24 hours rat alveolar macrophage production of (A) NO (nitrite) and (B) TNF- α *in vitro*. Rat alveolar macrophages were treated with 0, 5, 10, 20, 50, 100 $\mu\text{g}/\text{mL}$ of AGRPS and AQ extracts, acidic and neutral PS fractions for 24 hours. The culture supernatants were analyzed for NO and TNF- α by Griess reaction assay and ELISA, respectively. Cells treated with LPS (1 $\mu\text{g}/\text{mL}$) were used as positive controls. The experiments were performed in triplicates at three independent times and the data were shown as mean \pm SD. Datasets were evaluated by ANOVA. * Values $P < 0.05$ compared to the untreated (vehicle) control were statistically significant.

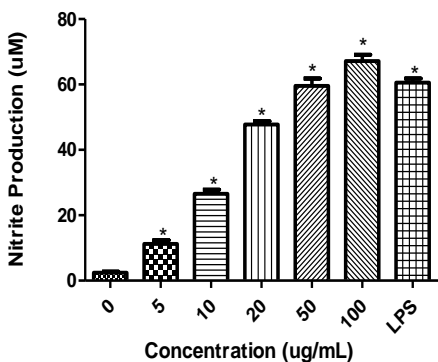
Figure 4.10 - Comparison of composite mixture (12% acidic PS fraction and 30% neutral PS yield), acidic PS, neutral PS fractions immunostimulatory effects on 24 hours macrophage production of NO (nitrite) *in vitro*

NO Production

(i) Composite Mixture (12% acidic PS fraction and 30% neutral PS yield)



(ii) Acidic PS fraction



(iii) Neutral PS fraction

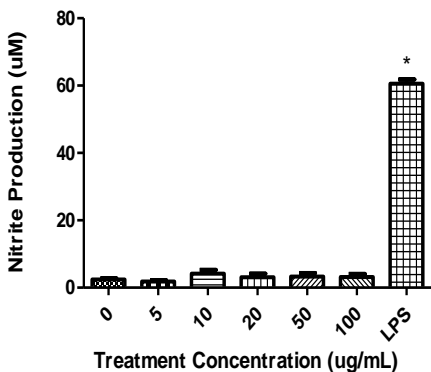


Figure 4.10 - Comparison of composite mixture (12% acidic PS fraction and 30% neutral PS yield), acidic PS, neutral PS fractions immunostimulatory effects on 24 hours macrophage production of NO (nitrite) *in vitro*. Murine macrophages (RAW 264.7 cells) were treated with 0, 5, 10, 20, 50, 100 $\mu\text{g}/\text{mL}$ of composite mixture, acidic PS, neutral PS fractions for 24 hours. The culture supernatants were analyzed for NO by Griess reaction assay, respectively. Cells treated with LPS (1 $\mu\text{g}/\text{mL}$) were used as positive controls. The experiments were performed in triplicates at three independent times and the data were shown as mean \pm SD. Datasets were evaluated by ANOVA. * Values $P < 0.05$ compared to the untreated (vehicle) control were statistically significant.

4.6.3.4 *In vitro* immunostimulatory effect of different molecular weight fractions isolated from Acidic PS

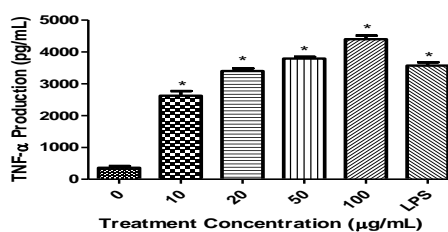
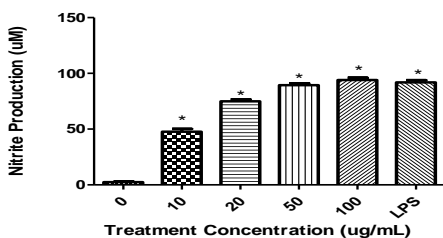
Since the acidic PS showed more immunostimulatory effect in the bioassay results as shown in Figures 4.8-4.9, ultrafiltration was used to fractionate the acidic PS into different molecular weight fractions (i.e. ≥ 100 kDa, 50 - 100 kDa, 30 - 50 kDa and 10 - 30 kDa) to evaluate the influence of molecular weight on AGRPS extract immunobioactivities. As a continuation of the mechanistic study, Figure 4.11 shows the influence of different molecular weight fractions of acidic PS on AGRPS extract modulation of macrophage function *in vitro*. The ≥ 100 kDa and 50 - 100 kDa molecular weight fractions of acidic PS enhanced macrophage production of NO (nitrite) and TNF- α compared to the untreated control. The 30 - 50 kDa and 10 - 30 kDa molecular weight fractions of acidic PS were devoid of stimulating NO production, though they did slightly exhibit some TNF- α production. Hence, high molecular weight (≥ 100 kDa and 50 - 100 kDa) is a vital property for AGRPS extract to exert an immunostimulatory effect.

Figure 4.11 - Immunostimulatory effects of acidic PS fractions ≥ 100 kDa, 50 - 100 kDa, 30 - 50 kDa, 10 - 30 kDa on 24 hours macrophage production of NO *in vitro*

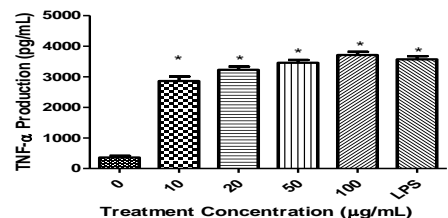
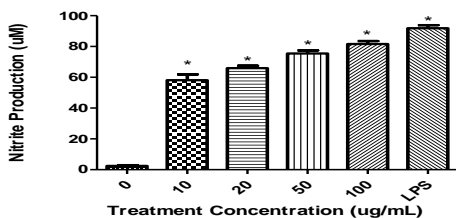
(A) NO Production

(B) TNF- α Production

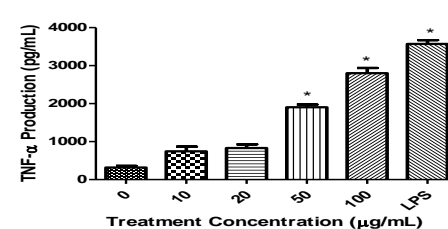
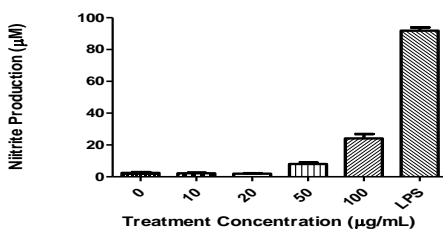
(i) ≥ 100 kDa Acidic PS fraction



(ii) 50 - 100 kDa Acidic PS fraction



(iii) 30 - 50 kDa Acidic PS fraction



(iv) 10 - 30 kDa Acidic PS fraction

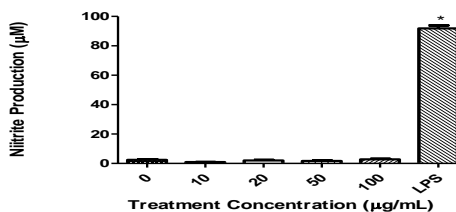


Figure 4.11 - Immunostimulatory effects of acidic PS fractions ≥ 100 kDa, 50 - 100 kDa, 30 - 50 kDa, 10 - 30 kDa on 24 hours macrophage production of NO *in vitro*. Murine macrophages (RAW 264.7 cells) were treated with 0, 10, 20, 50, 100 $\mu\text{g}/\text{mL}$ of acidic PS fractions ≥ 100 kDa, 50 - 100 kDa, 30 - 50 kDa, 10 - 30 kDa for 24 hours. The culture supernatants were analyzed for NO (nitrite) by Griess reaction assay. Cells treated with LPS (1 $\mu\text{g}/\text{mL}$) were used as positive controls. The experiments were performed in triplicates at three independent times and the data were shown as mean \pm SD. Datasets were evaluated by ANOVA. * Values $P < 0.05$ compared to the untreated (vehicle) control were statistically significant.

4.6.3.5 *In vitro* suppression of LPS-induced stimulation by AGRPS and AQ extracts, Acidic and Neutral fractions isolated from AGRPS

The mechanistic study data presented in Figures 4.12 and 4.13 shows the inhibitory effect of 24 hr pre-conditioning with ginseng on 24 hr accumulation of NO (nitrite) and TNF produced by murine cell line and rat alveolar macrophage induced by LPS challenge. In the absence of ginseng precondition, LPS induced a marked NO and TNF response. AGRPS extract, AQ extract and acidic PS significantly suppressed LPS stimulated 24-hour production of NO and TNF- α . Neutral PS had no significant effect suggesting that acidic PS is critical in AGRPS extract suppression of LPS induced immunological response.

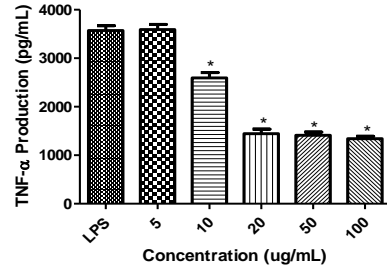
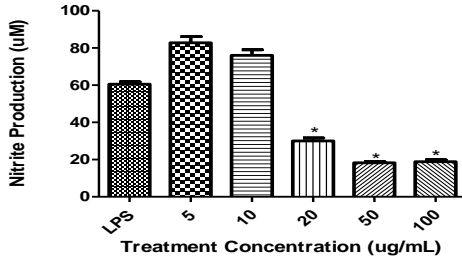
Bioassay of the composite mixture (simulating the 12% acidic PS fraction and 30% neutral PS yield obtained from the ion exchange chromatographic procedure) as seen in Figure 4.14, revealed that neutral PS enhances acidic PS suppression of LPS immunological response.

Figure 4.12 - Effects of AGRPS and AQ extracts, acidic and neutral PS fractions on LPS-stimulated 24 hours murine macrophage production of (A) NO (nitrite) and (B) TNF- α *in vitro*

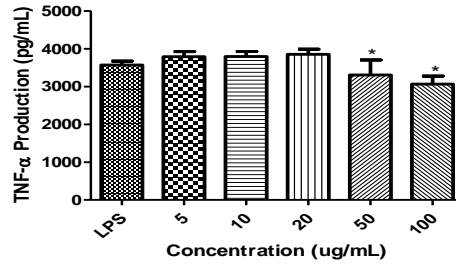
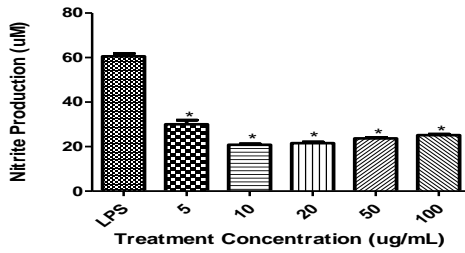
(A) NO Production

(B) TNF- α Production

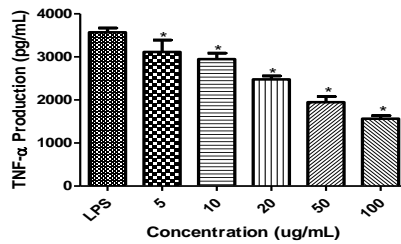
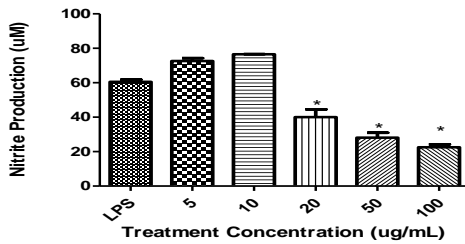
(i) AGRPS extract



(ii) AQ extract



(iii) Acidic PS fraction



(iv) Neutral PS fraction

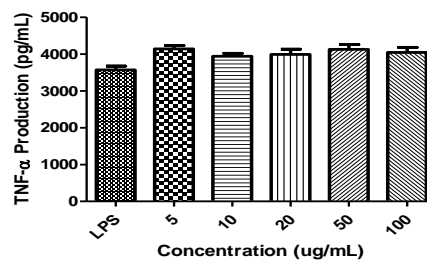
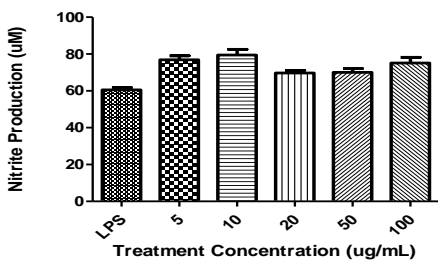


Figure 4.12 - Effects of AGRPS and AQ extracts, acidic and neutral PS fractions on LPS-stimulated 24 hours murine macrophage production of (A) NO (nitrite) and (B) TNF- α *in vitro*. Murine macrophages (RAW 264.7 cells) were pretreated AGRPS and AQ extracts, acidic and neutral PS fractions (0, 5, 10, 20, 50, 100 μ g/mL) for 24 hours and were washed before challenge with LPS 1 μ g/mL. The LPS-stimulated 24 hours production of NO and TNF- α were determined by Griess reaction assay and ELISA, respectively. The experiments were performed in triplicates at three independent times and the data were shown as mean \pm SD. Datasets were evaluated by ANOVA. * Values P < 0.05 compared to the LPS positive control were statistically significant.

Figure 4.13 - Effects of AGRPS and AQ extracts, acidic and neutral PS fractions on LPS-stimulated 24 hours rat alveolar macrophage production of (A) NO (nitrite) and (B) TNF- α *in vitro*

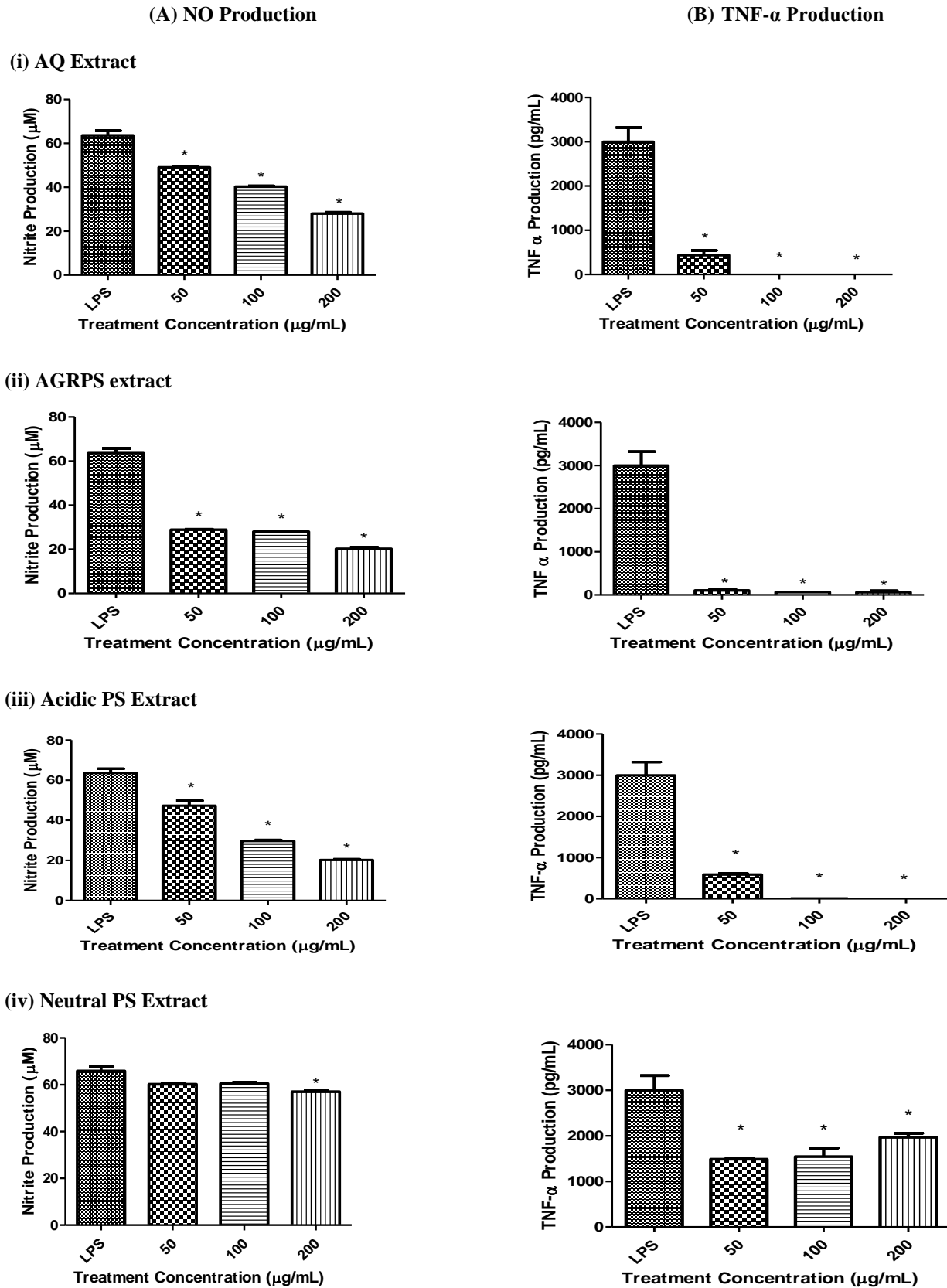
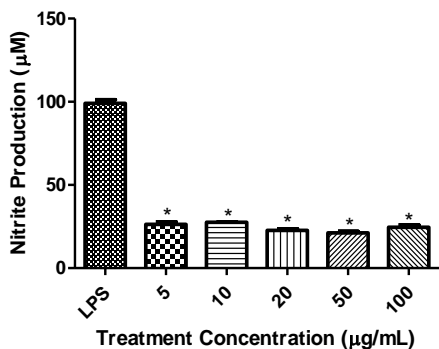


Figure 4.13 - Effects of AGRPS and AQ extracts, acidic and neutral PS fractions on LPS-stimulated 24 hours rat alveolar macrophage production of (A) NO (nitrite) and (B) TNF- α *in vitro*. Rat alveolar macrophages were pretreated AGRPS and AQ extracts, acidic and neutral PS fractions (0, 5, 10, 20, 50, 100 μ g/mL) for 24 hours and were washed before challenge with LPS 1 μ g/Ll. The LPS-stimulated 24 hours production of NO and TNF- α were determined by Griess reaction assay and ELISA, respectively. The experiments were performed in triplicates at three independent times and the data were shown as mean \pm SD. Datasets were evaluated by ANOVA. * Values $P < 0.05$ compared to the LPS positive control were statistically significant.

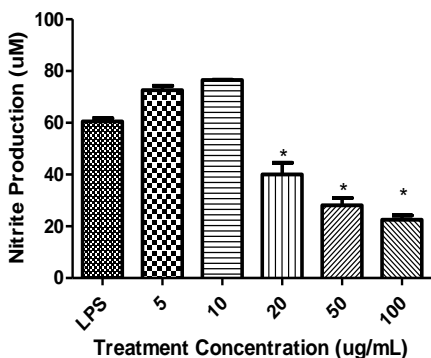
Figure 4.14 - Comparison of composite mixture (12% acidic PS fraction and 30% neutral PS yield), acidic PS, neutral PS fractions on LPS-stimulated 24 hours macrophage production of NO (nitrite) *in vitro*

NO Production

(i) Composite Mixture (12% acidic PS fraction and 30% neutral PS yield)



(ii) Acidic PS fraction



(iii) Neutral PS fraction

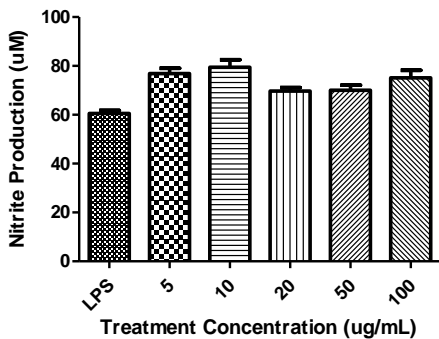


Figure 4.14 - Comparison of composite mixture (12% acidic PS fraction and 30% neutral PS yield), acidic PS, neutral PS fractions on LPS-stimulated 24 hours macrophage production of NO (nitrite) *in vitro*. Murine macrophages (RAW 264.7 cells) were pretreated composite mixture, acidic PS, neutral PS fractions (0, 5, 10, 20, 50, 100 μ g/mL) for 24 hours and were washed before challenge with LPS 1 μ g/mL. The LPS-stimulated 24 hours production of NO and TNF- α were determined by Griess reaction assay and ELISA, respectively. The experiments were performed in triplicates at three independent times and the data were shown as mean \pm SD. Datasets were evaluated by ANOVA. * Values $P < 0.05$ compared to the LPS positive control were statistically significant.

4.6.3.6 *In vitro* suppression of LPS-induced stimulation by different molecular weight

fractions isolated from Acidic PS

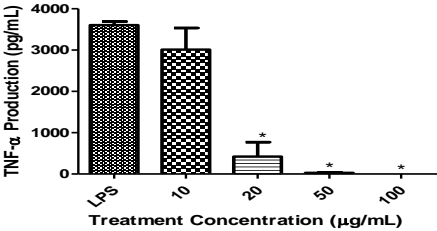
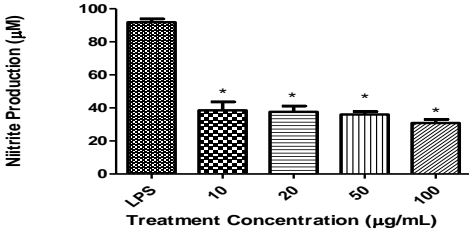
The mechanistic study data also indicates that ≥ 100 kDa and 50 - 100 kDa molecular weight fractions of acidic PS suppressed LPS induced macrophage immunological response. The 30 - 50 kDa and 10 - 30 kDa molecular weight fractions of acidic PS did not suppress LPS immunological response (Figure 4.15). Hence, high molecular weight (≥ 100 kDa and 50 - 100 kDa) is a vital property for AGRPS extract to suppress LPS induced immunological response.

Figure 4.15 - Effects of acidic PS fractions ≥ 100 kDa, 50 - 100 kDa, 30 - 50 kDa, 10 - 30 kDa on LPS-stimulated 24 hours macrophage production of (A) NO (nitrite) and (B) TNF- α *in vitro*

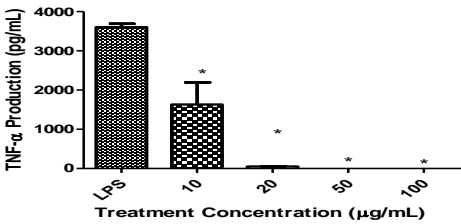
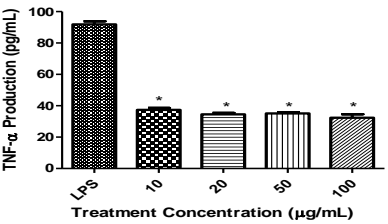
(A) NO Production

(B) TNF- α Production

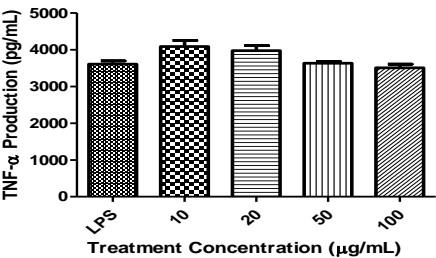
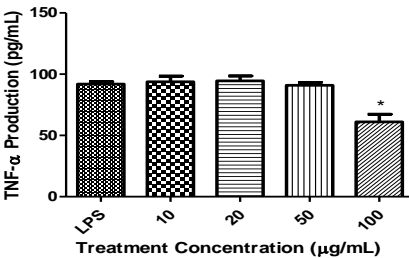
(i) ≥ 100 kDa Acidic PS fraction



(ii) 100 - 50 kDa Acidic PS fraction



(iii) 30 - 50 kDa Acidic PS fraction



(iv) 10 - 30 kDa Acidic PS fraction

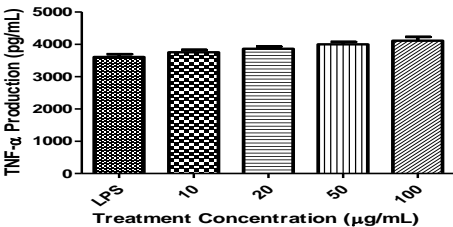
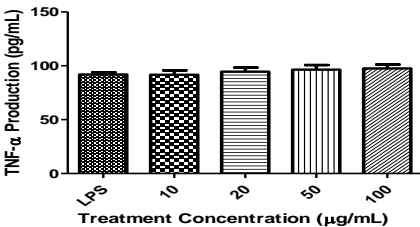


Figure 4.15 - Effects of acidic PS fractions ≥ 100 kDa, 50 - 100 kDa, 30 - 50 kDa, 10 - 30 kDa, on LPS-stimulated 24 hours macrophage production of (A) NO (nitrite) and (B) TNF- α *in vitro*. Murine macrophages (RAW 264.7 cells) were pretreated with acidic PS fractions ≥ 100 kDa, 50 - 100 kDa, 30 - 50 kDa, 10 - 30 kDa (0, 10, 20, 50, 100 $\mu\text{g}/\text{mL}$) for 24 hours and were washed before challenge with LPS 1 $\mu\text{g}/\text{mL}$. The LPS-stimulated 24 hours production of NO and TNF- α were determined by Griess reaction assay and ELISA, respectively. The experiments were performed in triplicates at three independent times and the data were shown as mean \pm SD. Datasets were evaluated by ANOVA. * Values $P < 0.05$ compared to the LPS positive control were statistically significant.

4.7 Discussion

Medicinal plants are used as prophylactic and therapeutic agents in herbal medicine. Polysaccharides have recently been recognized as a major contributor to the bioactivity of herbal medicines. Polysaccharides from plant sources with immunomodulatory, anti-oxidant, anti-hyperglycemic, anti-bacterial, anti-inflammatory, anti-viral and anti-tumor activities have been reported [9, 10, 16, 18, 25, 32, 35, 45]. Polysaccharides, including lentinan, a (1,3)- β -D-glucan, isolated and purified from *Lentinus edodes* has been licensed as an over-the-counter (OTC) dietary supplement and immunostimulatory drug in China [46]. In Canada, CVT-E002™ (sold commercially as COLD-FX®) a poly-furanosyl-pyranosyl polysaccharide-rich herbal product of the root of American ginseng was licensed in 2007 as a natural health product to ‘help reduce the frequency, severity and duration of cold and flu symptoms by boosting the immune system’ [20, 47]. The paradoxical immunomodulatory effect of AQ and AGRPS extracts was examined *ex vivo* and *in vivo*. In the *ex vivo* study, Figures 4.1-4.2 shows that there was an increase in proinflammatory production in cultured alveolar macrophages obtained from AGRPS and AQ extracts treated rats. The AGRPS and AQ extract treatments reduced responsiveness of alveolar macrophages collected from ginseng-treated animals to LPS *ex vivo* challenge (Figures 4.1-4.2). The extracts *ex vivo* immunomodulatory effects were dependent on the duration of exposure. As shown in Figure 4.3, the *in vivo* study suggests that orally administered AGRPS and AQ extracts possess *in vivo* immunostimulatory and suppression of LPS induced immunological effects. This was evidenced by the extracts up-regulation and down-regulation of plasma TNF- α cytokine production under basal and LPS endotoxic conditions respectively. Overproduction of early and primary cytokines like TNF- α in the inflammatory process contributes significantly to the pathological complication observed in infectious and inflammatory diseases such as endotoxemia

[48-50]. There was a significant difference in cytokine production between LPS challenge and ginseng treatments exposed to LPS. However, in the lung histochemical study described in Figures 4.4-4.5, no difference was observed between ginseng treatments exposed to LPS and the LPS challenge. It may take a longer duration of exposure or a higher dose of LPS to observe lung pathological complication observed in infectious and inflammatory diseases like sepsis induced acute lung injury.

An *in vitro* mechanistic study was performed to identify the underlying bioactive fractions responsible for AGRPS and AQ extracts *ex vivo* and *in vivo* immunobioactivities. For this study, AGRPS and AQ extracts and the fractions of AGRPS extract immunomodulatory effects were evaluated *in vitro* using murine cell line and rat alveolar macrophages. The relationship between physicochemical properties (ionic charge and molecular weight) of AGRPS fractions and their influence on AGRPS modulation of macrophage function was examined. The pharmacological effects of ginseng polysaccharides including immunomodulation can be attributed to its acidic and neutral polysaccharide components [25]. There have been different reports of ginseng acidic and neutral polysaccharide immunomodulatory effects in the literature. Kim et al. (1990) reported that acidic PS and neutral PS of *P. ginseng* (Asian ginseng) may stimulate B cells and macrophages [15]. While the study by Fan et al. (2010) showed that neutral PS of *P. ginseng* stimulated the proliferation of lymphocytes; increased natural killer cell cytotoxicity; enhanced the phagocytosis and NO production by macrophages and increased the level of TNF- α in serum [51]. The *in vitro* mechanistic study also delineated the influence of AGRPS (*Panax quinquefolius*) ionic charge fractions on AGRPS and AQ extracts modulation of macrophage function. The data revealed that acidic PS was the major bioactive species responsible for the immunostimulatory and suppression of LPS induced immunological effects of AGRPS and AQ

extracts (Figures 4.8-4.9 and 4.12-4.13). This outcome suggests that acidic (and not the neutral) PS physicochemical property is vital for AGRPS and AQ extracts macrophage-mediated immunomodulatory effects. Because neutral PS did not stimulate production of NO, but showed some significant stimulation of TNF- α production, investigation was done to determine if there was any interaction between acidic PS and neutral PS. Bioassay of a composite mixture of 12% acidic PS fraction and 30% neutral PS (Figures 4.10 and 4.14) revealed that neutral PS enhances acid PS macrophage-mediated immunostimulatory and suppression of LPS induced immunological effects. The observed enhancement of acidic PS immunostimulatory effect by neutral PS supports the previous finding by Fan et al. (2010) which reported that neutral PS of *P. ginseng* enhanced macrophage production of NO [51]. While the observed enhancement of acidic PS suppression of LPS immune response by neutral PS is a novel outcome. One study by Lemmon et al. (2012) [that used human peripheral blood mononuclear cells and ginseng concentrations similar to that utilized in this study] reported that the immunostimulatory effects of AGRPS and AQ extracts are mediated by PS with a molecular weight higher than 100 kDa [52]. Data from this study showed that ≥ 100 kDa and 100 - 50 kDa molecular weight fractions of acidic PS possess both immunostimulatory and suppression of LPS induced immunological effects which are a novel outcome. This indicates that the PS high molecular weight physicochemical property plays a significant role in AGRPS and AQ extracts macrophage-mediated immunostimulatory and suppression of LPS induced immunological effects (Figures 4.11 and 4.15).

The *in vitro* mechanistic study revealed the importance of the acidic nature and high molecular weight of the polysaccharide fractions in AGRPS and AQ extracts modulation of macrophage function. Outcomes of this study correlate well with what has been previously reported regarding

ionic charge and molecular weight physicochemical properties being critical factors that affect the biological activity of polysaccharides [36-38]. Macrophages are activated through their recognition and binding of plant polysaccharides via Toll-like receptor 4 (TLR4). The activation of these receptors leads to intracellular signaling cascades, resulting in transcriptional activation and the production of proinflammatory mediators [9]. Results from this study suggests that acidic nature and high molecular weight (≥ 100 kDa and 100 - 50 kDa) polysaccharide fractions are vital for AGRPS and AQ extracts activation and suppression of macrophage function. These physicochemical properties may be associated with the ability of the extract's polysaccharide fractions to bind to TLR4 receptor and up-regulate or down-regulate TLR4 receptor expression, which triggers or inhibits intracellular signaling cascades and the production of proinflammatory mediators under basal or LPS endotoxic conditions respectively. Data from this study opens the door for future investigation of the possible association between the extracts polysaccharide fractions physicochemical properties and the the extracts ability to up-regulate or down-regulate TLR4 receptor expression. TLR4 has been shown to be expressed in murine macrophages activated by *Carthamus tinctorius* polysaccharides [53]. Studies which used TLR 4-deficient mice indicates that TLR4 is involved in murine macrophage recognition and activation of *Platycodon grandiflorum* polysaccharides [54] and *Levan* polysaccharide fraction from fermented soybean mucilage [55]. The expression of Toll-like receptors including TLR2, TLR4 and the adaptor molecule MyD88 which were increased in murine macrophages stimulated with *Staphylococcus aureus* bacteria has been reported to be significantly reduced by ginseng polysaccharide pretreatment *in vitro*. Ginseng polysaccharide intravenous pretreatment was also reported to enhance macrophage-mediated bactericidal activity in mice by reducing the number of *S. aureus* bacteria present in the spleen, kidney, and blood [33-34]. The iNOS (inducible nitric

oxide synthase), MAPK (mitogen-activated protein kinase) kinases such as p38, ERK1/2 (extracellular signal-regulated kinases 1/2), PI3K (phosphoinositide 3-kinase), and NF- κ B (nuclear factor kappa B) signaling pathways may mediate the immunostimulatory effect observed in AGRPS and AQ extracts and high molecular weight acidic PS, as studies by Friedl et al. (2001) and Lemmon et al. (2012) using AQ and PS extracts of *P. ginseng* and American ginseng AQ and PS extracts [29, 52] demonstrated. The signaling pathways that mediate the extracts suppression of LPS induced NO and TNF- α production requires further investigation.

The ion exchange chromatography was used to fractionate AGRPS extract into fractions of neutral and acidic charge. The 30.0% recovery for the neutral fraction of AGRPS (*Panax quinquefolius*) obtained from this study is close to the 33.0% recovery reported for *Panax notoginseng* polysaccharides [56]. The AGRPS (*Panax quinquefolius*) neutral fraction recovery is different from the 60.5% recovery reported for *Panax ginseng* polysaccharides [23]. In this study 12.0% recovery obtained for AGRPS (*Panax quinquefolius*) acidic fraction is close to the 17.1% recovery reported for *Panax ginseng* polysaccharides [23]. This was different from the 31.0% recovery reported for *Panax notoginseng* polysaccharides [56]. The difference in species of the ginseng herb may account for the variations in the recovery data of the neutral and acidic fractions between this study and other studies [23, 56].

The study from chapter 3 of this thesis and that of Lemmon et al. (2012) suggests that concurrent treatment of AQ and AGRPS extracts did not inhibit LPS-induced immune response [29, 52]. While data from the *in vitro* study shows that direct treatment and 24hr pretreatment with AGRPS and AQ extracts exerts a direct immunostimulatory effect and an indirect suppression of LPS-induced immune response respectively as shown in Figures 4.8- 4.9 and 4.12-4.13. This suggests that the extracts *in vitro* immunomodulatory effects were dependent on the duration of

exposure. A similar situation was observed in the *ex vivo* study (Figures 4.1-4.2), where the 6 days AQ and AGRPS extract pretreatments was more effective than that of the 3 days in suppressing the LPS-induced immune response.

The *ex vivo* and *in vivo* immunomodulatory effects of AGRPS and AQ extracts (Figures 4.1, 4.2, 4.3) were validated *in vitro* (Figures 4.8-4.9 and 4.12-4.13). The *ex vivo*, *in vivo* and *in vitro* studies support the observations from chapter three of this thesis, indicating that bioactive AGRPS isolated from AQ extract and its parent AQ extract exerted immunostimulatory effects. The *in vivo* immunostimulatory effect of AGRPS and AQ extracts was very small as compared with LPS toxic stimulatory effect, suggesting that American ginseng extract and its natural health product containing polysaccharides like CVT-E002TM are safe with health benefits with regards to immunostimulation [47]. The results from this study suggests that under LPS mediated excessive stimulatory infectious condition like endotoxemia; pretreatment with AGRPS and AQ extracts will not cause additive immunostimulatory effects, but rather the extracts will reduce and neutralize LPS immunologic stimulatory effect. The ability of AGRPS and AQ extracts to neutralize LPS immunological is a desired requirement for future prophylactics against endotoxemia. A possible mechanism by which AGRPS suppresses LPS immunotoxic stimulatory effect may be through its ability to desensitize immune cells (e.g. macrophages) from LPS toxic stimulation, similar to the tolerance ability of LPS pre-exposure to desensitize subsequent LPS challenge [57]. AGRPS and AQ extracts are not antagonists of LPS because they have low stimulatory effect and also requires pretreatment before they can suppress LPS stimulation. The data from this study supports the reported CVT-E002TM immunostimulatory [21, 28, 47, 58-61] and suppression of proinflammatory effects [20, 62] under basal and induced proinflammatory conditions respectively. Therapies directed at the neutralization of

proinflammatory mediators or LPS that were promising in experimental models have been largely ineffective in clinical trials. Therefore, the development of new therapies is of major interest [63-65].

The *in vitro* stimulatory effect was stronger than that observed *in vivo* which may be due to low bioavailability of the orally-administered extracts. As described in the following chapter, a new method developed to analyze AGRPS in plasma detected low levels of AGRPS in the plasma of rats orally fed with AGRPS. The low plasma levels of AGRPS are pharmacologically in close agreement with the *in vitro* 50-200 µg/mL immunobioactive concentrations of AGRPS in cultured murine cell line and rat alveolar macrophages reported in this study and chapter 3. Evaluation of the total AGRPS concentration at C_{max} in total plasma volume revealed that an estimated 9.49 % of the orally administered AGRPS was available in systemic plasma. Polysaccharides of *Radix ophiopogonis* have been reported to have low bioavailability of 1.7 % in rats after oral administration respectively, and this can be attributed to their large molecular size and hydrophilic character [66]. The 125mg/kg daily oral dose of American ginseng used in the *ex vivo* and *in vivo* studies, is in agreement with the 9g of ginseng daily dose [67] recommended for human consumption (assuming an average adult weighs 70 kg).

The merit of this study is that a modern day approach of evaluating herbal medicines [68-69] was utilized via the phytochemical and immunopharmacological screening of AGRPS and AQ extracts from good agricultural practice quality American ginseng roots randomly collected from five different farms by the Ontario Ginseng Growers Association in Ontario, Canada. The acidic PS and its ≥ 100 kDa and 100 - 50 kDa molecular weight fractions were identified as the major bioactives responsible for the extracts immunomodulatory effects. AGRPS and AQ extracts *in vivo* stimulation was small in magnitude as compared to its suppression of LPS induced

immunologic response, suggesting attention should be focused more on their beneficial immunosuppressive effect which may be useful in the prophylaxis of LPS related disease conditions such as endotoxemia.

4.8 Conclusion

From this study it can be concluded that AGRPS and AQ extracts possess *ex vivo*, *in vivo* and *in vitro* immunostimulatory and immunosuppressive effects in basal immune function and LPS-induced endotoxic conditions respectively, which can be attributed primarily to acidic PS and its higher molecular weight fractions and not neutral PS or the lower molecular weight fractions of acidic PS. Hence AGRPS and AQ extracts may have beneficial suppressive effect against LPS related disease conditions such as endotoxemia.

4.9 References

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Chapter 5

ANALYSIS OF INTESTINALLY ABSORBED
AMERICAN GINSENG POLYSACCHARIDES IN
PLASMA BY HIGH PERFORMANCE SIZE
EXCLUSION CHROMATOGRAPHY

5.1 Introduction

Studies have shown that water-soluble polysaccharides used in natural health products are good biological response modifiers [1]. A good example is ginseng polysaccharides which exhibit a wide range of beneficial pharmacological effects in cell culture and animal models, including immunomodulation, anti-tumor, anti-oxidation, anti-depression, hypoglycemia and inhibition of gastric lesions [2-10]. Extractable polysaccharides constitute about 10% of the dry weight of ginseng root, a multi-action widely used herb which belongs to the Araliacea family [11].

CVT-E002™ (sold commercially as COLD-FX®) is a poly-furanosyl-pyranosyl polysaccharide-rich herbal product of the root of American ginseng (*Panax quinquefolium*), with purported beneficial effects on influenza and the common cold [2]. Ginseng polysaccharides are rich in L-arabinose, D-galactose, L-rhamnose, D-glucuronic acid and D-galacturonic acid monosaccharides [2, 4, 5, 12]. These polysaccharides are heterogeneous in nature and have been reported to contain neutral and acidic polysaccharides [6, 8, 12], with widely varying molecular weights ranging from 3.5 to 2000 KDa as reported by different investigators and using different methodologies [3, 5, 7, 12-15]. Chapter four of this thesis showed previously by using ion exchange chromatography and ultrafiltration, that American ginseng polysaccharides are heterogeneous in nature, consisting of both neutral and acidic polysaccharides with polydisperse molecular weight distributions.

In the past, non-chromatographic studies such as radioactive labeling [16-17] and bioassay [18] have been used for the *in vivo* analysis of dermatan sulphate, dextran, and α -D-glucan polysaccharides in plasma and the intestinal tract, after intravenous, subcutaneous, oral and intra-intestinal administration. Chromatographic methods have been reported for the determination of

glucans, chitosan ester, dermatan sulfate and *Ophiopogon japonicus* polysaccharides in plasma after intravenous [19-22] and intra-intestinal [23] administration. The polysaccharides used in these chromatographic studies were tagged with a fluorescent label prior to the *in vivo* treatments or subjected to a post column fluorescence derivatization method, which then required the use of fluorometric detection for their analysis in plasma. However, due to the heterogeneous-complex structures of ginseng polysaccharides, labeling can be nonspecific in that it may occur at different sites of the polysaccharides with not all active components labeled. Since the safety of fluorescently-labeled polysaccharides has not been established, the development of a chromatographic method to analyze unlabeled polysaccharides in plasma would be useful for both animal and humans. Here, the knowledge of bioactive concentration levels in body fluids, such as serum would allow pharmacotherapy to be optimized and provides the basis for studies on patient compliance, bioavailability, pharmacokinetics, genetics, organ function and the influences of co-medication [24]. There is currently no available method to detect and quantify ginseng polysaccharides in plasma.

Analytical size-exclusion chromatography (SEC) combined with light scattering detection is a rapid method to analyze and characterize complex biopolymers like polysaccharide–protein complexes [25]. The light scattering detector provides an absolute measure of the biopolymers weight-average molecular weight (M_w) with the intensity from the detector being proportional to important variables such as molecular mass, concentration, and the specific refractive index increment [26]. The light scattering detector response of an analyte requires its corresponding refractive index (RI) detector response to generate the analyte's absolute molecular weight. In the absence of the corresponding RI detector response, the approximate molecular weight can be determined from the calibration curve of standards of known molecular weights using light

scattering detector response. As previously described in chapter three of this thesis, SEC combined with a light scattering detector gave the best characterization of polysaccharides (PS) in ginseng aqueous and PS extracts with regards to the formation of a Gaussian peak distribution, when compared to data generated from the refractive index and viscometer detectors.

In the previous chapter of this thesis, a study with ginseng orally treated rats showed that American ginseng root polysaccharides (AGRPS) exerts both an *ex vivo* and *in vivo* stimulatory and suppression of lipopolysaccharide induced proinflammatory effects. However, there is no confirmatory data that substantiates the absorption of AGRPS into systemic circulation. Chromatographic analysis of human and rat plasma samples have shown that heparin ($M_w = 9$ KDa), marine sulfated polysaccharide ($M_w = 11$ KDa) and *Radix ophiopogonis* ($M_w = 4.8$ KDa) polysaccharides are absorbed from the gastrointestinal tract (GIT) into systemic circulation after oral administration [27-29].

The primary purpose of this study was to develop a high performance size-exclusion chromatography (HPSEC) method that can analyze AGRPS in plasma of rats after oral administration to ascertain if AGRPS can be absorbed from the GIT into systemic circulation. To confirm that the peaks detected in rat plasma are from AGRPS, enzymatic hydrolysis of the plasma samples using pectinase and cellulase enzymes was examined prior to HPSEC analysis.

5.2 Materials and Methods

5.2.1 Materials

Methods for the preparation of AGRPS and aqueous (AQ) extracts used in this study were previously provided in chapter four of this thesis. CVT-E002™ (sold commercially as COLD-FX®) was purchased from a commercial source (Lot number: 0805093, Afexa Life Sciences Inc. Mississauga, ON). According to the manufacturer's description, each capsule of COLD-FX® is certified to contain 200 mg of CVT-E002™, a proprietary ChemBioPrint® (CBP®) product rich in poly-furanosyl-pyranosyl-saccharides. These polysaccharides were extracted by an aqueous method from the root of *Panax quinquefolius* (American ginseng) as described by Shan et al (1999) [5].

Pectinase (E.C.3.2.1.1) and cellulase (E.C.3.2.1.1) from *Aspergillus niger* were purchased from Sigma-Aldrich (USA) and used as received. Pullulan polysaccharide reference standards with different molecular weights 2150, 11800, 22800, 48000, 112000, 404000, 1220000 Da were purchased from PolyAnalytik (USA).

5.2.2 Animals

Adult male Sprague-Dawley rats weighing 240g (purchased from Charles River, St. Constant, QC, Canada) were used in accordance with the guidelines of the Canadian Council on Animal Care. The Animal Ethics Review Committee of the University of Western Ontario approved this study (Protocol No: 2009-070).

5.2.3 Methods

5.2.3.1 HPSEC Method Validation

Validation of the HPSEC method for the determination of AGRPS analyte in plasma samples used in this study was evaluated according to the US Food and Drug Administration (FDA) guideline for Bioanalytical Method Validation [30]. Specificity, linearity, accuracy, precision, sensitivity and recovery performance characteristics of the HPSEC method were evaluated according to the acceptance criteria and recommendations stated in this guideline.

5.2.3.1.1 Specificity and Determination of AGRPS Molecular Weight

The specificity of the method to measure AGRPS was investigated by comparing chromatograms of blank rat plasma, AGRPS and blank rat plasma spiked with AGRPS. The degree of interference by endogenous substances was assessed by the inspection of chromatograms derived from processed blank plasma, AGRPS and blank sample spiked with AGRPS. No peaks from endogenous or exogenous materials should interfere with AGRPS. To calculate the molecular weight of AGRPS, a standard calibration curve of known pullulan polysaccharide standards was constructed. The logarithm of the different molecular weights of pullulan polysaccharide (2150, 11800, 22800, 48000, 112000, 404000, 1220000 Da) was plotted against their retention (hydrodynamic) volumes as shown in the Appendix 1.

5.2.3.1.2 AGRPS Calibration Curve (Linearity)

An eight point calibration curve was prepared of AGRPS in blank rat plasma with final concentrations of 125, 250, 500, 1000, 2000, 4000, 8000 and 16000 µg/mL. Replicate analysis (n = 5) was constructed by plotting the mean peak area against the mean concentrations of AGRPS

as shown in the Appendix. A correlation coefficient value of $R^2 \geq 0.99$ is statistically considered a goodness of fit. Linearity of the calibration curve was evaluated by linear regression analysis using the equation $Y = mX + c$ where Y is the peak area, X is the concentration of AGRPS; unknown concentrations were determined from the linear regression equation of the peak area against concentration for the calibration curve. To quantify the concentration of AGRPS in the plasma samples, the peak area of AGRPS was related to the calibration curve of AGRPS in blank plasma.

5.2.3.1.3 Accuracy and Precision

Accuracy and precision were evaluated from replicate analysis ($n = 5$) of test samples at three different concentrations (125, 1000 and 2000 $\mu\text{g/mL}$). Test samples were analyzed against calibration curves.

The accuracy was calculated from the nominal concentration (C_{nom}) and the mean value of the observed concentration (C_{obs}) as:

$$\text{Accuracy} = [C_{\text{obs}}/C_{\text{nom}}] \times 100 \% \quad (5.1).$$

The precision (relative standard deviation, RSD) was calculated from the standard deviation and observed concentration as:

$$\text{Precision (RSD, \%)} = [\text{standard deviation SD}/C_{\text{obs}}] \times 100 \% \quad (5.2)$$

Accuracy was required to be within $\pm 15\%$ bias from the nominal values and the precision to be within $\pm 15\%$ relative standard deviation (RSD).

5.2.3.1.4 Sensitivity

The limit of detection and the limit of quantification were determined based on the standard deviation of the response and the slope of the calibration curve as stated in the International Conference on Harmonization (ICH) guideline for validation of analytical procedures [31].

The limit of detection is expressed as $= 3.3 \sigma/S$, where σ = the standard deviation of the response and S = the slope of the calibration curve.

The limit of quantification is expressed as $= 10 \sigma/S$.

5.2.3.1.5 Recovery

The recovery of AGRPS from plasma was determined for different standard concentrations (at concentrations of 125, 1000 and 2000 $\mu\text{g/mL}$) from replicate analysis ($n = 5$) by spiking the AGRPS into blank plasma. The percentage (%) recovery was calculated from the mean peak area of AGRPS spiked before extraction (PA) and the mean peak area of AGRPS spiked post-extraction (PZ) as:

$$\% \text{ Recovery} = [\text{PZ/PA}] \times 100 \% \quad (5.3).$$

According to the acceptance criteria, the recovery of the analyte does not need to be 100%, but should be consistent and precise.

5.2.3.2 Animal studies

5.2.3.2.1 Determination of the absorption of AGRPS following oral administration

To determine the absorption of orally administered AGRPS, rats (n = 3 rats per time point of 0.5, 1, 2, 4, and 8 hr) were fasted overnight with free access to water, and were given a single dose of 125 mg/kg AGRPS in saline by oral gavage (30mg/1mL/0.240kg). The sham control group (n = 3 rats) received saline (1mL/0.240kg) orally. To provide a positive control for the detection of AGRPS in systemic circulation, an additional group of fasted rats (n = 3 rats per time point of 0.5, 1, 2, 4, and 8 hr) were administered with the same dose of 125 mg/kg AGRPS by IP injection (30mg/1mL/0.240kg). IP injection will allow the AGRPS to by-pass the GIT barrier allowing entrance to the systemic circulation from hepatic-portal circulation.

5.2.3.2.2 Determination of the absorption of AQ extract following oral administration

To determine the applicability of the chromatographic method, plasma samples from rats that received a 125 mg/kg single dose of AQ extract were dissolved in saline by oral gavage (30mg/1mL/0.240kg) for 6 days. These were collected 24 hr after the last dose.

5.2.3.2.3 Determination of the absorption of CVT-E002TM following oral administration

To determine the applicability of the chromatographic method, plasma samples from rats that received a 125 mg/kg single dose of CVT-E002TM (COLD-FX®) dissolved in saline by oral gavage (30mg/1mL/0.240kg) for 6 days were also collected 24 hr after the last dose.

5.2.3.2.4 Sample collection and processing to determine AGRPS plasma profile

Animals were anesthetized with intramuscular injection of ketamine 80 mg/kg and xylazine 5 mg/kg. Blood was collected from the anesthetized rats by cardiac puncture at time points of 0.5, 1, 2, 4, and 8 hr post dose (n = 3 rats per time point) into tubes coated with 3.8% trisodium citrate solution by intracardiac puncture and then placed on dry ice. Blood samples were centrifuged at 3500×g (Beckman Model TJ-6, USA) for 10 minutes and the plasma was separated, aliquoted and stored at -80 °C until use. Plasma samples were removed from storage and warmed to room temperature prior to processing. All plasma samples, spiked calibration working solutions, or spiked test samples were treated in the same manner as described below. To 800 µL portion of each plasma sample, 320 µL of 1 M perchloric acid was added. The mixture was vortexed and then centrifuged at 6000 rpm (VWR Galaxy Mini Centrifuge, South Korea) for 10 min to precipitate denatured plasma proteins. After neutralizing 600 µL of the supernatant by the addition of 180 µL of 1 M sodium hydroxide, it was vortexed and submitted to HPSEC analysis.

5.2.3.2.5 Enzymatic Hydrolysis of plasma samples

To ascertain whether AGRPS was found in plasma of the AGRPS-treated rats, an enzyme hydrolytic method was developed by incubating AGRPS solution with pectinase or cellulase enzyme which was then subjected to HPSEC analysis. After identifying the enzyme and incubation time suitable for AGRPS hydrolysis, it was then applied to the plasma samples followed by HPSEC analysis. For this, 2000µg/mL of AGRPS was dissolved in 800µL distilled water and incubated with or without pectinase (100µL of 3800 Units) or cellulase (100µL of 2000 Units) enzyme for 1hr, 8hr and 24hr at 37°C, pH was not adjusted. The samples were then

subjected to HPSEC analysis. After identifying the enzyme and incubation time suitable for enzymatic hydrolysis of AGRPS, it was then applied to 600 μ L of plasma samples obtained from 4 hr oral and IP AGRPS treated rats. The enzymes were then inactivated with 240 μ L of 1 M perchloric acid. The mixture was vortexed and then centrifuged at 6000 rpm (VWR Galaxy Mini Centrifuge, South Korea) for 10 min to precipitate denatured enzyme and plasma proteins. After neutralizing 450 μ L of the supernatant by the addition of 135 μ L of 1 M sodium hydroxide, it was vortexed and then submitted to the HPSEC analysis.

5.2.3.3 Size-Exclusion Chromatography Analysis

5.2.3.3.1 Instrumentation and Chromatographic Conditions

Processed plasma samples were analyzed with AquaGel PAA-203 and PAA-204 columns (8 \times 300 mm, PolyAnalytik, USA) connected in series to a Viscotek HPSEC system (Varian Instruments, USA) with OmniseC software (version 4.5, Viscotek, USA) for data acquisition at 40°C. Processed plasma samples were filtered with a 0.2 μ m nylon filter prior to analysis. Each sample (100 μ L) was injected and eluted using a 0.1M sodium nitrate (NaNO₃) mobile phase at a flow rate of 0.7 mL/min and monitored using HPSEC system with right angle light scattering detection. Microsoft Excel 2010 software was used to process the HPSEC data and generate chromatograms presented in this study.

5.3 Statistical analysis

All statistical analysis, the plotting and calculation of AGRPS plasma concentration-time profiles, peak plasma concentration (C_{\max}), time to reach C_{\max} (T_{\max}) and area under the plasma concentration-time curve ($AUC_{0.5-8}$) from time 0.5 hr to 8 hr of oral and IP AGRPS administered rats were performed with GraphPad prism 4.0a Software (GraphPad Software Inc., USA). Data were expressed as means \pm standard derivation (SD). Student's t-test statistical analysis was used to compare oral and IP routes C_{\max} , T_{\max} and $AUC_{0.5-8}$ values. Statistically significant differences are indicated by p-values of <0.05 .

5.4 Results

5.4.1 HPSEC Method Validation (Chromatographic Performance)

5.4.1.1 Specificity and Determination of AGRPS Molecular Weight

The specificity of the method was investigated for detection of AGRPS and assessment of potential interference of AGRPS from endogenous substances respectively. Typical chromatograms of the HPSEC analysis of blank plasma, AGRPS before plasma spiking and AGRPS added to plasma followed by extraction are shown in Figure 5.1. The AGRPS peaks are well shaped with no interfering peaks found at the retention volume region. These results indicate that the HPSEC method is specific with no interference from plasma constituents observed. The identity of the parent (unchanged) AGRPS in plasma of AGRPS treated rats is based on the retention volume matched with the AGRPS spiked into blank plasma.

The weight average molecular weight of AGRPS was determined from the pullulan polysaccharide calibration curve plot, i.e. ($R_v = -2.9506 \text{ Log } M_w + 29.203$), where R_v is the retention volume and M_w is the molecular weight (see Appendix 4). AGRPS eluted with a retention volume of 11.22 mL which corresponds to a weight-average molecular weight (M_w) = 1240 kDa based on the calibration curve. The refractive index detection did not give any signal profile for AGRPS, which is needed by the HPSEC system software along with the light scattering signal profile to generate the absolute molecular weight of AGRPS. Hence the weight average molecular weight polysaccharide samples in this study were determined from the pullulan polysaccharide calibration curve plot.

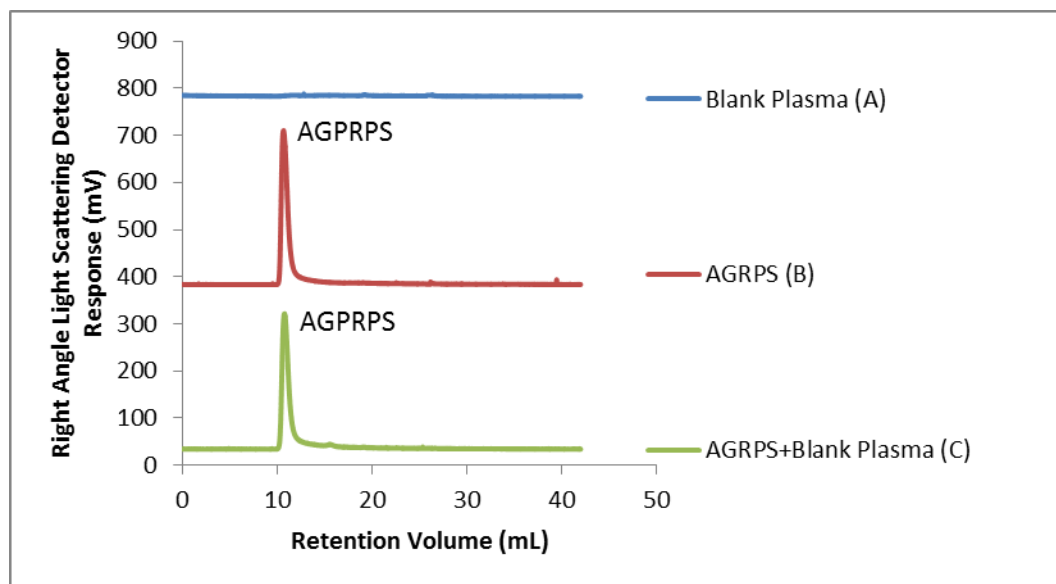


Figure 5.1 – Chromatograms obtained from high performance size exclusion chromatography analysis of (A) blank plasma (B) 2000 $\mu\text{g}/\text{mL}$ AGRPS and (C) blank plasma spiked with 2000 $\mu\text{g}/\text{mL}$ AGRPS. Each processed sample was injected and eluted with 0.1 M NaNO_3 mobile phase on a series of AquaGel PAA-203 and PAA-204 columns (8×300 mm, PolyAnalytik, USA), this was monitored with right angle light scattering detector at 1 mL/min flow rate. AGRPS eluted with retention volume of 11.22mL in both chromatograms of B and C. The y-axis is the right angle light scattering detector response (mV), while the x-axis represents the retention volume (mL).

5.4.1.2 AGRPS Calibration Curve (Linearity)

The peak areas were linearly related to concentrations of AGRPS spiked into blank plasma over the range of 125–16000 µg/mL. The equation of the obtained calibration curve was: $Y = 0.16473X - 6.9623$. The correlation coefficient value was found to be $R^2 = 0.9915$ which is statistically considered a goodness of fit, with Y being the peak area and X is the AGRPS concentration.

$$Y = 0.16473X - 6.9623$$

$$R^2 = 0.9915$$

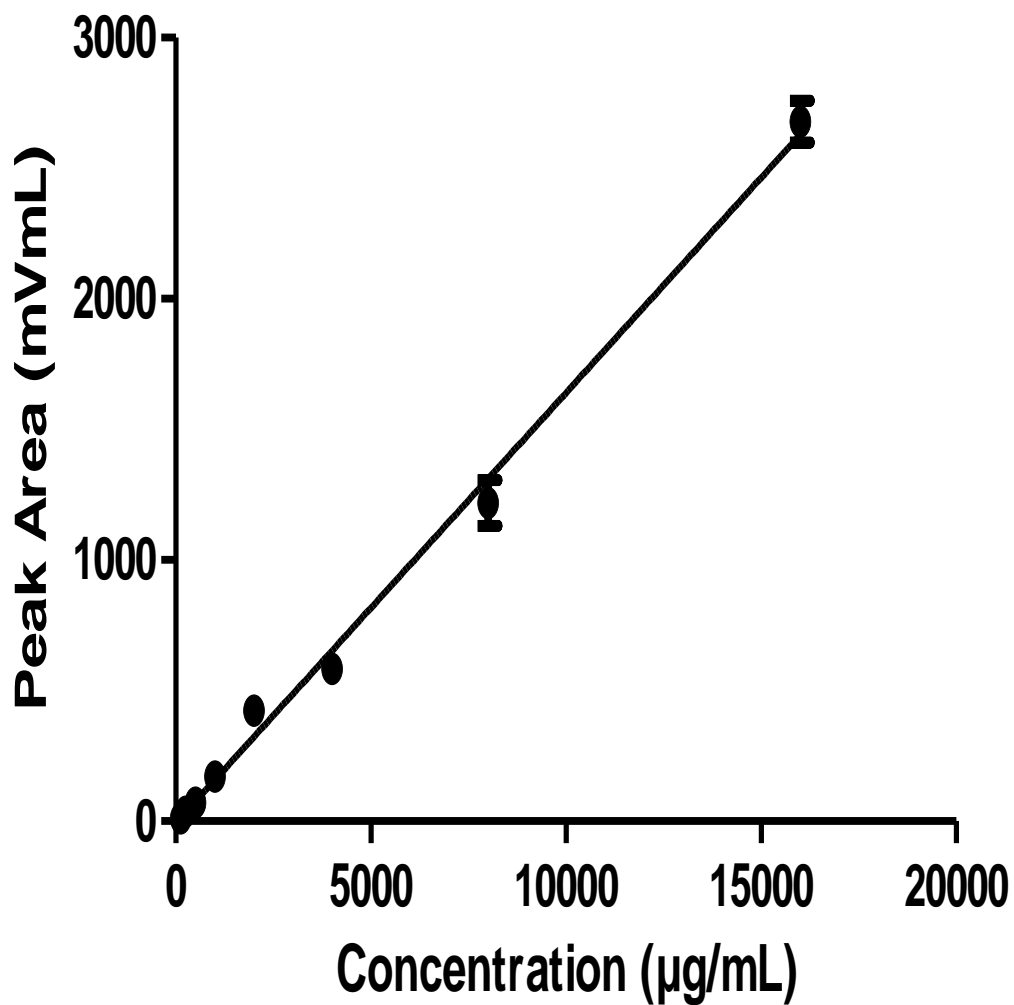


Figure 5.2 – Calibration curve of peak area against AGRPS concentration used in chromatographic analyses of plasma samples.

5.4.1.3 Accuracy and Precision

The accuracy at 125, 1000 and 2000 $\mu\text{g/mL}$ of test samples of AGRPS was calculated as 83.00 % \pm 0.01, 103.00 % \pm 0.13 and 111.00 % \pm 0.15 respectively based on equation 5.1. The precision (RSD %) at the above concentrations was calculated as 6.90 % \pm 0.01, 12.20 % \pm 0.13 and 5.00 % \pm 0.13 respectively based on equation 5.2. For accuracy, the mean value for 1000 $\mu\text{g/mL}$ and 2000 $\mu\text{g/mL}$ was within acceptable limits of \pm 15% (85-115%) of the actual value, while the mean value of the 125 $\mu\text{g/mL}$ (lowest concentration) was also within acceptable limits of \pm 20 % (80-120 %) of the actual value. For precision, 125, 1000 and 2000 $\mu\text{g/mL}$ test samples of AGRPS were within \pm 15% (85-115%) of the coefficient of variation. The HPSEC method was found to be within the limits of accuracy and precision for the quantitative measurement of AGRPS in plasma.

5.4.1.4 Recovery

The mean recovery (extraction efficiency) of AGRPS from plasma at 125, 1000 and 2000 $\mu\text{g/mL}$ was calculated as 71.00 % \pm 0.01, 92.00 % \pm 0.11 and 107.00 % \pm 0.03 respectively based on equation 5.3.

5.4.1.5 Sensitivity

The limits of detection and quantitation of the HPSEC method for the analysis of AGRPS in plasma were 14.4 $\mu\text{g/mL}$ and 43.7 $\mu\text{g/mL}$ respectively based on the standard deviation of the response and the slope of the calibration curve as stated in the ICH guideline for validation of analytical procedures [31].

5.4.2 Determination of the absorption of orally administered AGRPS into systemic circulation in rats

The aim of this study was to investigate whether orally administered AGRPS can be absorbed into the systemic circulation in rats by measuring the AGRPS plasma concentration. After oral administration of AGRPS, a small peak with a retention volume of 11.22 mL appeared 0.5 hr post dose (Figure 5.3A) that increased after 4 hr duration of exposure (Figure 5.3B). A second peak appeared with a larger retention volume of 16.03 mL after 4 hr treatment, this peak was interpreted as a biotransformed endproduct of AGRPS in the plasma sample (Figure 5.3B). This additional peak identified after 4 hr treatment was absent in the plasma sample after 0.5hr oral administration (Figure 5.3A). The AGRPS and its biotransformed end product eluted with retention volumes of 11.22 mL and 16.03 mL, which have molecular weights of 1240 kDa and 29 kDa, respectively based on the pullulan polysaccharide calibration curve.

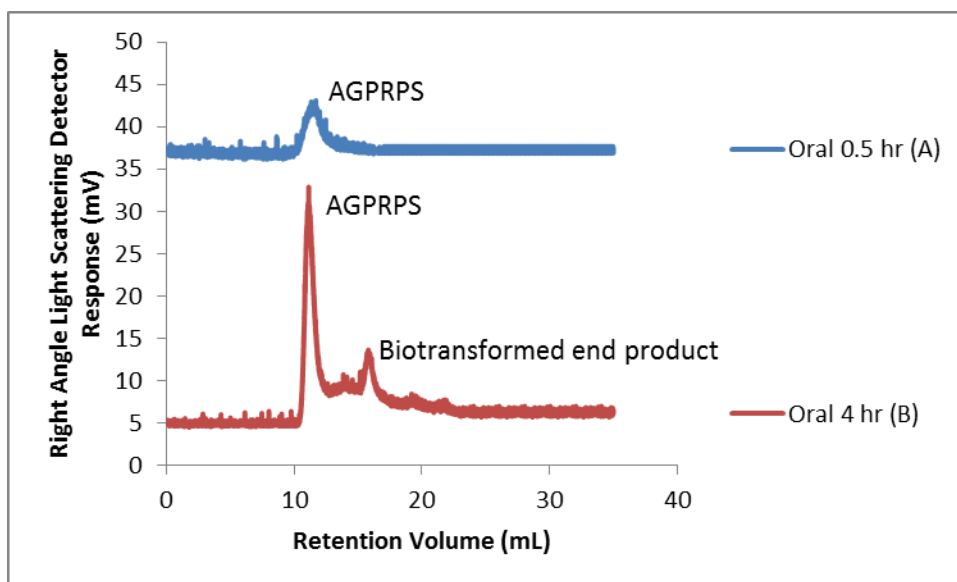


Figure 5.3 – Chromatograms obtained from high performance size exclusion chromatography of AGRPS analyte (11.22 mL) and its biotransformed end product (16.03 mL) in plasma samples collected from rats orally fed with 125 mg/kg single dose of AGRPS for (A) 0.5 hr and (B) 4 hr. Each processed sample was injected and eluted with 0.1 M NaNO₃ mobile phase on a series of AquaGel PAA-203 and PAA-204 columns (8 × 300 mm, PolyAnalytik, USA), this was monitored with right angle light scattering detector at 1 mL/min flow rate. The y-axis is the right angle light scattering detector response (mV), while the x-axis represents the retention volume (mL).

To substantiate the identity of the purported AGRPS peak observed after oral administration, the plasma profile after intraperitoneal (IP) administration of AGRPS was also investigated as this route of administration is known to bypass the gastrointestinal barrier. As shown in Figure 5.4A, a large peak, which has the same retention volume as the purported AGRPS peak, appeared 0.5 hr post dose. This peak showed a progressive decrease reaching a minimum level at 4 hr after treatment with the appearance of a second peak (biotransformed end product peak) as seen in Figure 5.4B. The AGRPS and its biotransformed end product eluted with retention volumes of 11.22 mL and 16.03 mL, which have molecular weights of 1240 kDa and 29 kDa, respectively based on the pullulan polysaccharide calibration curve.

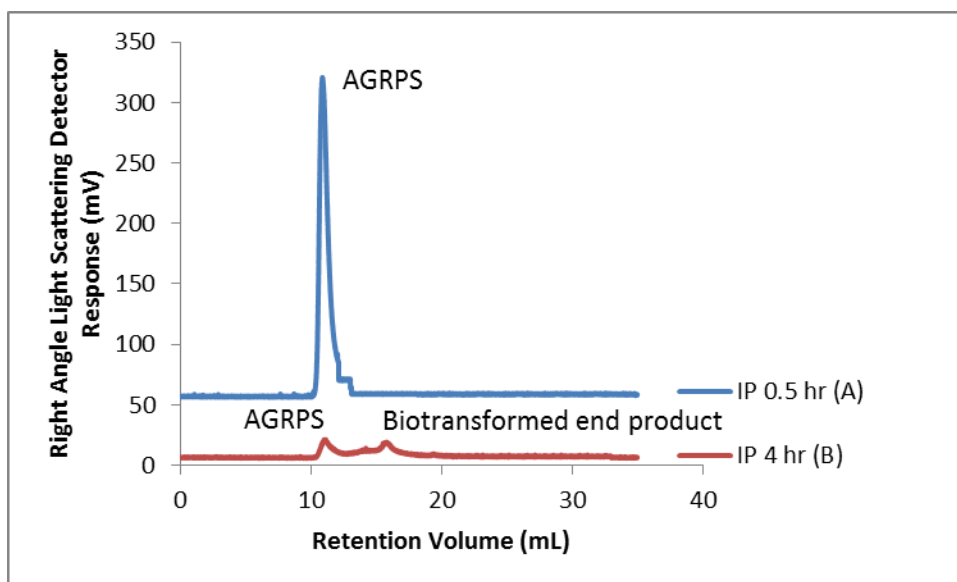


Figure 5.4 – Chromatograms obtained from high performance size exclusion chromatography of AGRPS analyte (11.22 mL) and its biotransformed endproduct (16.03 mL) in plasma samples collected from rats treated IP with 125 mg/kg single dose of AGRPS for (A) 0.5 hr and (B) 4 hr. Each processed sample was injected and eluted with 0.1 M NaNO₃ mobile phase on a series of AquaGel PAA-203 and PAA-204 columns (8 × 300 mm, PolyAnalytik, USA), this was monitored with right angle light scattering detector at 1 mL/min flow rate. The y-axis is the right angle light scattering detector response (mV), while the x-axis represents the retention volume (mL).

5.4.3 Verification of Polysaccharide peak in the plasma of AGRPS treated rats

Previous studies have reported the use of pectinase enzyme to hydrolyze and release pectin polysaccharides from plant polysaccharides including ginseng polysaccharides [32-41]. Cellulase enzyme was also investigated being used as a negative control in the verification of AGRPS. Cellulase is not known to hydrolyze ginseng polysaccharides, but has been used to enhance the release of ginsenosides from ginseng root during extraction [42-43].

To verify if the purported AGRPS peak from the plasma of the AGRPS treated rat is actually a polysaccharide peak, an enzyme hydrolytic method accompanied with HPSEC analysis was investigated. Two different enzymes (pectinase and cellulase) and incubation time points (1 hr, 8 hr and 24 hr) were examined. The enzymatic condition that hydrolyzed AGRPS the most was then applied to plasma samples of AGRPS treated rats. Pectinase enzymatic hydrolysis was used as a positive control, while cellulase enzymatic hydrolysis was used as a negative control for the enzymatic hydrolysis study.

HPSEC analysis of AGRPS solution incubated with pectinase for 1 hr showed the presence of a single peak as seen in Figure 5.5A, while 8 hr incubation of AGRPS solution with pectinase enzyme (Figure 5.5B) showed the presence of two peaks; with a decrease in the first peak detector intensity response. The 24 hr incubation of AGRPS solution with pectinase enzyme (Figure 5.5C) showed the presence of two peaks. The observed significant decrease in the first peak intensity and increase in the second peak intensity suggests the occurrence of hydrolysis of AGRPS by the pectinase enzyme. Only a single peak was observed after 1, 8 and 24 hr incubation with the cellulase enzyme (Figures 5.6A, 5.6B and 5.6C), which was used as a negative control for polysaccharide hydrolysis. The results clearly indicate the AGRPS peak of

interest was susceptible to hydrolysis by pectinase but not cellulase, substantiating the polysaccharides composition of AGRPS.

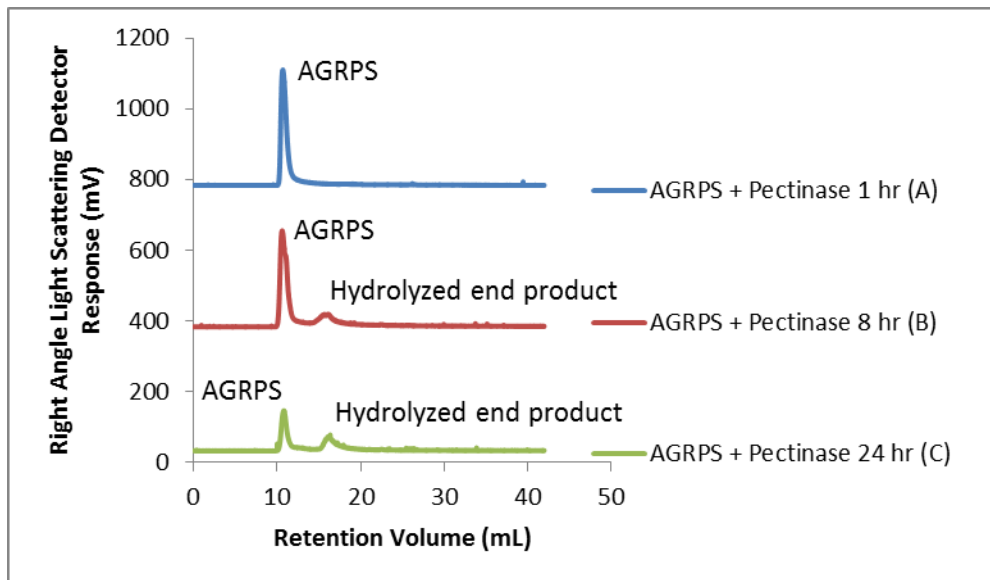


Figure 5.5 – Chromatograms obtained from high performance size exclusion chromatography analysis after (A) 1 hr (B) 8 hr and (C) 24 hr incubation of AGRPS solution with pectinase enzyme. Each processed sample was injected and eluted with 0.1 M NaNO₃ mobile phase on a series of AquaGel PAA-203 and PAA-204 columns (8 × 300 mm, PolyAnalytik, USA), this was monitored with right angle light scattering detector at 1 mL/min flow rate. The y-axis is the right angle light scattering detector response (mV), while the x-axis represents the retention volume (mL).

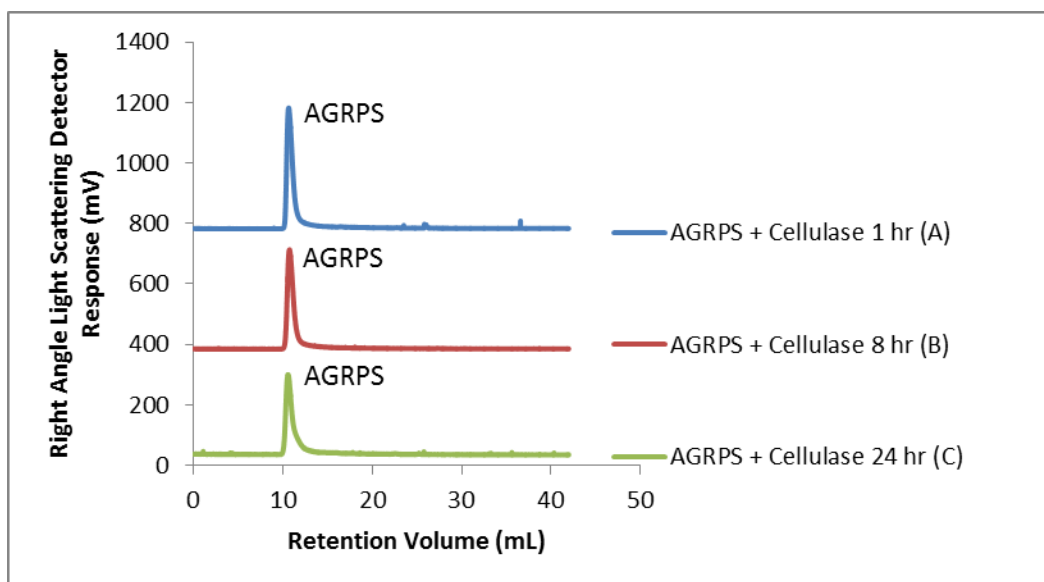


Figure 5.6 – Chromatograms obtained from high performance size exclusion chromatography analysis after (A) 1 hr, (B) 8 hr and (C) 24 hr incubation of AGRPS solution with cellulase enzyme. Each processed sample was injected and eluted with 0.1 M NaNO₃ mobile phase on a series of AquaGel PAA-203 and PAA-204 columns (8 × 300 mm, PolyAnalytik, USA), this was monitored with right angle light scattering detector at 1 mL/min flow rate. The y-axis is the right angle light scattering detector response (mV), while the x-axis represents the retention volume (mL).

The 24 hr incubation time with pectinase enzyme which gave the most significant hydrolytic outcome of the three investigated time points was then applied to the plasma samples obtained from rats treated 4hr orally and IP with AGRPS. As seen in Figures 5.7B and 5.8B, the HPSEC analysis of plasma samples incubated with pectinase enzyme showed the presence of two peaks. The detector response of the first peak and second peaks retention volumes decreased and increased respectively, suggesting that the pectinase enzyme was able to hydrolyze AGRPS in plasma due to its pectin polysaccharide composition. These data suggest that AGRPS is absorbed from the GIT barrier after oral administration.

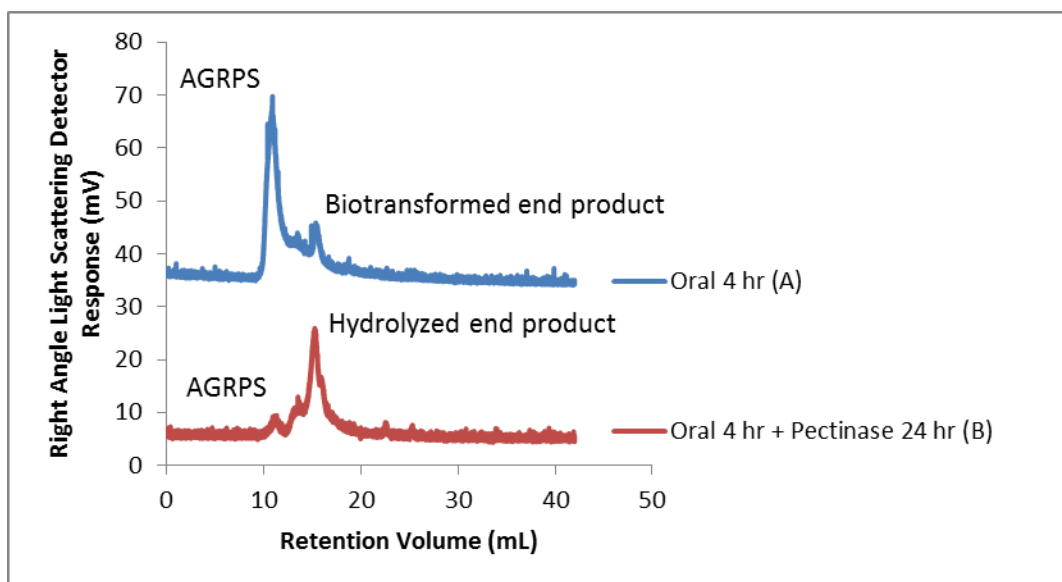


Figure 5.7 – Chromatograms obtained from high performance size exclusion chromatography analysis after 24 hr incubation of rat plasma obtained from 4 hr oral AGRPS treatment (A) without pectinase enzyme and (B) with pectinase enzyme. Each processed sample was injected and eluted with 0.1 M NaNO₃ mobile phase on a series of AquaGel PAA-203 and PAA-204 columns (8 × 300 mm, PolyAnalytik, USA), this was monitored with right angle light scattering detector at 1 mL/min flow rate. The y-axis is the right angle light scattering detector response (mV), while the x-axis represents the retention (hydrodynamic) volume (mL).

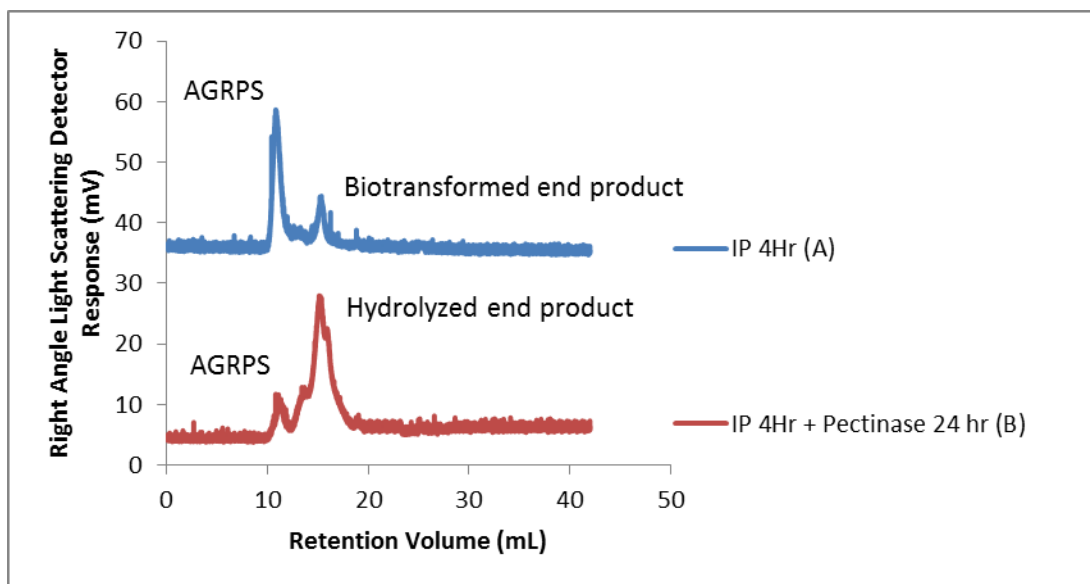


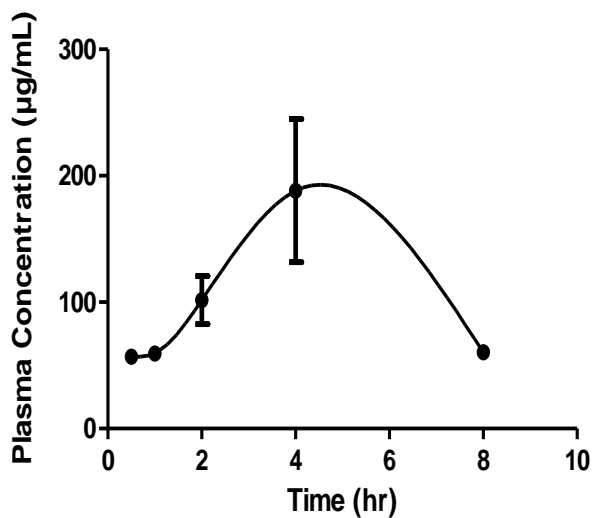
Figure 5.8 – Chromatograms obtained from high performance size exclusion chromatography analysis after 24 hr incubation of rat plasma obtained from 4 hr IP AGRPS treatment (A) without pectinase enzyme and (B) with pectinase enzyme. Each processed sample was injected and eluted with 0.1 M NaNO₃ mobile phase on a series of AquaGel PAA-203 and PAA-204 columns (8 × 300 mm, PolyAnalytik, USA), this was monitored with right angle light scattering detector at 1 mL/min flow rate. The y-axis is the right angle light scattering detector response (mV), while the x-axis represents the retention (hydrodynamic) volume (mL).

5.4.4 Determination of AGRPS at different times in plasma of rats treated with AGRPS

The HPSEC method was applied to study the AGRPS plasma profile in rats, after 0.5, 1, 2, 4 and 8 hr oral and IP administration of 125 mg/kg AGRPS (single doses), these data were identical to those presented in Figures 5.3-5.4. Plasma concentrations of AGRPS in rat at different time points after administration were estimated from the AGRPS calibration curve which was constructed by plotting the mean peak area against the mean concentrations of AGRPS as shown in Figures 5.2. The peak areas of AGRPS were derived from the software of the HPSEC system and related to the equation of AGRPS spiked into plasma calibration curve ($Y = 0.16473X - 6.9623$), with Y being the peak area and X is AGRPS concentration. Following oral administration of AGRPS, the plasma concentration increased gradually until it peaked and then declined. Figure 5.9A shows the time-dependent change in rat plasma concentration of AGRPS after oral administration. Plasma profile parameters including peak plasma level (C_{max}), time to reach the peak plasma concentration (T_{max}), area under the concentration–time curve ($AUC_{0.5-8}$) were evaluated. The C_{max} , T_{max} , and $AUC_{0.5-8}$ parameters of AGRPS after oral and IP administration are listed in Table 5.1. AGRPS reached the peak concentration in 4 hr (T_{max}) with a concentration of $188 \pm 52 \mu\text{g/mL}$ (C_{max}). After IP injection, AGRPS peaked in 0.5 hr (T_{max}) with a concentration of $2112 \pm 23 \mu\text{g/mL}$ (C_{max}) in plasma. The area under the curve ($AUC_{0.5-8}$) of AGRPS was observed to be $896 \pm 39 \mu\text{g}\cdot\text{hr/mL}$ and $2930 \pm 13 \mu\text{g}\cdot\text{hr} / \text{mL}$ for oral and IP routes respectively. The administration of AGRPS via the IP injection route resulted in a continuous decline as shown in Figure 9B. The plasma concentrations after oral administration were smaller than those measured after IP administration. As seen in Table 5.1, the total plasma volumes calculated from the Lee and Blaufox equation [44], estimates that $1.85 \pm 52 \text{ mg}$ of AGRPS are present at C_{max} in plasma after AGRPS oral treatment. After IP injection, 20.82 ± 23

mg estimates of AGRPS were calculated from this equation to be present in plasma at C_{\max} .

(A) Oral Administration



(B) IP Administration

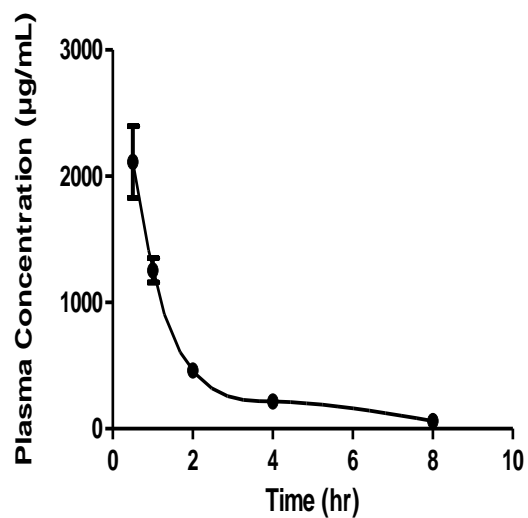


Figure 5.9 – Plasma concentration–time curve of AGRPS in rats after 0.5–8 hr (A) oral and (B) IP administration of 125 mg/kg single doses of AGRPS. Each data point represents the mean \pm SD ($n = 3$). The y-axis is AGRPS plasma concentration ($\mu\text{g/mL}$) while the x-axis represents the time post dose (hr).

Table 5.1. Plasma profile of AGRPS in rats administered by Oral and IP routes

Route	Body Weight (g)	Estimated Plasma Volume ** (mL)	Dose of AGRPS Treatment (mg)	T _{max} (hr)	AUC _{0.5-8} (µg/mL.hr)	C _{max} ** (µg/mL)	Estimated Amount of AGRPS @ C _{max} in Plasma (Conc x plasma volume) ** (mg)	Theoretical Amount of AGRPS @ C _{max} in Plasma (Assuming 100 % bioavailability) ** (mg)
Oral	240	9.86	30	4.0*	896 ± 39*	188 ± 52*	1.85± 52*	19.49
IP	240	9.86	30	0.5	2930 ± 13	2112 ± 23	20.82± 23	19.49

AUC_{0.5-8} (Area under the concentration–time curve from time 0.5 hr to 8 hr), C_{max} (maximum plasma concentration), T_{max} (time to attain C_{max}).

Data were expressed as means ± SD (n = 3). Statistical analyses were assessed using the Student's t-test. *Values of oral route compared to the IP route that are statistically significant differences were indicated by p-values of <0.05

** According to reported physiological parameter values, the AGRPS plasma concentrations at C_{max} are 188 µg/mL (oral) and 2112 µg/mL (IP) and the estimated total AGRPS plasma concentrations are 1.85 mg (oral) and 20.82 mg (IP) in total plasma volume, whereas the theoretical maximum amount of AGRPS in total plasma is 19.49 mg (assuming 100% absorption and zero first pass elimination). This will give an estimated 9.49 % (1.85/19.49 x 100%) and 106.82 % (20.82/19.49 x 100%) absorption of AGRPS for oral and IP routes respectively (assuming 100 % bioavailability).

5.4.5 Determination of the absorption of orally administered AQ extract into systemic circulation in rats

To further test the applicability of the HPSEC method, chromatographic analysis was done on the AQ extract and rat plasma samples collected 24 hr after the last day of a 6 day oral treatment with 125 mg/kg of AQ extract. Only one peak (Figure 5.10) eluted with retention volume similar to that of the peak interpreted as the biotransformed endproduct of AGRPS was detected. The absence of a measurable AGRPS peak may be due to the low levels of AGRPS in the sample, since AQ extract is known to contain about 10% of AGRPS by weight.

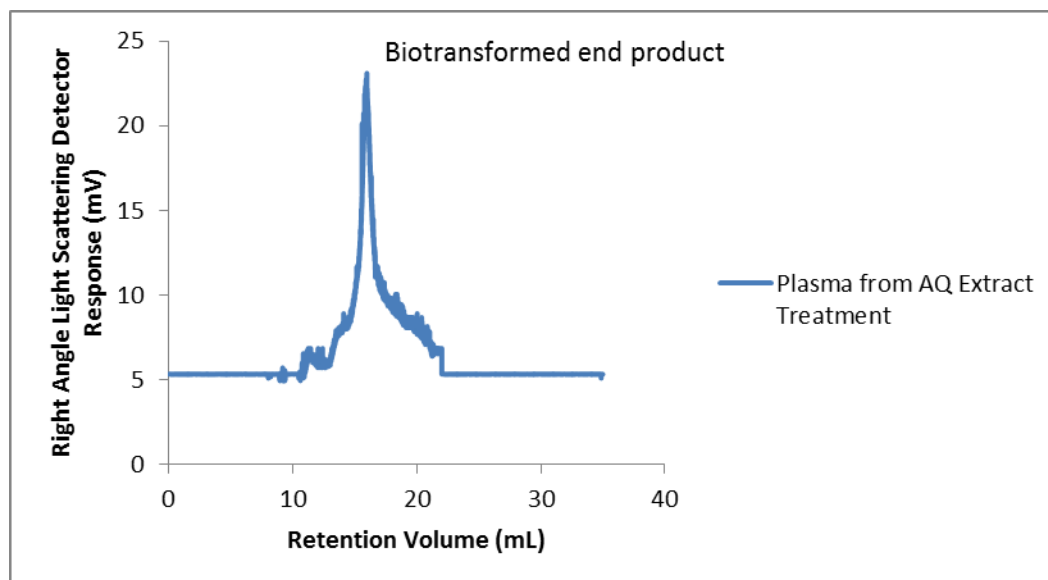


Figure 5.10 – Chromatogram obtained from high performance size exclusion chromatography of rat plasma sample collected 24 hr on the last day after a 6 days oral treatment with 125 mg/kg of AQ extract. Each processed sample was injected and eluted with 0.1 M NaNO₃ mobile phase on a series of AquaGel PAA-203 and PAA-204 columns (8 × 300 mm, PolyAnalytik, USA), this was monitored with right angle light scattering detector at 1 mL/min flow rate. The y-axis is the right angle light scattering detector response (mV), while the x-axis represents the retention volume (mL).

5.4.6 Determination of the absorption of orally administered CVT-E002TM into systemic circulation in rats

To further test the applicability of the HPSEC method, chromatographic analysis was done on CVT-E002TM and rat plasma samples collected 24 hr after the last day of a 6 day oral treatment with 125 mg/kg of CVT-E002TM. Peaks obtained from the chromatographic analysis eluted with retention volumes of 11.22 mL, 14.00 mL and 16.03 mL which correspond to 1240 kDa, 142 kDa and 29 kDa molecular weights respectively, based on pullulan polysaccharide calibration curve. The first peak in the plasma sample of the CVT-E002TM treated rats has an elution profile similar to that of CVT-E002TM (Figure 5.11) and AGRPS (Figures 5.1, 5.3-5.4), which demonstrates the applicability of the HPSEC method for plasma analysis of ginseng polysaccharides product.

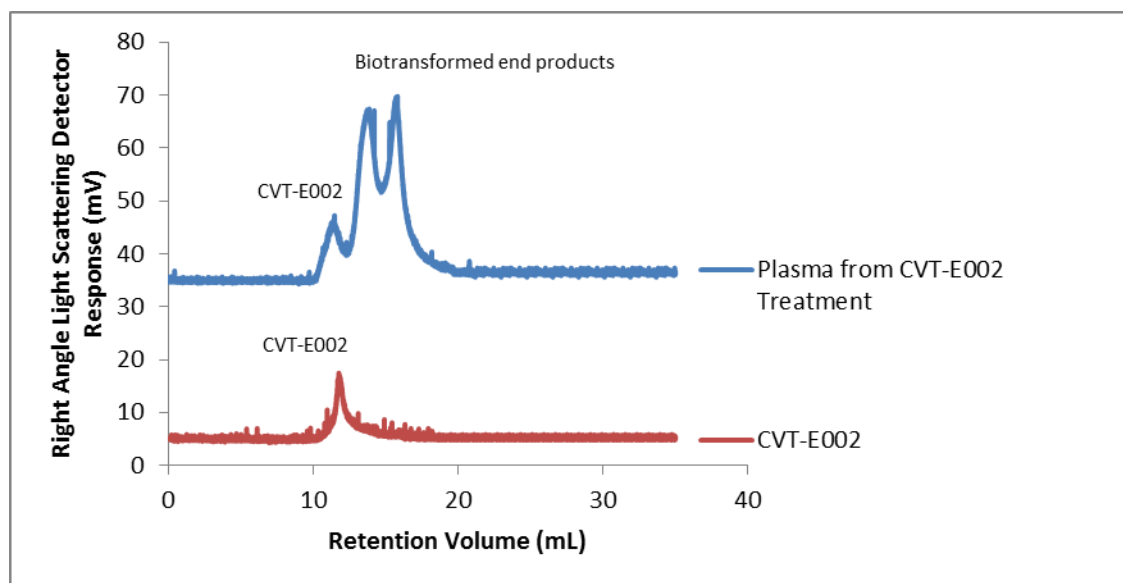


Figure 5.11 – Chromatograms obtained from high performance size exclusion chromatography of (A) CVT-E002TM (11.22 mL) and its biotransformed end products (14.00 mL and 16.03 mL) in rat plasma sample collected 24 hr on the last day after a 6 days oral treatment with 125 mg/kg of CVT-E002TM and (B) 2000 μ g of CVT-E002TM. Each processed sample was injected and eluted with 0.1 M NaNO₃ mobile phase on a series of AquaGel PAA-203 and PAA-204 columns (8 \times 300 mm, PolyAnalytik, USA), this was monitored with right angle light scattering detector at 1 mL/min flow rate. The y-axis is the right angle light scattering detector response (mV), while the x-axis represents the retention volume (mL).

5.5 Discussion

Despite polysaccharides such as AGRPS being soluble in water, their weak chromophore group and the complexity of the plasma matrix are barriers to overcome in their analysis in plasma. Various chromatographic and spectroscopic analytical methods have been used to analyze plant polysaccharides tagged with a fluorescent label or subjected to a post column fluorescence derivatization method with fluorometric detection in biological matrices (e.g. plasma) after *in vivo* treatments [19-23, 27-29]. The limitations of the use of labeled polysaccharides in previous chromatographic studies in plasma include; the difficulty of distinguishing metabolites from the parent of polysaccharide by these methods, polysaccharides may undergo isomerization, degradation or change of conformation during the labeling procedure, the isolation of the labeled polysaccharide from the reaction mixture is often complicated and time-consuming [20].

The development of a chromatographic method to analyze native (unlabeled) polysaccharides in plasma will be useful in human studies. Perchloric acid-protein precipitation of plasma and HPSEC with right angle light scattering detection used in this study served as a new approach to analyze unlabeled polysaccharides such as AGRPS in the plasma of rats. The chromatographic performance of HPSEC analytical method was validated and found to be specific, sensitive and within the limits of accuracy and precision with a linear detector response for the analysis of AGRPS in plasma. The method was also selective in that it was able to distinguish AGRPS from its biotransformed end product, which gave a different retention volume (Figure 5.1 compared to Figures 5.3 and 5.4). The data generated in chapter four of this thesis using ion exchange chromatography and ultrafiltration methodologies revealed that American ginseng polysaccharides are heterogeneous with a wide range of different molecular weights and ionic charge (neutral and acidic polysaccharides). Though the HPSEC method was able to separate

(resolve) native AGRPS from its hydrolyzed end product by molecular weight (Figures 5.5, 5.7-5.8), it was limited in that it could not separate native AGRPS into multiple species of acidic and neutral fractions.

The HPSEC data of Figure 5.3 indicates that orally administered AGRPS is absorbed from the GIT into systemic circulation in rats. This relates well with the objective of this study which was to ascertain if orally administered AGRPS can be absorbed from the GIT into systemic circulation. Figures 5.5 and 5.7 provide supportive evidence of AGRPS intestinal absorption, in that it verified the identity of the purported AGRPS chromatographic peak, which was susceptible to 24hr pectinase enzyme (but not cellulase) enzyme hydrolysis, thereby substantiating the polysaccharide composition of AGRPS. The time-dependent change in AGRPS rat plasma concentration after oral administration of the AGRPS (Figure 5.9) provided additional evidence of the intestinal absorption of AGRPS into systemic circulation and its elimination in rats. This result is consistent with the concept of absorption and elimination of biochemical compounds in biological systems.

There was a relative difference in the C_{max} of oral treatment compared to IP injection which can be attributed to the gastrointestinal barrier: the latter was higher than the former by 11 fold. Evaluation of total AGRPS concentration at C_{max} in total plasma volume was based on dose and total plasma volume using the Lee and Blaufox equation [44], which revealed that 9.49 % of orally administered AGRPS was available in systemic plasma 4 hr post dose. Since an intravenous route was not examined in this study, this value does not provide absolute bioavailability data, but serves as a useful predictor. One can infer that the oral route resulted in low AGRPS plasma level predicting a possible low bioavailability.

The polysaccharide analyte peak from the plasma of rats orally fed with AGRPS (Figure 5.3) had an elution profile similar to AGRPS (Figure 5.1B) and AGRPS spiked in blank plasma (Figure 5.1C) with a retention volume of 11.22 mL and a corresponding molecular weight of 1240 kDa. The measured AGRPS molecular weight of 1240 kDa is close to the 1500 kDa, 1800 kDa and 1900 kDa molecular weights that have been previously reported for ginseng polysaccharides [14-15, 45].

The validated HPSEC analytical method from this work, which does not require labeling, was able to analyze AGRPS in plasma of rats orally fed with AGRPS (Figures 5.3 and 5.7). As seen in Figures 5.10 and 5.11, this promising new approach was found to be applicable in the plasma analysis of rats orally treated with AQ extract and CVT-E002TM. The achieved results from this study will be highly instructive for future use in human studies of AGRPS pharmacokinetics and the pharmaceutical development of AGRPS natural health products.

The low levels of AGRPS in the plasma of orally fed rats are pharmacologically in close agreement with the *in vitro* 50-200 µg/mL immunobioactive concentration of AGRPS in cultured murine cell line and rat alveolar macrophages reported in chapters 3 and 4. Polysaccharides of *Radix ophiopogonis* ($M_w = 4.8$ KDa) have been reported to have low bioavailability of about 1.7 % in rats after oral administration, which was attributed to their large molecular size and hydrophilic characteristics [29]. The low plasma profile of orally administered AGRPS compared to its IP treatment (Figure 5.9) can be attributed to several factors including low membrane permeability, poor or incomplete absorption, high molecular weight and hydrophilicity (water solubility) of AGRPS, poor blood flow from the gut compared to that of the peritoneal cavity, hepatic, gastric and/or intestinal first-pass metabolism (enzymatic, chemical, bacterial) [46-50] and difference in the route of administration. Orally administered

ginsenosides (bioactive component of ginseng root) have been reported to demonstrate low bioavailability due to their low membrane permeability across the intestinal mucosa, active biliary excretion, decomposition in the stomach, metabolism in the large intestine and elimination in the liver. Of these factors, low membrane permeability and active biliary excretion has been attributed to be the two major factors that limits the absorption of orally administered ginsenosides from the gastrointestinal tract into systemic circulation [51-54].

A possible mechanism by which AGRPS is absorbed via the GIT may be endocytosis, whereby molecules are engulfed by the membrane and move through the intestinal cell in vesicles, which would be subsequently released on the membrane's other side [55]. Of recent, one chromatographic study reported that orally administered polysaccharides were detected in rat serum by high performance liquid chromatography analysis. These polysaccharides were found to be internalized in human intestine mucosa epithelial cells and the underlying mechanism of their intestinal absorption was found via clathrin-mediated endocytosis [56].

The natural function of the GIT is to digest and absorb nutrients which sustain the living system. By the oral route, biochemical compounds pass sequentially from the GIT lumen, through the gut wall and then the liver before entering the systemic circulation. Passive diffusion is the predominant mechanism for the permeation of biochemical compounds through the GIT membrane barrier. A compound must have favorable physicochemical properties (i.e. lipophilicity and low molecular weight) to undergo passive diffusion. Endogenous biochemical compounds that are necessary for life (including polysaccharides) do not have physicochemical properties that allow for sufficient passive diffusion to occur, so there are membrane transporters that greatly enhance their permeability [55]. It's possible that AGRPS is a substrate of uptake membrane transporters which enables AGRPS absorption from the GIT into systemic circulation,

despite its high molecular weight and hydrophilic physicochemical properties. This possibility has not been reported for AGRPS.

Chromatographic data from this study provides novel evidence and answers to the question of whether orally administered AGRPS extract can be absorbed from the GIT into systemic circulation. It also lends credence to the fact that orally administered AGRPS extract can be absorbed from the GIT into systemic circulation to exert the *in vivo* and *ex vivo* immunomodulatory effects observed in chapter 4 of this thesis.

5.6. Conclusion

A new approach using acid protein precipitation and HPSEC with light scattering detection was developed for the analysis of AGRPS in plasma and outcome from its application indicates that AGRPS is absorbed from the GIT into systemic circulation after oral administration. This new approach does not require labeling and is suitable for the analysis of AGRPS in plasma during a single analytical run.

5.7 References

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Chapter 6

DISCUSSION AND CONCLUSIONS

6.1 Summary and Discussion

6.1.1 Chapter Three: *In vitro* Immunostimulatory and Anti-inflammatory Effects of American Ginseng Aqueous and Alcoholic Extracts

In view of the reported ginseng inconsistent immunostimulatory and immunosuppressive effects [1-12], which may be attributed to different extraction solvent matrix, the aims of chapter three were to; (i) evaluate the immunostimulatory and immunoinhibitory effects of ginseng aqueous (AQ) and alcoholic (ALC) extracts and (ii) identify what bioactive fractions in these extracts cause the immunomodulatory effects. The immunobioactivities of the ginsenoside and macromolecule fractions of ginseng ALC extract was also examined to address the gap in knowledge of the immunoinhibitory fractions of the extract.

The hypothesis was that ginseng AQ extract is immunostimulatory while its ALC extract is immunoinhibitory. Bioassay of the ginseng extracts revealed that the direct treatment of AQ extract up-regulate macrophage production of NO, TNF- α and IL-6 mediators while that of the ALC extract did not. The ALC extract was found to suppress lipopolysaccharide (LPS) induced macrophage NO, TNF- α and IL-6 production, while the AQ extract did not suppress LPS proinflammatory response. These outcomes support the stated hypothesis that ‘ginseng AQ extract is immunostimulatory while its ALC extract is immunoinhibitory’. A novel finding was achieved in chapter three in that the macrophage-stimulating activity of the AQ extract was inhibited in the presence of the ALC extract. Fractionation of the AQ extract revealed the presence of two major peaks with average molecular weights of ~73 kDa and ~37 kDa. The first fraction had a similar elution volume as the crude polysaccharide (PS) fraction isolated from the AQ extract, and it was the only bioactive species. A parallel fractionation study of the ALC

extract yielded novel macromolecules (devoid of PS) which are overlooked by many investigators who focus mainly on ginsenoside biological activities. To investigate the immunobioactive fractions that contribute to the ALC extract immunoinhibitory effect, the extract was fractionated into the macromolecule and ginsenoside fractions by ultrafiltration. Their suppression of LPS induced production of NO, TNF- α and IL-6 was studied in murine macrophages (RAW 264.7 cell line) using an *in vitro* model that emulates predominately LPS endotoxic condition. The macromolecule and ginsenoside fractions exerted immunoinhibitory effect. Hence, the macromolecule and ginsenoside fractions contribute to the immunoinhibitory effect of ginseng ALC extract.

The ALC extract of American ginseng, its macromolecule and ginsenoside fractions which are devoid of PS are immunoinhibitory. The AQ extract and its PS phytochemical are immunostimulatory. Bioassay revealed that ALC extract suppressed AQ extract up-regulation of macrophage NO production, suggesting that concurrent consumption of both AQ and ALC extracts will lead to the loss of AQ extract immunostimulatory health benefit. Findings from this chapter provide evidence highlighting the important role of extraction solvent on ginseng bioactive phytochemicals and immunomodulatory effects. This has significant implications with regards to the production, the immunomodulatory efficacy and use of ginseng extracts and natural health products. The outcomes from chapter three provide a pathway for identifying immunobioactive fractions of ginseng. The immunobioactivities of ginseng are primarily associated with the presence of its polysaccharides, macromolecule and ginsenoside fractions which are dependent on the extraction solvent used in preparing ginseng extracts and its natural health products.

6.1.2 Chapter Four: Stimulation and Suppression of Innate Immune Function by American

Ginseng Polysaccharides: Biological Relevance and Identification of

Bioactives

Polysaccharides which are the key immunostimulatory phytochemicals of American ginseng AQ extract have recently been shown to suppress response to proinflammatory challenge [13-20]. Physicochemical properties such as water solubility, ionic charge, molecular weight, are known to affect the biological activity of polysaccharides, including immunomodulation [21-23]. The aims of chapter four were to; (i) determine whether AGRPS extract stimulates basal innate immune function and at the same time can suppress LPS proinflammatory challenge and (ii) identify the bioactive fractions responsible for AGRPS extract immunobioactivities. The relationship between the ionic charge and molecular weight of AGRPS fractions and their influence on AGRPS modulation of macrophage function under basal and LPS proinflammatory conditions knowledge gap was also investigated.

The first hypothesis was that AGRPS extract possess both immunostimulatory and immunosuppressive effects under basal and LPS endotoxic conditions respectively. While the second hypothesis was that the ionic charge and molecular weight variations contribute to AGRPS immunomodulatory activities. As shown in chapter four, culture of alveolar macrophages obtained from AGRPS extract treated rats resulted in an increase of *ex vivo* production of NO and also reduced the responsiveness of the alveolar macrophages to *ex vivo* LPS challenge. AGRPS extract *ex vivo* immunomodulatory effects were dependent on the duration of exposure. Oral treatment with AGRPS extract elevated plasma TNF- α concentration *in vivo*. This treatment also suppressed LPS-induced elevation of plasma TNF- α *in vivo*. AGRPS

extract also exerted *in vitro* immunostimulatory and immunosuppressive effects, under basal and LPS endotoxic conditions respectively. This outcome is in agreement with the first hypothesis that ‘AGRPS extract possess both immunostimulatory and immunosuppressive effects under basal and LPS endotoxic conditions respectively’. The *in vitro* mechanistic study revealed that the immunostimulatory and immunosuppressive effects of AGRPS extract are mediated primarily by its acid polysaccharides components, particularly the species with molecular weights ≥ 100 kDa and 100 - 50 kDa. This outcome supports the second hypothesis that ‘the ionic charge and molecular weight variations contribute to AGRPS immunomodulatory activities’. The ability of AGRPS pretreatment to suppress LPS proinflammatory response suggests that for a person who consumes ginseng polysaccharides health products e.g. CVT-E002™ (sold commercially as COLD-FX®), exposure to LPS related stimulatory infectious condition will not cause additive toxic stimulatory effects, but rather there will be a suppression of LPS stimulatory effect. Such beneficial immunomodulatory response is desired for future prophylactics against LPS related disease conditions like endotoxemia.

6.1.3 Chapter Five: Analysis of Intestinally Absorbed American Ginseng Polysaccharides

in Plasma by High Performance Size Exclusion Chromatography

Though scientific studies have reported that orally administered ginseng polysaccharides exerts numerous beneficial pharmacological effects namely; anti-depressant [24], anti-ulcer [25-26], anti-tumor effects [27] and immunomodulation [28-31] which was observed in chapter four, there is no method available presently to analyze ginseng polysaccharides in plasma. Such a method would serve as a key that opens the door to investigating the absorption of AGRPS from

gastrointestinal tract (GIT) into systemic circulation after oral administration of AGRPS.

Chromatographic analytical methods have been used to analyze plant polysaccharides tagged with a fluorescent label or subjected to a post column fluorescence derivatization method with fluorometric detection in biological matrices (e.g. plasma) after oral treatment [32-37]. The development of a chromatographic method to analyze unlabeled polysaccharides in plasma will serve as a useful tool in human studies, and for ascertaining the gastrointestinal (GIT) absorption of orally administered ginseng polysaccharides into systemic circulation. The aims of chapter five were to; (i) develop and apply a method for the determination of AGRPS in plasma and (ii) ascertain that orally administered AGRPS can be absorbed from the gastrointestinal tract into systemic circulation. The hypothesis was that orally administered AGRPS is absorbed from the GIT into systemic circulation. Perchloric acid-protein precipitation of plasma and high performance size exclusion chromatography (HPSEC) with right angle light scattering detection was used in chapter five as; (i) a new approach to analyze orally administered unlabeled AGRPS in rat plasma and (ii) a tool to address the gap in knowledge with regards to the absorption of orally administered AGRPS from the GIT into systemic circulation.

The HPSEC analytical method was applied to analyze unlabeled AGRPS in plasma and study its plasma profile in rats. The low plasma level of AGRPS obtained upon the application of the newly developed and validated HPSEC analytical method supports the stated hypothesis that 'orally administered AGRPS is absorbed from the GIT into systemic circulation'. The low plasma profile after oral treatment indicates that AGRPS is poorly absorbed from the GIT into systemic circulation may be due to its low membrane permeability, poor or incomplete absorption, high molecular weight and hydrophilicity (water solubility) of AGRPS, poor blood flow from the gut compared to that of the peritoneal cavity, hepatic, gastric and/or intestinal first-

pass metabolism (enzymatic, chemical, bacterial) [38]. Polysaccharides of medicinal plants such as *Radix ophiopogonis* have been reported to have low oral bioavailability (1.7%) in rat which can be attributed to their large molecular size and hydrophilic character [37]. The verification of the identity of AGRPS from plasma of treated animals using an enzyme hydrolytic method accompanied with HPSEC analysis provided supportive evidence of the absorption of AGRPS from GIT into systemic circulation. The time-dependent change in plasma concentration after oral administration of the polysaccharides in rats provided additional evidence of the intestinal absorption of AGRPS into systemic circulation.

HPSEC analysis of plasma samples from rats treated with CVT-E002™ (a polysaccharide herbal product of American ginseng root sold commercially as COLD-FX®) suggests the applicability of this new approach to the analysis of AGRPS in plasma. This work provides a new opportunity for human study of AGRPS pharmacokinetics. The knowledge of AGRPS plasma concentrations will enhance their prophylactic and therapeutic use in herbal medicine. The promising application of this new approach and the achieved results will be highly instructive for future pharmaceutical development of AGRPS natural products. Results from this study indicate that AGRPS is absorbed from the GIT into systemic circulation after oral administration. A possible mechanism by which AGRPS is absorbed via the GIT may be endocytosis; a recent chromatographic study which detected orally administered polysaccharides in rat serum has discovered the underlying mechanism responsible for their intestinal absorption to be via clathrin-mediated endocytosis [39].

6.2 Research Significance

The bioactive components and the extract solvent matrix (aqueous or alcoholic) play a crucial role in the biological effects of medicinal plants (e.g. American ginseng) and their natural products. This is especially true of immunotherapeutics, where their up-regulatory and down-regulatory interaction with innate component of the immune system (e.g. macrophages) determines their observed immunopharmacological effects. Identification of the bioactive(s) and extract of medicinal plants and natural products associated with immunomodulatory effect(s) is vital to provide appropriate prophylaxis or therapy [40]. Ginseng has been used for thousands of years as a tonic to improve overall health, restoration of homeostasis, body healing and reduction of overall stress, the medicinal use of American ginseng traces back about 400 year [16, 41-42].

The merit of the studies performed in this thesis is that a scientific approach of evaluating medicinal plants used in traditional medicine [40, 43] was utilized; via the phytochemical and immunopharmacological characterization of extracts and their bioactives from good agricultural practice quality American ginseng roots randomly collected from five different farms by the Ontario Ginseng Growers Association in Ontario, Canada. The outcomes of the studies performed in this thesis will be useful for future development of American ginseng products that may prevent and reduce risk of immune diseases such as endotoxemia. With the aid of new technology a novel approach was used for the analysis of AGRPS in plasma of rats orally fed with AGRPS and CVT-E002™, thereby enhancing its plausible future use in human study of AGRPS pharmacokinetics. The promising application of this novel approach and the achieved results will be highly instructive for future development of AGRPS natural health products.

6.3 Future Directions

The phytochemical and immunopharmacological characterization of American ginseng provided valuable information on the immunoinhibitory activity of novel macromolecules in American ginseng ALC extract. This provides a new opportunity for future *in vivo* and clinical investigations into their use as plausible medicinal agents against endotoxic LPS related inflammatory diseases. As a follow up for investigating the *in vivo* immunomodulatory effects of these novel macromolecules, an analytical method should also be developed and validated for the detection and quantification of these novel macromolecules in plasma to ascertain their absorption into systemic plasma following oral administration.

Investigation of the *in vitro* immunomodulatory effects of the acidic and neutral polysaccharide fractions of AGRPS extract revealed that ionic charge and molecular weight are key physicochemical properties that influence AGRPS extract immunomodulatory effects. It will be worthwhile for a further *in vivo* study to be carried out, since there is a paucity of information with respect to *in vivo* data in this regard. Such a study can be used to correlate the observed *in vitro* immunomodulatory effect. Future studies which examine the relationship between AGRPS ionic charge and molecular weight physicochemical properties and its ability to up-regulate and down-regulate TLR4 receptor expression under basal and LPS endotoxic conditions should be done. Chromatographic and spectrometric analytical techniques should be used in the future to elucidate structural information of the acidic and neutral polysaccharide fractions of AGRPS and that of ginseng ALC extract macromolecule fractions.

The novel approach used in this thesis to analyze AGRPS in rat plasma, indicates that orally administered AGRPS is absorbed through the GIT into systemic circulation in rats. Hence, a

tissue distribution study in rats and a human pharmacokinetic study of AGRPS in plasma should also be carried out.

In vitro bioavailability study should be done as a follow-up investigation in human epithelial Caco-2 cell line to determine the time and concentration dependent uptake and metabolism profiles of AGRPS. HPSEC should be applied in the proposed *in vitro* study to determine AGRPS and metabolite concentration in cells. Investigation should also be performed to determine whether clathrin-mediated endocytosis is an underlying mechanism responsible for the intestinal absorption of AGRPS. While perchloric acid-protein precipitation was used to isolate AGRPS from plasma in this study, a recent study has reported the use of a solid phase extraction (SPE) methodology to isolate oligo- and polysaccharides from maltodextrins, sugar syrup and honey [44]. The use of SPE methodology may have a favorable application and should be investigated for future use in the analysis of AGRPS in plasma.

Sepsis is a leading cause of in-hospital mortality and morbidity in Canada, and its prevention and control are essential for patient safety and quality of life [45]. A prospective observational study of 12 Canadian community and teaching hospital critical care units found that mortality for patients with severe sepsis was slightly over 38% [46]. Sepsis is the most common cause of acute lung injury (ALI), since 40–60% of patients with sepsis develop lung injury, regardless of the anatomic site of infection [47-48]. Sepsis is a severe, systemic inflammatory response to overwhelming infection that can be triggered by LPS endotoxins of Gram-negative bacteria e.g. *Escherichia coli* [49].

Sepsis-induced ALI and its most severe form, the acute respiratory distress syndrome (ARDS) is characterized by increased activation, influx and adhesion of inflammatory cells to pulmonary

microvascular endothelial cells (PMVEC) barrier, and their production of inflammatory mediators and oxidant stress [50-53]. These septic hallmark events cause vascular lung injury and increased EC barrier permeability with protein leaks, resulting in edema and lung inflammation [50]. Therapeutic options are limited once ALI develops, making prevention paramount [54]. Many treatments identified in preclinical studies have failed to improve patient outcomes despite compelling preclinical data [55-58]. Inadequate and delayed recognition of patients at risk and the subsequent development of the full blown septic ALI syndrome before therapy which obscured the therapeutic window have been linked as contributory factors [54]. Preliminary data suggests that ALI is rarely present at the time of hospital admission but develops over a period of hours to days in patients with predisposing conditions such as sepsis [59-66]. Patients admitted and treated for sepsis in the intensive care unit (ICU) are often transferred from operating rooms (ORs) and emergency departments (EDs) [67-68]. Early prevention and intervention with potential prophylactic pretreatment, targeted at these high risk septic ALI patients (in the ORs and EDs) at the time of hospital admission could improve the patient's quality of life and reduce health costs.

Based on the outcomes of this thesis it will be worthwhile in the future to examine whether American ginseng possesses any protective effect against sepsis-induced ALI by;

1. Evaluating the *in vitro* protective effects of AGRPS extract and novel high molecular weight macromolecules of ALC extract against simulated septic ALI inflammatory cell activation, PMVEC barrier dysfunction and oxidant stress.
2. Investigating if AGRPS extract and novel high molecular weight macromolecules of ALC extract exhibit *in vivo* protective effect against septic ALI, and examine their beneficial effects in

improving survival in septic ALI.

6.4 Conclusions

The ALC extract of American ginseng and its novel high molecular weight macromolecule fractions (devoid of polysaccharides) exert a direct immunoinhibitory effect. AGRPS possess *ex vivo*, *in vivo* and *in vitro* direct immunostimulatory and in-direct immunosuppressive effects in basal immune function and LPS proinflammatory conditions respectively. These immunobioactivities can be attributed to the acidic polysaccharides (PS) and its higher molecular weight fractions and not neutral PS or the lower molecular weight fractions of acidic PS. Hence AGRPS may have a beneficial suppressive effect against LPS related disease conditions such as endotoxemia. These unique pharmacological properties of American ginseng will hopefully be translated into future development of novel immunotherapeutics. The *in vivo* pharmacological effects including immunomodulation of orally administered AGRPS can be attributed to its absorption from the GIT into systemic circulation which enables its distribution to different pharmacological sites of action. The novel approach outlined in this thesis suggests that orally administered AGRPS has a low plasma profile. The promising application of the novel approach used in this thesis and the achieved results will be highly instructive for future human study of AGRPS pharmacokinetics and development of AGRPS natural health products.

6.5 References

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Department of Physiology and Pharmacology
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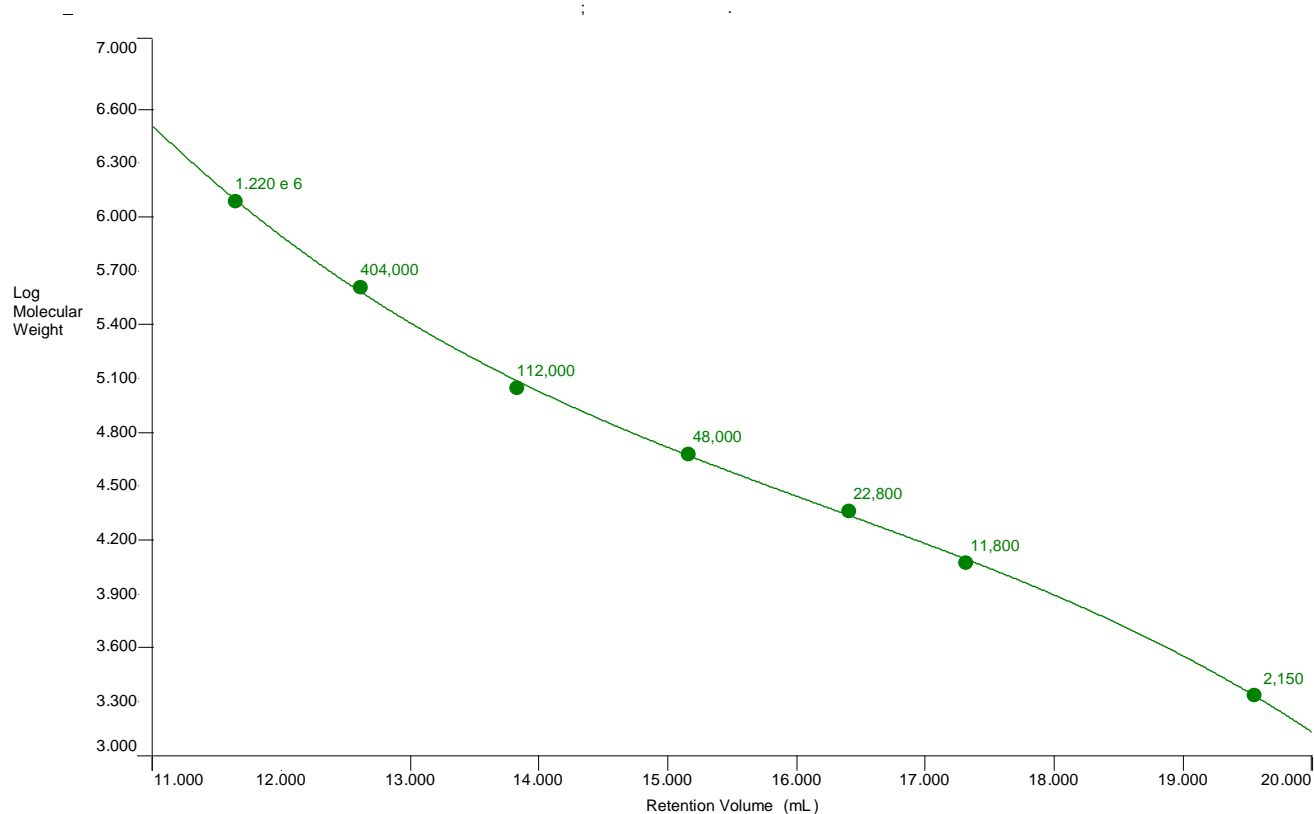
I await your kind response.

Best Regards,

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Appendix 4: Standard curve of pullulan polysaccharide Log Molecular weight versus

Retention volume for determination of molecular weight for Chapter 5



The pullulan polysaccharide reference standards with molecular weights 2150, 11800, 22800, 48000, 112000, 404000 and 1220000 Da eluted with corresponding retention volumes of 19.55 mL, 17.31 mL, 16.40 mL, 15.16 mL, 13.83 mL, 12.61 mL and 11.64 mL. The weight average molecular weight of AGRPS was determined from the pullulan polysaccharide calibration curve plot, i.e. ($R_v = -2.9506 \text{ Log } M_w + 29.203$), where R_v is the retention volume and M_w is the molecular weight.

Appendix 5: Calculation of AGRPS Maximum Plasma Concentrations in Chapter 5

The use of an arbitrary value of 7% (0.07) for estimation of blood volume can lead to significant errors. Blood volume of rat can be related to body weight using the experimentally determined equation of Lee and Blaufox (1985); $BV = 0.06 \times BW + 0.77$. In which blood volume = BV in mL and BW = body weight in grams. Lee and Blaufox (1985) also reported that plasma constitutes ~ 65 % of rat total blood volume [1].

Dose of AGRPS = 125 mg/kg.

For 240g rat treated with 30 mg (30000 μ g) used in this study; $BV = 0.06 \times 240 + 0.77 = 15.17$ mL total blood volume.

Plasma constitutes ~ 65 % of rat total blood volume, hence plasma volume will be

$$= 65/100 \times 15.17 \text{ mL} = 9.86 \text{ mL.}$$

Assuming 100% bioavailability, 15.17 mL rat total blood volume will contain 30mg of AGRPS, while 9.86mL rat total plasma volume will contain = $9.86/15.17 \times 30\text{mg} = 19.49\text{mg}$ of AGRPS.

For Oral route, $C_{\text{max}} = 188 \mu\text{g/mL}$ of AGRPS;

At C_{max} , 9.86 mL of rat plasma volume will contain = $9.86 \text{ mL} \times 188 \mu\text{g/mL}$

$$= 1853 \mu\text{g of AGRPS.}$$

$$= 1.85 \text{ mg of AGRPS}$$

At C_{max} , after Oral dose, percentage of AGRPS in plasma = $1.85 \text{ mg}/19.49 \times 100\% = 9.49 \%$

For IP route, $C_{\max} = 2112 \mu\text{g/mL}$ of AGRPS;

At C_{\max} , 9.86 mL of rat plasma volume will contain $= 9.86 \text{ mL} \times 2112 \mu\text{g/mL}$

$$= 20824 \mu\text{g of AGRPS}$$

$$= 20.82 \text{ mg of AGRPS.}$$

At C_{\max} after IP dose, percentage of AGRPS in plasma $= 20.82 \text{ mg} / 19.49 \times 100\% = 106.82 \%$.

Reference

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CURRICULUM VITAE

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EDUCATION:

Doctor of Philosophy (PhD), Pharmacology and Toxicology, University of Western Ontario, London, Ontario, Canada – 2014.

Master of Science (MSc), Pharmaceutical Analysis with Merit Award, University of Strathclyde, Glasgow, United Kingdom – 2006.

Bachelor of Pharmacy (BPharm) with Distinction Award, Obafemi Awolowo University, Ile-Ife, Nigeria – 2003.

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-Phytochemical and *In vivo* & *In vitro* Pharmacological Characterization of American Ginseng Extracts.

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-Bioassay Screening of Medicinal Plants in the Drug Research and Production Unit.

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Lui EMK, Azike CG, Guerrero-Analco JA, Kalda SJ, Romeh AA, Pei H, Arnason JT, Charpentier PA (2012). Bioactive Polysaccharides of American Ginseng *Panax quinquefolius* L. in Modulation of Immune Function: Phytochemical and Pharmacological Characterization. *The Complex World of Polysaccharides, InTech Publications, Chapter 19, 513-534.*

Azike CG, Charpentier PA, Hou J, Pei H, Lui EMK (2011). The Yin and Yang actions of North American ginseng root in modulating the immune function of macrophages. *Chinese Medicine, 6, 1-12.*

Zhang T, Watson DG, Azike C, Tettey JN, Stearns AT, Binning AR, Payne CJ (2007). Determination of Vancomycin in Serum by Liquid Chromatography. *Journal of Chromatography B, 857, 352–356.*

Iwalewa EO, Adewunmi CO, Omisore NO, Adebajji OA, Azike C, Adigun AO, Adesina OA, Olowoyo OG (2005). Pro- and Antioxidant Effects and Cytoprotective Potentials of Nine Edible Vegetables. *Journal of Medicinal Food, 8, 539-544.*

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Azike CG, Charpentier PA, Lui EMK (2014). Stimulation and Suppression of Innate Immune Function by American Ginseng Polysaccharides: Biological Relevance and Identification of Bioactives. *Manuscript in preparation.*

Azike CG, Charpentier PA, Xu ZW, Romeh AA, Lui EMK (2014). Analysis of Intestinally Absorbed American Ginseng Polysaccharides in Plasma by High Performance Size Exclusion Chromatography. *Manuscript in preparation.*

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Poster presented: Anti-inflammatory effect of North American (NA) ginseng.

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Poster Presented: Paradoxical Immuno-modulating Effect of North American Ginseng Aqueous and Polysaccharide Extracts on Macrophage Immune Function.

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