ISOLATION AND CHARACTERISATION OF THE POTENTIAL IMMUNOMODULATORY PRINCIPLES FROM *ASTRAGALUS MEMBRANACEUS*

Robert James Watson

Bachelor of Applied Science

Darling Downs Institute of Advanced Education

Toowoomba, Queensland

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Certification of Thesis

I hereby certify that the research, experimental work, analysis, findings, and conclusions reported in this thesis are entirely my own effort, except where acknowledged. I also certify that the work is original and has not been submitted for any other award.

Signature of candidate Date

Endorsement

Principle Supervisor……	Associate Supervisor……
T K S Mukkur	Ray Marshall
……..	…….
Position	Position
……..	…….
Date	Date

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Principal Supervisor:

Associate Professor T.K.S. Mukkur _____

Associate Supervisor:

Dr R. Marshall _____

Abstract

This thesis describes an *in-vivo* evaluation of ethanolic extracts of *Astragalus membranaceus* and fractions derived from this extract administered either orally by gavage or by intraperitoneal injection to Balb/c mice. Total antibody titre was used as an indicator of humoral immune response. Cell mediated immune response was determined using Interferon-γ and Interleukin-12 as indicators. Additionally, mice were vaccinated with a killed *Salmonella typhimurium* vaccine, previously demonstrated to induce humoral response but not cell mediated immunity, to determine whether the acquired immune response was enhanced or suppressed. Serum was analysed for total antibody titre using an enzyme-linked immunosorbent assay. Serum and splenocyte culture supernatants were analysed for levels of interferon-γ and interleukin-12. No statistically significant differences were observed between groups either orally gavaged or intraperitoneally injected with extracts of *Astragalus membranaceus*, or orally gavaged with fractions derived from this extract when compared with the control groups.

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Chapter 1: Introduction

Astragalus membranaceus has been used in Traditional Chinese Medicine (TCM) for conditions such as diarrhoea, fatigue and loss of appetite as well as a tonic for conditions of depleted qi. Western herbal applications of this herb have been predominantly as an immune response potentiator. However, little experimental evidence to support the therapeutic claims has been published. In this study, the immunomodulatory potential of ethanolic extracts of the root of *Astragalus membranaceus* was investigated.

Select previous studies have claimed that *Astragalus membranaceus* may potentiate immune responses (Yoshida et al, 1997). although these studies have involved administration of *Astragalus membranaceus* extracts via an intraperitoneal injection. This is at odds with the accepted use of this traditional Chinese medicine for modulating the immune response where the extracts of this herb are administered by exclusively the oral route. Furthermore, these products are in general ethanolic extracts rather than the aqueous preparations reported in a number of previous investigations (Rittenhouse et al, 1991; Sun et al, 1983)

The aim of this investigation was to determine whether oral administration of ethanolic extracts of the roots of *Astragalus membranaceus* promoted antibody response and/ or induced cell mediated immunity (CMI) in mice vaccinated with killed cell *Salmonella typhimurium* vaccine. This vaccine has been reported to induce only antibody response but no CMI (Harrison, 1997; Mukkur et al, 1991; Mukkur et al, 1997). Antibody responses were measured using an enzyme linked immunosorbent assay whereas cell mediated immunity was measured using interferon-gamma (IFN-γ) as an indirect indicator of the cellular arm of the immune response and interleukin-12 (IL-12) as an indicator of innate immunity.

Chapter 2: Literature Review

2.1 Astragalus membranaceus

2.1.1 Botanical Aspects.

The genus Astragalus contains more than 2000 species distributed worldwide and is a member of the Leguminosae family. *Astragalus membranaceus* is commonly referred to as the membranous milk-vetch (English), huang qi (Chinese), ogi (Japanese) and hwanggi (Korean).

2.1.2 Traditional Use.

Used in Traditional Chinese medicine (TCM), *Astragalus membranaceus* (hereafter referred to as Astragalus) is identified as a herb which tonifies the qi and is indicated in conditions of spleen qi deficiency such as diarrhoea, fatigue and loss of appetite. It is also stated to raise the yang qi of the spleen and stomach and address prolapse of organs such as the uterus, stomach and anus, as well as assisting in control of uterine bleeding. Astragalus has been stated to tonify the lung qi and is therefore used in cases of colds, spontaneous sweating and shortness of breath. Other traditional uses have included wasting disorders, night sweats, chronic ulceration and sores, numbness and paralysis of the limbs and oedema (from deficiency). The part of Astragalus traditionally used is the root, with only one investigation found during the course of this study of

the use of the aerial parts of the plant (Yan et al, 1999). It is classically prescribed in TCM in combination with other Chinese medicinal herbs as a dried root, powdered or as a decoction, with the combination depending on the desired therapeutic effect and the specific TCM diagnosis.

2.1.3 Chemistry

Principle constituents of *Astragalus membranaceus* stated in published literature include polysaccharides, saponins, flavonoids, amino acids and various trace elements. Levels of Astragalosides, a cycloartane triterpene glycoside, are often used as indicators of herb quality. The astragalosides are a series denoted as I- VII based on a aglycone cycloastragenol and containing from one to three sugars attached at the 3-,6- and 25-positions. In astragalosides I-III the 3- glucose is acetylated. (Anonymous, Alternative Medicine Review, 2003)

Hairy root cultures were used to isolate Agroastragaloside II, a new astragaloside, structure of this was established as 3-O-β-(2'-O-acetyl)-D-xylopyranosyl-6-O-β-D-glucopyranosyl-(24S)-β,6α,16β,24,25-pentahydroxy-9,19-cyclolanostane. The previously reported astragalosides, namely astragaloside II, isoastragaloside I and 3-O-β-D-

xylopyranosyl-cycloastragenol were also isolated from the culture of hairy roots (Hirotani et al. 1994).

2.1.4 Hepatoprotective effects of *Astragalus membranaceus*

Administration of an ethanolic extract of astragalus root has been reported to alleviate liver damage induced by the administration of stilbenemidine. It was reported that pre-administration of astragalus extract to mice reduced elevated serum glutamic-pyruvic transaminase levels and subacute toxicity of stilbenemidine exposure, decrease pentobarbital induced loss of righting reflex and protected hepatic cells from pathological changes (Zhang et al. 1990).

2.1.5 *In vitro* immunological studies of *Astragalus membranaceus*

Studies utilising human melanoma cell lines A375P and HS294T demonstrated that concomitant addition of *Astragalus membranaceus* extract with IL-2 to cell cultures showed a ten-fold potentiation in IL-2 activity manifested by tumour cell killing activity. A significant reduction in the number of effector LAK cells following LAK cell generation with *Astragalus membranaceus*, fractionated on a Sephadex Sephacryl S-200 column and retaining the fraction with an estimated molecular weight of 20000 – 25000, plus IL-2 were required for equicytotoxic reaction, when

compared to the addition of IL-2 without the Astragalus fraction in isolation was observed (Chu et al, 1988).

Tumour cells are known to produce substances which suppress the function of macrophages (Israel et al, 1981). Macrophage suppression by urological tumour lines was demonstrated to be reversed *in-vitro* by the addition of aqueous extracts of *Astragalus membranaceus* or *Ligustrum lucidum* (Rittenhouse et al, 1991).

Aqueous extracts of *Astragalus membranaceus* were evaluated using the local xenogenic graft-versus-host reaction. This study utilised the crude extract (designated Fraction 7) in addition to a number of purified fractions. Greater immunomodulating response characterised by local xenogenic graft-versus-host reaction from two of the fractions examined (fractions 3 and 8) were observed than that exhibited by the precursor crude extract (Chu et al, 1988).

Yoshida et al (1997) studied the immunomodulating potential of a variety of chinese medicinal herbs by assessing the ability of the extracts to stimulate the proliferation of murine spleen cells and reported that extracts of *Astragalus membranaceus* and *Oldenlandia diffusa* in particular promoted the proliferation of murine spleen cells.

2.1.6 *In-vivo* immunological studies on *Astragalus membranaceus*

Chu et al (1988) injected fraction F3 intravenously into rats whose immune response had been suppressed by administration of cyclophosphamide. Administration of the F3 fraction was found to markedly enhance the ability of rats to reject the xenogenic graft and was therefore taken as a strong indicator of the extract's immune potentiating activity *in vivo* (Chu et al, 1988).

Zhao et al (1990) administered *Astragalus membranaceus* extracts, prepared according to the methods of Chu et al (1988), by intraperitoneal injection to both normal mice or mice immunosuppressed by cyclophosphamide or radiation or by ageing. The authors reported an enhanced antibody response to a trinitrophenol–horse red blood cell antigen, a T-dependant antigen that was associated with an increase in T-helper cell activity in both the normal and immunosuppressed mice. Further, it was reported that the observed immunomodulatory activity of the extracts was associated with the carbohydrate content of the extract.

Ying-Zhen et al (1990) injected a refined aqueous extract, prepared by precipitation in alcohol of *Astragalus membranaceus,* intraperitoneally to

determine its effect on experimentally-induced coxsackie B-3 viral myocarditis in 4 week old male Balb/c mice. Examination showed that the mice treated with extract exhibited lesser severity and reduced area displaying myocardial lesions. Virus titre was also reduced in the treated group.

The effect of Astragalus extracts on inhibition of tumour cells has also been studied *in-vivo* (Lau et al, 1994). Balb/c mice were transplanted intraperitoneally with renal adenocarcinoma cell line (Renca). The treatment group was then injected with an aqueous extract of *Astragalus membranaceus* and *Ligustrum lucidum* by the intraperitoneal route for 10 days. Survival was enhanced with administration of the extract with a cure rate of 57% when the tumour load was $2x\ 10^5$ and 100% when the tumour load was $1x\ 10^5$ when compared with the control mice administered saline. Splenocytes from mice transplanted with the Renca cell line were also shown to have a depressed capability for responding to IL-2 in generating lymphokine-activated killer cells (LAK cells). It was suggested that the anti-tumor activity of the extract may be a result of augmentation of the phagocyte and LAK cell activity

Khoo and Ang (1995) fed Wistar rats with cyclophosphamide-induced myelosupression a dose of 240mg of a suspension of a commercially

available dried extract of *Astragalus membranaceus or Ligustrum lucidum*. Daily neutrophil count and platelet count levels were monitored. No differences were observed between the study and control groups (Khoo and Ang, 1995). In contrast, Yan et al (1999) evaluated an extract of the stems and leaves of *Astragalus membranaceus* for immunomodulatory potential and showed the extract to promote the proliferation of lymphocytes, raise the T-cell count and elevate LAK activity induced by IL-2 in mice.

Hairy roots of *Astragalus membranaceus* were produced using a large-scale culture technique. These cultured roots were assessed to have a similar quality to traditionally produced roots with respect to levels of polysaccharides, saponins and astragalosides. Aqueous extracts of the cultured hairy root was dosed orally into cyclophosphamide suppressed rats and determined the immune response by assessment of spleen weight, thymus weight, carbon clearance, transformation of lymphatic cells and hemolysin antibody formation. Immune response triggered by the cultured material was reported as similar to those observed with root material, using spleen weight, thymus weight, clearance of carbon particles, transformation of lymphatic cells and hemolysin antibody formation as indicators (Zhiren et al, 1998).

2.2 Whole-cell formalin-killed *Salmonella typhimurium* vaccine

It was decided to use killed *S. typhimurium* vaccine that has been reported to induce only antibody response and cell mediated immunity in mice to determine whether oral administration of *A. membranaceus* extracts or fractions could induce a cell mediated immunity or immune response (CMI). The choice of this antigen system is also significant since humoral immunity mediated by antibodies does not protect mice against challenge with virulent *Salmonella* species, whereas a live attenuated *Salmonella* species that induce production of antibodies and CMI are highly effective in protecting mice against salmonellosis. (Mukkur et al. 1991, Mukkur et al. 1997)

Chapter 3: Materials and Methods

3.1 Herbs

The *Astragalus membranaceus* root material was obtained from Greenridge Botanicals, A Division of Thursday Plantation Laboratories Ltd (Ballina, NSW). The herb was sourced from crops grown at the Greenridge farm located at Lobwein's Rd, Kingsthorpe Queensland. Following harvest, the roots were hot air dried at 70°C and stored as coarsely cut sections. All herb used in these studies was randomly drawn from commercially harvested stocks.

3.2 Preparation of Herbal Extracts

3.2.1 Confirmation of authenticity

Confirmation of authenticity of the herbal starting material used in these studies was accomplished by the use of thin-layer chromatography (Appendix A: Thin Layer Chromatography method) Additionally, observation of the crop prior to harvest was consistent with published descriptions of the growth habits of *Astragalus membranaceus*. The plant is a perennial herb 25-40cm high. Leaves are oblong and 3-6 cm long,

petiole obsolete, stipules free, cauline, green, triangular ovate, sparingly vested on the outside with white hair. Leaflets oblong-ovate, oval or oblong-oval (World Health Organisation, 1999).

3.2.2 Preparation of *Astragalus membranaceus* Extract

The extraction process employed in this study was selected to be equivalent to that utilised by Greenridge Botanicals, a Division of Thursday Plantation Laboratories Ltd., in the preparation of commercial extracts of *Astragalus membranaceus*.

Dried *Astragalus membranaceus* roots were hammer milled to a coarse powder. Two hundred grams of the milled herb was macerated in eight hundred millilitres of 60% v/v ethanol-water for seven days in a glass vessel. Following maceration, the supernatant was decanted off and the residual wetted herb mass was pressed in a hand operated garlic press to maximise recovery of the applied extraction solvent. A total of five hundred and sixty millilitres of solvent was recovered. The volume of the recovered liquid was reduced to 143.6mL using a rotary evaporator. The concentrated liquid was diluted to 200mL using 14.3mL distilled water and 42.1mL of ethanol (95%). The resultant extract was thus a 1:1 extract (1mL of the final liquid being equivalent to 1g of dried herb) prepared at

a final ethanol content of 20% v/v. This extract is equivalent to that used by Greenridge Botanicals, a Division of Thursday Plantation Laboratories Ltd in the product AstraForte, a blend of extracts of *Astragalus membranaceus, Ganoderma lucidum* and *Ligustrum lucidum*. Extracts were filter sterilised by passing through a 0.45 micron membrane filter prior to administration.

3.3 Separation of herbal fractions

Preparation of fractions of components of Astragalus extract was carried out to allow identification of the fractions containing the immunostimulatory or immunoinhibitory actions of compounds present in the herb.

100mL of the whole extract prepared as described in Section 3.2 was placed into a 250mL separating funnel. To this was added 50ml of n-hexane. The flask was agitated and the phases allowed to separate. The aqueous phase was drained from the separating funnel and the organic phase collected. This process was repeated a further two times and the organic phases combined. The 150mL of hexane was then rotary evaporated to dryness and the residue suspended in 100mL of 20% v/v

ethanol by sonication. This liquid represented the lipophilic compounds fraction.

An aliquot of 20mL of the aqueous phase from the above described procedure was chilled to 4°C and 200mL of ethanol (95% v/v) chilled to 4°C was added. The combined liquids were mixed thoroughly and allowed to stand in a bath of iced water for ten minutes. The liquid was then filtered through a Whatman No 4 filter paper and the collected residue washed with three lots of 10mL of 95% ethanol chilled to 4°C. The collected residue was washed from the filter followed by sonication in 20mL of 20% v/v ethanol: water. This was considered to represent the hydrophilic polysaccharide fraction of the herb and is referred to as the polysaccharide fraction in the text.

The filtrate from the above procedure was retained and rotary evaporated to dryness. The residues obtained were suspended in 20mL of 20% v/v ethanol by sonication. This fraction was referred to as the hydrophilic fraction that represented the non-polysaccharide water soluble components of the whole Astragalus extract.

3.4 Animals

Mice used to conduct these studies were sourced from the Animal Resource Centre, Perth WA. Six week old female, specific pathogen-free Balb/c mice were allowed to acclimatise for one week prior to being separated into groups of 10 mice each for the conduct of the studies. All mice were housed 10 per cage in the general housing area of the animal house at the University of Southern Queensland. Mice received unlimited water and mouse/rat chow. The temperature was maintained at a constant 22°C and a 12 hours light/ 12 hours dark cycle was in operation throughout the duration of the studies. All experiments carried out in this investigation were approved by the USQ Animal Ethics Committee.

3.5 Bacterial Strains

Salmonella typhimurium aroA (CS322) was used from stocks held by the Department of Biological and Physical Sciences, University of Southern Queensland. The cultures used had been stored at -70°C. Subculturing onto Miller's Luria-Bertani (LB) agar and growth in Miller's Luria-Bertani (LB) broth was accomplished as required.

3.6 Preparation of vaccines

Ten microlitres of the stock culture of *Salmonella typhimurium* was streaked onto Miller's LB agar and incubated overnight to obtain isolated colonies. A single colony was inoculated into 200mL of Miller's LB broth and incubated at 37°C for 18 hours with agitation at 150 rpm in a Bioline Shaker Incubator (Edwards Instrument Co., Australia). A series of dilutions over a range from 10^{-4} to 10^{-8} were prepared in LB broth and plated on LB agar and incubated overnight at 37°C in order to determine the number of colony forming units per mL in the culture. Formalin was added to the broth at a final concentration of 1% and the broth further incubated at 37°C for 4 hours with agitation at 150 rpm. An aliquot of 100 microlitres of the broth was then streaked on Miller's LB agar and incubated at 37°C overnight as a check for sterility. The broth culture was centrifuged at 7000 rpm for twenty minutes at ambient temperature. The supernatant was discarded and the pellet washed twice with sterile phosphate buffered saline. The final pellet was then suspended in 50mL of phosphate buffered saline and stored under refrigeration at 4°C prior to use.

3.7 Preparation of whole-cell *aroA Salmonella typhimurim* (CS322) antigen

Salmonella typhimurium strain CS322 *aroA* was prepared as described in Section 3.5. The final pellet was suspended in filter sterile coating buffer and adjusted to an optical density of 0.4 at 610nm. This antigen was stored refrigerated at 4°C prior to use.

3.7.1 Preparation of *Salmonella typhimurium* lysate for splenocyte stimulation.

Lysate of *Salmonella typhimurium* was prepared according to a modification of the procedure detailed by Villarreal (1992). *Salmonella typhimurium* was grown on motility test agar overnight at 37°C. Bacteria with the highest motility was then inoculated into 400mL of tryptic soy broth and incubated overnight at 37°C. The broth was then centrifuged at 13000 x g for 10 minutes and the supernatant discarded. The pellet was then washed with phosphate buffered saline containing 5mM EDTA, centrifuged and re-washed. The pellet was then resuspended in 1/150 of the original volume and the cells disrupted by three 2 minute bursts of

sonication (60 duty cycle,output 7). Disruption of cells was confirmed by gram stain. The resultant lysate solution was then diluted to 1/3 of the original volume in sterile PBS and centrifuged at 13000 x g for 10 minutes to remove insoluble matter. The supernatant was passed through a 0.2 μ syringe filter and placed in a sterile tube for storage at 4°C. The protein concentration was determined using a Coomassie® Plus Protein Assay Reagent Kit.

3.8 Procedure for collection of samples from mice and anaesthesia of mice

3.8.1 Serum Samples:

On the final day of each experiment, serum was collected as follows. Mice were anaethesised with a mixture of 40 microlitres of ketamine (100mg/mL) and 40 microlites of xylazine (20mg/mL). Blood samples were obtained by cardiac puncture using a 23G ¾" needle. (Becton-Dickinson, Eight Mile Plains, Qld). Collected blood was placed in a 1.5mL microfuge tube and immediately capped. Immediately following collection of the blood sample, cervical cordotomy was performed on the mice to ensure a rapid and painless euthanasia.

The collected blood sample was allowed to clot for 15 minutes and then centrifuged at 3000xg for five minutes (Mikro 12-24 centrifuge, Hettich Zentrifugen, Tuttlingen). The supernatant serum was removed using a glass pastuer pipette and stored at -20°C prior to analysis.

3.8.2 Collection of spleens for preparation of splenocyte supernatants

Spleens were excised from five of the mice cadavers in different groups and placed in 10mL of tissue culture media and splenocyte culture supernatants prepared as described below.

3.8.3 Preparation of Splenocyte culture supernatants

Immediately following collection of the blood samples by cardiac puncture as described, the spleen was dissected from five mice per group. These spleens were placed into tissue culture media (refer Appendix B). The collected spleens were held for no longer than fifteen minutes prior to proceeding with the culture preparation. The spleens and tissue culture media were then poured into a sterile cell strainer (Becton-Dickinson, Eight Mile Plains Qld) on top of a sterile 50mL tube. The cells were mashed through the grid of the strainer using the plunger from a sterile 1mL syringe (Becton-Dickinson, Eight Mile Plains Qld). Two washes of

approximately 5mL of tissue culture media was used to wash the cells through the strainer. The cells were then pelleted by centrifuging for 10 minutes at 1000rpm at ambient temperature. The supernatant was then decanted and the cell pellet was washed with 10mL of tissue culture media. The cell pellet was washed again with 10mL of tissue culture media and centrifuged for 10 minutes at 1000rpm and ambient temperature. The final cell pellet was then suspended into 10mL of the tissue culture media. An aliquot of 10μL of the cell suspension was added to 90μL of the tissue culture media and mixed thoroughly. 10μL of this was added to 10μL of tryptan blue and mixed thoroughly. A volume of 10μL of this was transferred to a haemocytometer and the white blood cells were counted. The concentration of cells was then adjusted to 5×10^6 cells per mL and 2mL of this suspension was dispensed into 9 wells of a 24 well culture plate (TPP, Switzerland). 100μL of *Salmonella typhimurium* lysate (refer 3.10) which was equivalent to approximately 2μg of protein per millilitre was added to each well. The plate was then covered with a loose fitting lid and incubated at 37°C. Supernatant was recovered from wells at 72 hours. Collected supernatant was stored at -20°C prior to analysis. Tissue culture procedures were conducted in a Class II Biological Safety Cabinet.

3.9 Estimation of antibody levels by enzyme-linked immunosorbent assay (ELISA)

The method used was an indirect enzyme-linked immunosorbent assay (ELISA) as described elsewhere (Mukkur et al., 1995). One hundred microlitres of whole cell *S. typhimurium* antigen (refer 3.7.2) suspended in coating buffer (refer Appendix B) was dispensed into columns 2 – 10 of a 96 well plate. The plate was then covered with a plastic film and incubated at 4°C overnight. Following this, 100μL of 0.2% gelatin in coating buffer was added to the wells in order to block the plate. 200μL of 0.2% gelatin in coating buffer was added to the wells in column 11 to act as the antigen control. The plate was covered with plastic film and incubated at 4°C overnight. Following this incubation the film was removed, the plate emptied, tapped dry on absorbent towelling and irrigated with wash buffer. The plate was allowed to stand for 3 minutes and the procedure repeated a further two more times.

Serial dilutions of serum in phosphate buffered saline were prepared of each sample over a range from 1/100 to 1/12800. Following washing of

the plate, 100μL of the dilutions were added to the plates. The plate was incubated for one hour at 37°C covered with plastic film.

Following this the plate was washed three times with wash buffer, allowing the wash buffer to stand for three minutes with each wash. 100μL of the secondary antibody, represented by goat anti-mouse polyvalent immunoglobulins (Sigma, St Louis, Mo) was then added to the wells of column 2 to 11. Minimum titre quoted by the manufacturer for the secondary antibody was 1:10,000.

The plate was washed three times with wash buffer again and Sigma-Fast™(Sigma, St Louis, Mo) made up according to the manufacturer's directions. 100μL was added to all wells of the plate used in the test. The plate was then covered with aluminium foil and incubated for 30 minutes. Absorbances for the contents of the wells was then read on a micro-plate reader (Bio-Rad Model 550, Hercules Ca) at 450nm. The antibody titre was then able to be established by plotting the absorbance value and corrected for non-specific binding followed by extrapolation to establish the antibody titre.

3.10 Interleukin-12 determination by enzyme-linked immunosorbent assay (ELISA)

Assay for interleukin-12 levels was conducted using Cytoscreen Mouse IL-12+p40 kit (Biosource International, Camarillo Ca). Analysis was carried out according to the manufacturer's directions for use of the kit (Appendix C). Samples of serum (50μL) were aliquoted into the pre-coated wells of the plate with the biotin conjugate. The plate was incubated for two hours at ambient temperature. Following this period of incubation, the plate was washed four times with wash buffer. Streptavidin-HRP was added and the plate incubated for 30 minutes at ambient temperature. The plate was then washed four times with the wash buffer. Colour substrate was added to each well and the plate incubated for 30 minutes. Stop solution was then added and the plate read in a micro-plate reader (Bio-Rad Model 550, Hercules Ca) at 450nm. IL-12 levels were calculated using the generated standard curve.

3.11 Determination of Interferon-γ (IFN-γ) levels by enzyme-linked immunosorbent assay (ELISA)

Interferon-γ levels were determined using a Quantikine® Mouse IFN-γ test kit (R&D Systems Inc, Minneapolis MN). Analysis was conducted

according to the manufacturer's directions for use of the kit (Appendix C). 50μL of splenocyte supernatant samples was aliquoted into the wells along with 50μL of the assay diluent. The plate was covered and incubated at ambient temperature for 2 hours. Following incubation the plate was washed five times with wash buffer. 100μL of conjugate was added to the wells, the plate covered and incubated at ambient temperature for 2 hours. The plate was washed five times with wash buffer. 100μL of substrate solution was added to each well and the plate incubated in the dark for 30 minutes at ambient temperature. 100μL of stop solution was added to each well and the plate read in a micro-plate reader (Bio-Rad Model 550, Hercules Ca) at 450nm. Interferon-γ levels in the samples were calculated using the generated standard curve.

3.12 Evaluation of Immunomodulatory Properties of the Crude Extract or Fractions Derived from *Astragalus membranaceus*

3.12.1 Experimental design for determination of total antibody titre and Interferon-γ levels in mice administered *Astragalus membranaceus* via oral gavage

For this study, forty female specific pathogen free Balb/c mice were randomly assigned into four groups of ten mice per cage. The assigned groups were treated as follows:

Group1: Mice in this group were dosed daily with 1.71 microlitres per day of the extract of *Astragalus membranaceus* prepared as described in 3.2.2. This is equivalent to 1.71mg of the dried root matter. The extract was suspended in phosphate buffered saline to a total volume of 250 microlitres and administered orally using a flexible plastic gavage tube (the plastic sheath of a 24G ¾", Insyte™ Catheter, Becton-Dickinson, Eight Mile Plains, Qld). Extract was administered for a period of 28 days. On the seventh day, mice were vaccinated with a single dose of whole cell killed *Salmonella typhimurium vaccine* (2.5×10^7 colony forming units). This vaccine was administered by a single intraperitoneal injection. On day 28 of the study, serum was collected as detailed.

Methods used for collection of serum samples and spleens from euthanased mice and preparation of splenocyte supernatants is described in Sections 3.8.1, 3.8.2 and 3.8.3

Group 2: Mice were dosed orally with 250 microlitres per day of sterile phosphate buffered saline. No vaccination was performed. Methods used for collection of serum samples and spleens from euthanased mice and preparation of splenocyte supernatants is described in Sections 3.8.1, 3.8.2 and 3.8.3.

Group 3: Mice were dosed orally with 250 microlitres per day of sterile phosphate buffered saline. After seven days, the mice were vaccinated with a single dose of whole cell killed *Salmonella typhimurium vaccine* (2.5×10^7 colony forming units). This vaccine was administered by a single intraperitoneal injection. Methods used for collection of serum samples and spleens from euthanased mice and preparation of splenocyte supernatants is described in Sections 3.8.1, 3.8.2 and 3.8.3.

Group 4: Mice were dosed orally with 1.71 microlitres of extract suspended in phosphate buffered saline to a total volume of 250 microlitres as described for group 1. Methods used for collection of

serum samples and spleens from euthanased mice and preparation of splenocyte supernatants is described in Sections 3.8.1, 3.8.2 and 3.8.3.

3.12.2 Experimental design for the determination of total antibody titre and interferon-γ levels in mice administered *Astragalus membranaceus* extract by the intraperitoneal route

For this study, thirty female specific pathogen free Balb/c mice were randomly assigned into three groups of ten mice per cage. The assigned groups were treated as follows:

Group 1. Mice were administered a single dose of 48 microlitres of *Astragalus membranaceus* 1:1 extract suspended to a total volume of 100 microlitres in sterile phosphate buffered saline on day one of the study. On day twenty-eight, samples were collected as described in Sections 3.8.1, 3.8.2 and 3.8.3.

Group 2: Mice were administered a single dose of 48 microlitres of *Astragalus membranaceus* 1:1 extract suspended to a total volume of 100 microlitres in sterile phosphate buffered saline on day one of the study. On the seventh day, mice were vaccinated with a single dose of whole

cell killed *Salmonella typhimurium vaccine* with an equivalent of 2.5 x 10^7 colony forming units. This vaccine was administered by a single intraperitoneal injection. On day twenty-eight, samples were collected as described in Sections 3.8.1, 3.8.2 and 3.8.3.

Group 3: Mice were administered a single dose of 100 microlitres of sterile phosphate buffered saline by intraperitoneal injection on day one of the study. On the seventh day, mice were vaccinated with a single dose of whole cell killed *Salmonella typhimurium vaccine* at a level of 2.5 x 10^7 colony forming units by the intraperitoneal route. On day twenty-eight, samples were collected as described in Sections 3.8.1, 3.8.2 and 3.8.3

3.13 Statistical analysis

All statistical analysis was conducted using Instat+ for Windows, version 3.024. Non-parametric analysis of variance (ANOVA) was conducted for the purpose of group comparisons in all studies (Steel et al, 1980).

Chapter 4: Results

4.1 Authentication of herbal material

Herbal material used in the preparation of extracts was drawn from plants grown at the Greenridge Botanicals (Aust) Pty Ltd farm located at Lobweins Road Kingsthorpe Queensland, Australia. Inspection of the crop during the growing period revealed plants consistent with the published descriptions and photographs of *Astragalus membranaceus* (World Health Organisation, 1999).

Root stock used in the preparation of extracts was subsequently drawn from commercial harvests of these plants. Macroscopic assessment of the harvested root material in comparison with a verified reference sample of *Astragalus membranaceus* showed no major differences. Thin layer chromatography of the dried plant matter was used to confirm authenticity of the herb (Figure 1). Comparison was made against botanically verified *Astragalus membranaceus* root (Howard Hollows, Adelaide, Australia).

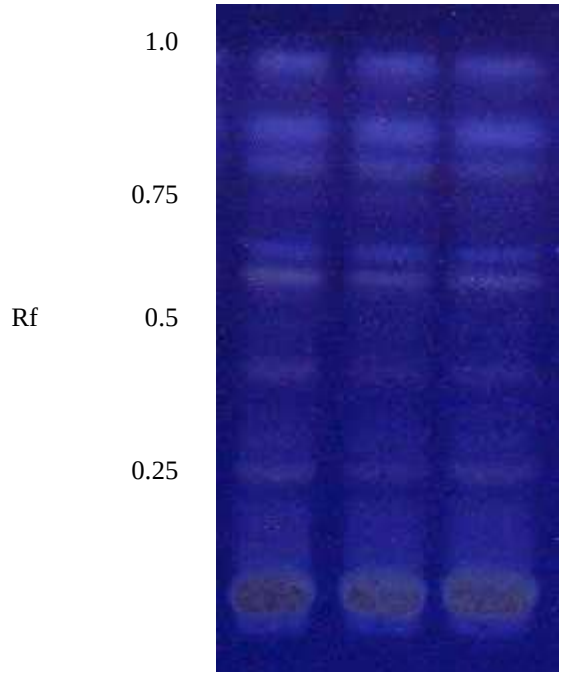

Figure 4.1: Thin layer chromatography of *Astragalus membranaceus*. Lane 1 authenticated *Astragalus membranaceus*, lanes 2 and 3 harvested herb. (Silica C_{60} F_{264} plates, Solvent system CH_3COOH: Formic Acid: H_2O: Ethyl Acetate 11:11:27:100; Plate development spray 1% w/v methanolic diphenylboric acid followed by 5% ethanolic polyethylene glycol. Viewed under UV light 365nm.

Review of the TLC plates showed a high degree of consistency between the band patterns of the herb used in preparation of the extracts and the reference material. Two major bands were observed at approximately Rf 0.8 and a further two at Rf 0.6, Single bands were observed at 0.25 and 0.4.No visible differences were observed between the sample and the reference material. It was determined that the macroscopic identification and assessment of the thin layer chromatography provided sufficient confirmation of starting material identity to be satisfied that the prepared extracts were of *Astragalus membranaceus*.

4.2 Imunomodulatory Properties of the Crude Extract or Fractions Derived from *Astragalus membranaceus*

4.2.1 Total antibody titres in mice following oral gavage with *Astragalus membranaceus*

In this study, four groups of mice were gavaged with *A. membranaceus* extract by the oral route. Two groups were vaccinated with killed *Salmonella typhimurium* vaccine whereas the remaining groups were unvaccinated. Serum antibody titres at 28 days post-immunisation are shown in Figure 4.2. Statistical analysis for this study is presented in Appendix D.

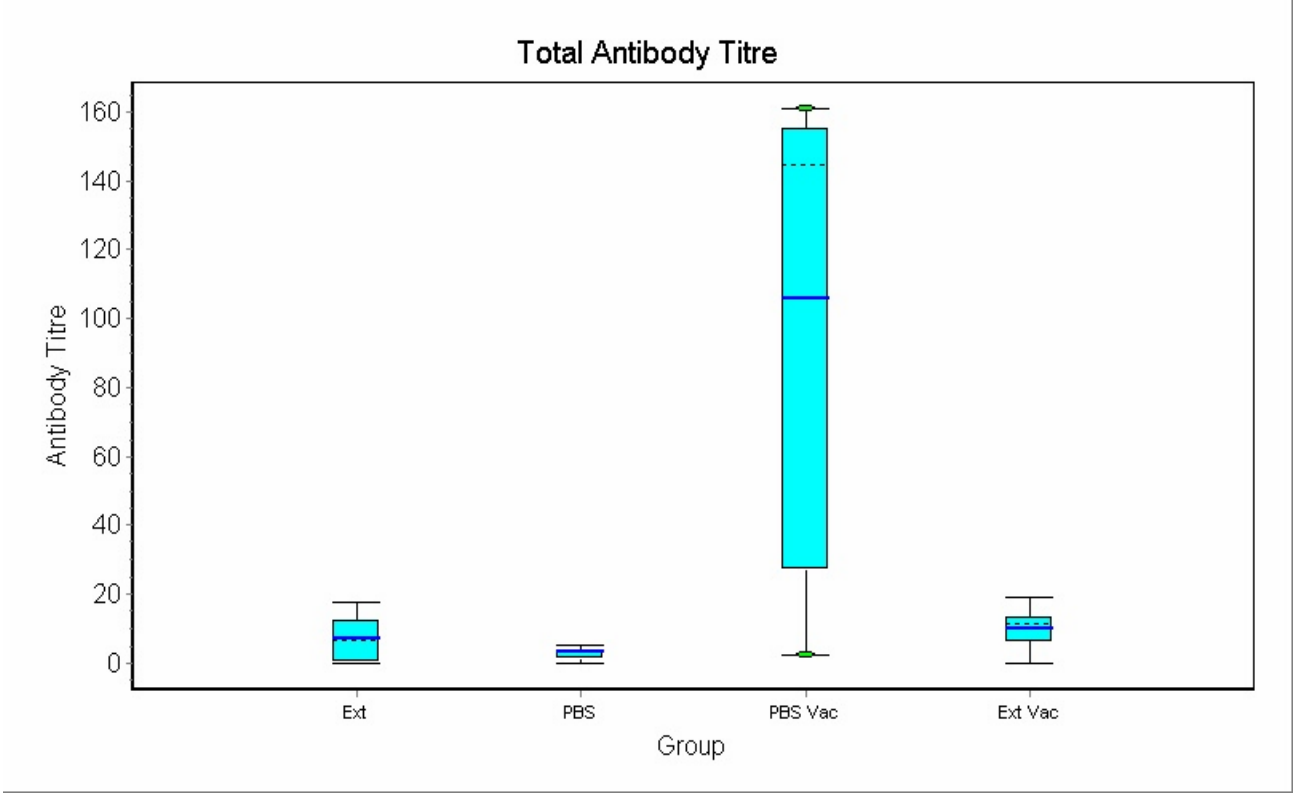

Figure 4.2 Histogram of total antibody titres for oral administration of *Astragalus membranaceus* extract or placebo (Phosphate buffered saline) Legend: Ext – *Astragalus membranaceus* extract, PBS- phosphate buffered saline, PBS Vac – Phosphate buffered saline and vaccinated, Ext Vac- *Astragalus membranaceus* extract and vaccinated.

Mice vaccinated with the killed *Salmonella typhimurium* vaccine yielded a high antibody titre that was statistically significant in comparison with the PBS control group ($P \leq 0.05$). Interestingly, there was no differences between the vaccinated group dosed with the extract versus unvaccinated groups. It would appear that the antibody titre in vaccinated mice treated with the extract was suppressed.

4.2.2 Total antibody titres in vaccinated versus non-vaccinated mice administered *Astragalus membranaceus* extract by intraperitoneal route

In this study, three groups of mice were treated with the extract of *A. membranaceus*. Two of these groups were vaccinated with killed *Salmonella typhimurium* vaccine, with one injected with sterile phosphate buffered saline and the other filter sterilised *A. membranaceus* extract. An un-vaccinated group was also injected with filter sterilised *A. membranaceus* extract. Serum was collected from the mice after 28 days and antibody titres determined by ELISA. Statistical analysis of the data is presented in Appendix D. Presentation of the results as a histogram is presented as Figure 4.3.

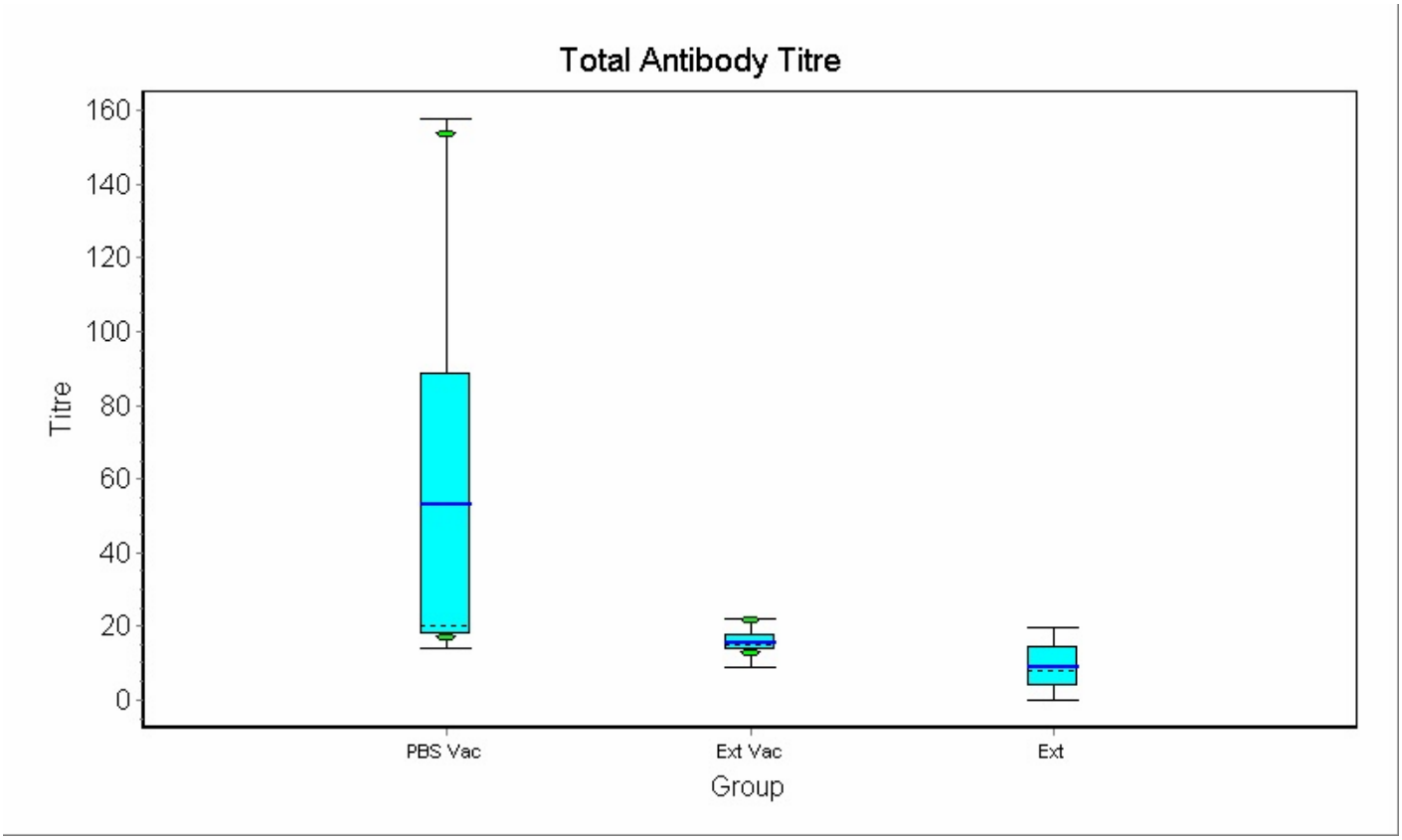

Figure 4.3 Histogram of antibody titres for intra-peritoneal injection of *Astragalus membranaceus* extract or placebo (Phosphate buffered saline). Legend: PBS Vac – PBS control, vaccinated. Ext Vac – *Astragalus membranaceus* extract, vaccinated, Ext – *Astragalus membranaceus* extract.

The results obtained demonstrate no statistically significant difference in the anti-salmonella antibody titres when compared with the control group receiving only the extract. However, the mean antibody titre of the group

immunised with the killed *Salmonella* vaccine was substantially greater than of the vaccinated group injected with the extract. This indicated potential suppression of antibody response by *A. membranaceus* extract when administered by the intraperitoneal route.

4.2.1 Determination of Interferon-γ levels in vaccinated versus non-vaccinated mice administered *Astragalus membranaceus* extract via the oral route

In addition to the determination of total antibody titre in the initial dosage round, determination of interferon-γ levels was conducted on stimulated splenocyte supernatants. Statistical analysis of the results is presented in Appendix D. A histogram of the obtained results is reported as Figure 4.4.

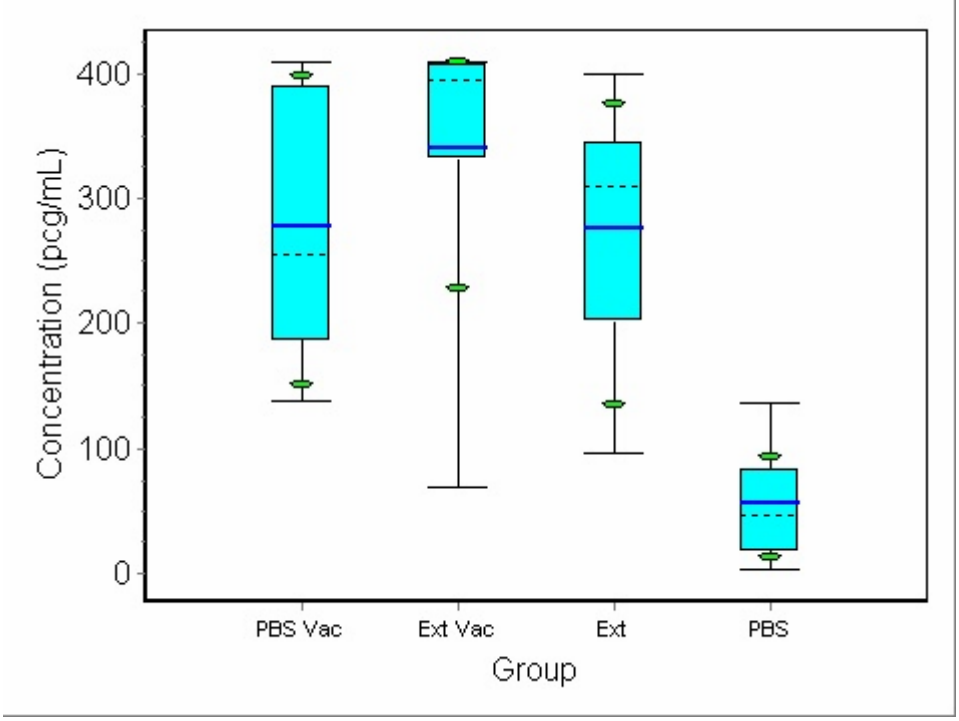

Figure 4.4 Histogram of interferon-γ levels of stimulated splenocyte supernatants following dosage of mice orally with *Astragalus membranaceus* extract or placebo (Phosphate buffered saline) and vaccination with killed *Salmonella typhimurium* vaccine.

The obtained results for this study indicate no statistically significant differences between the groups. The control group dosed with phosphate buffered saline and not vaccinated with killed *Salmonella typhimurium* vaccine has shown a marginally lower mean when compared with the other groups.

4.2.2 Interferon-γ levels in vaccinated versus non-vaccinated mice administered *Astragalus membranaceus* extract by intraperitoneal route.

Splenocytes from the different groups of mice described in section 4.2.2 were stimulated with the *Salmonella* antigen for 72 hours and supernatants collected. The concentrations of interferon-γ were determined on this collected supernatant. Analysis of this data is presented as a histogram in Figure 4.5.

Analysis of variance of the results revealed that there was no statistically significant difference between the groups. It was clear that there was no stimulation of CMI following administration of *Astragalus membranaceus* extract by the intraperitoneal injection.

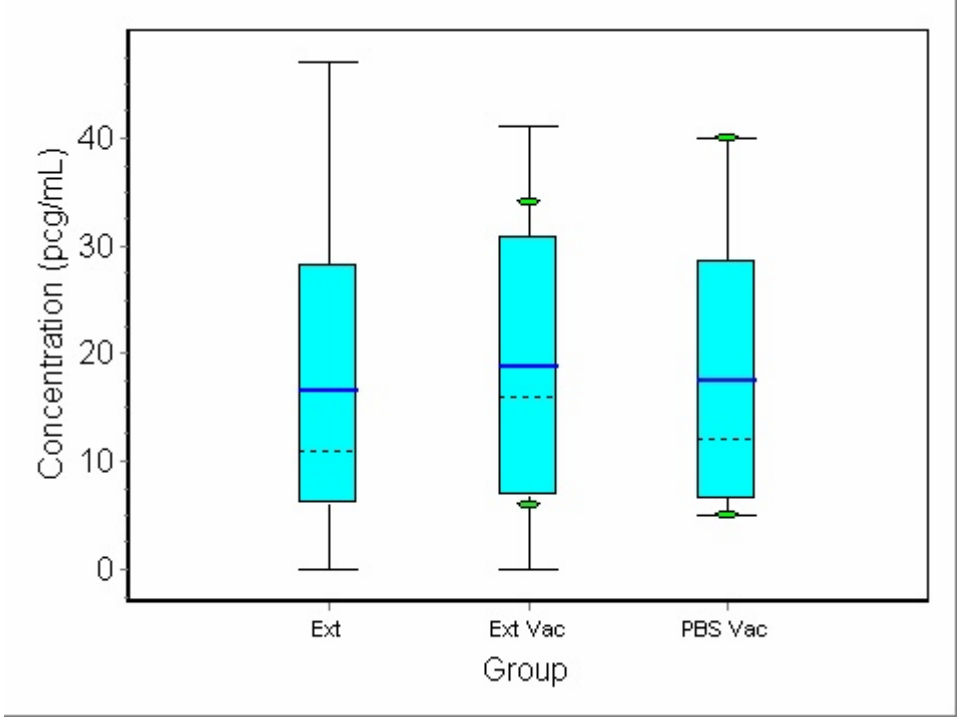

Figure 4.5 Histogram for interferon-γ levels of stimulated splenocyte supernatants following intraperitoneal injection with *Astragalus membranaceus* extract or placebo (Phosphate buffered saline) and vaccination with killed *Salmonella typhimurium* vaccine. Ext: *A.membranaceus* extract, Ext Vac- *A.membranaceus* extract and vaccination with *Salmonella typhimurium* vaccine, PBS Vac- phosphate buffered saline with *Salmonella typhimurium* vaccine.

4.2.5 Determination of serum interferon-γ levels in mice administered *Astragalus membranaceus* extract by the oral route.

This study, and those reported subsequent to this, were performed following assessment of the results of the initial studies conducted. Serum interferon-γ levels were assessed for mice dosed orally with *A.*

membranaceus extract. In addition, groups were dosed with three fractions derived from whole extract in an attempt to identify likely classes of compound involved in the production of any observed immunological response.

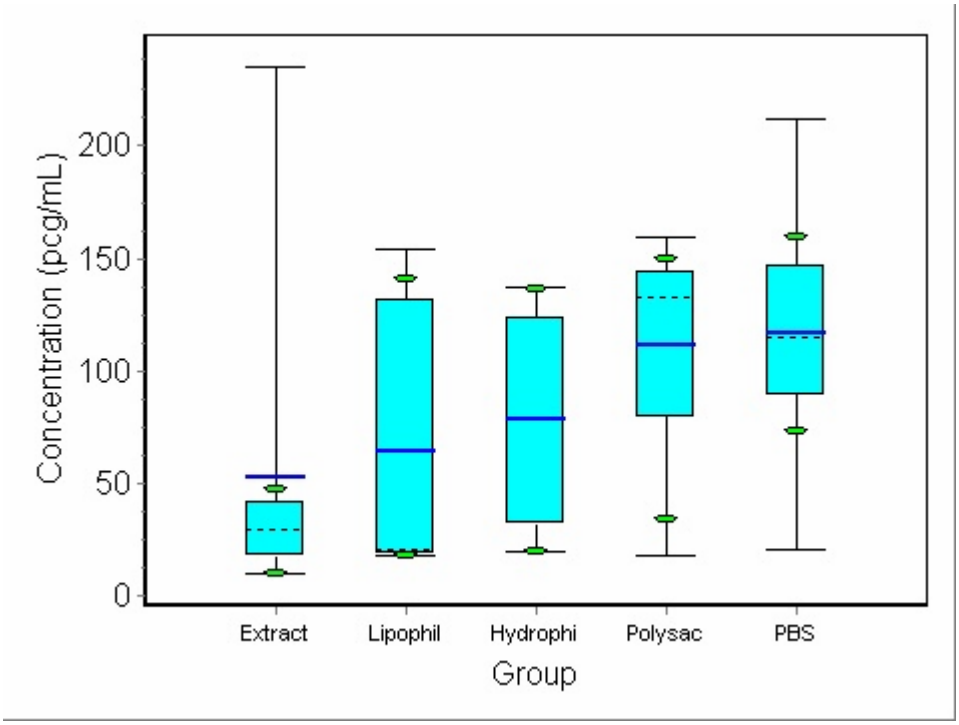

Figure 4.6 Histogram for interferon-γ levels of serum following oral administration of *Astragalus membranaceus* extract, fractions derived from *A. membranaceus* or placebo (Phosphate buffered saline). Legend: Extract- *A. membranaceus* extract, Lipophil – Lipophilic fraction, Hydrophi – Hydrophilic fraction, Polysac – Polysaccharide fraction, PBS – phosphate buffered saline.

No statistically relevant differences were observed to be present between groups. Analysis of variance revealed no evidence to support the hypothesis that the *Astragalus membranaceus* extract, or fractions derived from *A. membranaceus* enhanced the production of interferon-γ in mice.

4.2.3 Interferon-gamma levels in mice administed *Astragalus membranaceus* extract or fractions derived from *Astragalus membranaceus* by the oral route.

Stimulated splenocyte supernatants derived from mice dosed as in 4.2.5 were assessed for production of interferon-γ. Statistical analysis of the results is presented in Appendix D. The results are presented as a histogram in Figure 4.7.

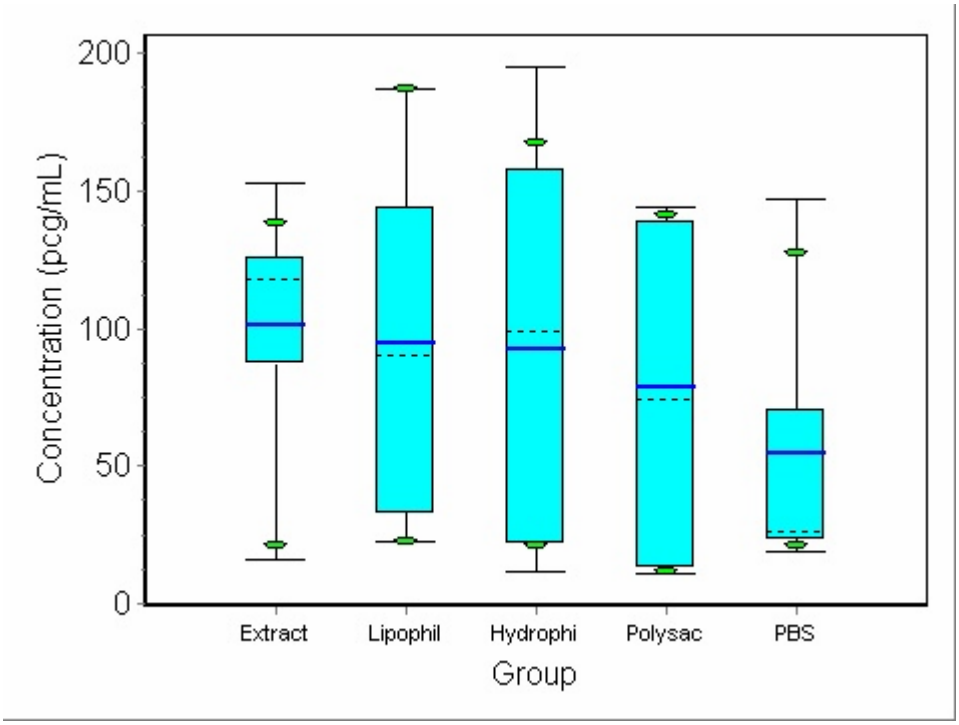

Figure 4.7 Histogram of interferon-γ levels of stimulated splenocyte supernatants following oral administration of *Astragalus membranaceus* extract, fractions derived from *A. membranaceus* or placebo (Phosphate buffered saline). Extract- *A. membranaceus* extract, Lipophil – Lipophilic fraction, Hydrophi – Hydrophilic fraction, Polysac – Polysaccharide fraction, PBS – phosphate buffered saline.

Analysis of variance on the data presented as a histogram in Figure 4.7 showed no statistically relevant differences in the levels of interferon-γ produced by the different groups. Hence there was no evidence to support the hypothesis that the extract of *A. membranaceus* or fractions derived from the extract enhanced cell mediated immunity.

4.2.4 Serum Interleukin-12 levels in mice following oral administration of *Astragalus membranaceus* extract or fractions derived from *Astragalus membranaceus*.

In this study serum interleukin-12 levels were assessed in mice dosed orally with *A. membranaceus* extract or with three fractions derived from whole extract in an attempt to identify likely classes of compound involved in the production of any observed immunological response. Statistical analysis of the results for this data is presented in Appendix D. A histogram representing the serum interleukin-12 levels in the different groups of mice is shown in Figure 4.8.

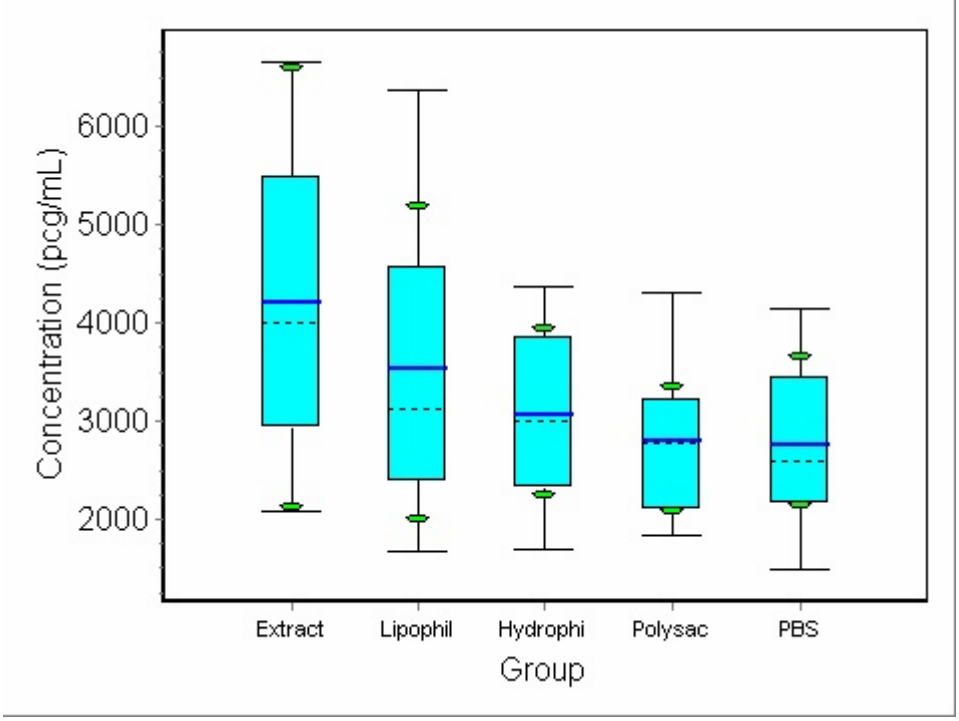

Figure 4.8 Histogram for interleukin-12 levels in serum following oral administration of *Astragalus membranaceus* extract, fractions derived from *A. membranaceus* or placebo (Phosphate buffered saline).

Legend: Extract- *A. membranaceus* extract, Lipophil – Lipophilic fraction, Hydrophi – Hydrophilic fraction, Polysac – Polysaccharide fraction, PBS – phosphate buffered saline.

Analysis of variance performed on the serum interleukin-12 levels revealed no statistically relevant differences between the different groups. These results provided further evidence to support the hypothesis that the extract of *A. membranaceus* or isolated fractions did not enhance innate immunity in mice.

Chapter 5: Discussion and Conclusions

The use of *Astragalus membranaceus* as a traditional Chinese medicine in a variety of applications for support of immune function is well documented (Anonymous , Alternative Medicine Review, 2003). The transition of this herb into contemporary western usage has resulted in the presentation of extracts, which in some cases are quite markedly different in methods of preparation in comparison to the presentation as a traditional dosage form. Although the use of cycloartane triterpene glycoside, Astragaloside IV is widely reported as a characteristic and active constituent (Li et al, 2001), standardisation of extracts to a recommended level of this compound, or any other of the phytochemical components, is not accepted as a rule or conducted.

5.1 Rationale for this study

In the studies published to date on the immunomodulatory activity of *Astragalus membranaceus*, much of the work has involved aqueous extracts as opposed to the ethanolic extracts utilised in the course of this study Current use of Astragalus in Australia is as a herbal medicine available as a Listed medicine. The listing process for products on the Australian Register of Therapeutic Goods requires evidence to be available supporting the quality and safety of the medicine, but no

assessment of the efficacy for the claimed therapeutic uses of the product. Safety is established by way of review of available supportive documentation, not by way of assessment of the claimed therapeutic properties of the individual products (Australian Regulatory Guidelines for Complementary Medicines, 2005)

Products assessed using these protocols are assigned an AUST L number and are available over the counter for the purpose of self-medication by the public. These products are taken via the oral route. Many of the papers published to date indicating the expression of positive immunostimulation as a consequence of administration of extracts of *Astragalus membranaceus* were the result of administration via the intraperitoneal route. This approach is at odds with the general use of Astragalus extracts in Australia. Additionally, much of the body of work previously conducted relates to the use of aqueous extracts. Although no profile of the phytochemical constituents was prepared as a part of this study, it is reasonable to expect that the components present in this extract, which is widely available in an over the counter preparation of Greenridge Botanicals in Australia, are likely to be substantially different to that found in an aqueous extract. It was therefore of interest to determine the immunomodulatory potential of the ethanolic extracts of *A. membranaceus* on adaptive immunity by using an inactivated *Salmonella*

vaccine which has been demonstrated to induce only an antibody response but no cell mediated immunity. The potential of the *A. membranaceus* extract or fractions derived there from to stimulate innate immunity was also investigated.

5.2.1 Antibody response

Determination of the total antibody titre following administration of ethanolic extracts of *Astragalus membranaceus* to mice by either of the oral or intraperitoneal routes demonstrated that there was no enhancement of humoral immune response. There was suppression of response instead. Elevation of titres in the PBS control groups vaccinated with the killed *Salmonella typhimurium* vaccine was consistent with previous studies (Mukkur et al, 1992). Failure to induce an antibody response during these initial determinations was the reason why no determination of antibody titres in experiments involving the use of the isolated fractions were carried out.

Given that the extract used in the course of these investigations was prepared by initially macerating the root matter in 60% v/v ethanol in water, it is highly probable that the content of polysaccharides and other water soluble compounds would be markedly lower than what would be

expected in an aqueous extract. This is a fundamental point of difference between the extracts used in these studies in comparison to the majority of previously published data where aqueous extract preparations were used. Regardless, results obtained in this investigation do raise an issue with the use of *A membranaceus* as a component of “AstraForte” that is currently marketed as a potential immunostimulant.

5.2.2 Cell-mediated immune response

Results of the initial determinations of IFN-γ in splenocyte culture supernatants from mice, vaccinated with killed Salmonella vaccine previously shown to induce only an antibody response, and simultaneously gavaged with the extract showed no significant difference in the levels of this cytokine when compared with the control groups.

In order to further explore the possibility that *A. membranaceus* extract may modulate innate immunity, further determinations were then made on the potential of the crude extract as well as the isolated hydrophilic, lipophilic and polysaccharide fractions administered by the oral route. No statistically significant difference between the PBS control groups, crude extract or fractions derived therefrom. It therefore appeared that there was no evidence that a 60% v/v ethanol in water extract of *Astragalus membranaceus* modulated CMI response.

5.2.3 Determination of Interleukin-12 Levels

As part of the examination of the potential for the extract of *Astragalus membranaceus* to promote and/or modify the immune response, it was decided to investigate the potential impact of this extract on innate immunity as judged by induction of IL-12.

This is significant because of the role of IL-12 in the activation and/or generation of Tc and NK cells (Coico et al., 2003), production of which of may ensure that there was no decreased production of IFN-γ, the most potent macrophage-activating agent *in vivo* (Marodi, 2006). Since there were no statistically significant differences in the IL-12 levels between the groups dosed orally with whole *A. membranaceus* extract or fractions derived versus the PBS control group, the hypothesis that the extract modulates CMI responses was not substantiated.

5.4 Future research directions

Given that there was potential suppression of antibody response and no promotion of acquired cell-mediated immunity response or innate immunity, potential of this herb as an immunosuppressant in autoimmune diseases mediated by antibodies to self-antigens warrants further investigation. However, prior to such trials in humans it is imperative to

re-evaluate the immunomodulatory properties of the aqueous preparations of *Astragalus membranaceus* which are relatively rich in polysaccharides, administered by the oral route, using the mouse model described in this investigation.

5.5 Conclusions

Investigation on the immunomodulatory potential of *Astragalus membranaceus*, presented in this thesis, have not supported the previously reported communications on the enhancement of immune response by extracts of *Astragalus membranaceus*, notwithstanding the fact that the extract in most investigations was administered by a systemic rather than the genuine oral route of administration. Analysis of the data on the immune parameters obtained in this investigation revealed that oral administration of the *Astragalus membranaceus* extract did not enhance either the antibody or cell-mediated immune response to the Salmonella vaccine antigens used for immunisation of mice but instead there was a potential suppression of the antibody response. In traditional medicine, this herb is not recommended for use in acute infections [Bone, 2000]. Given its potential immunosuppressive property, this herb clearly cannot be recommended as an immunostimulant.

If the immunomodulatory potential of *Astragalus membranaceus* is due to a polysaccharide component, as has been reported to be the case with Echinacea species (Wagner et al, 1985) then further experiments using extraction with solvents containing a lower concentration of ethanol or aqueous extracts will be warranted to obtain a resolution to this important question. On the other hand, if the results obtained in this investigation are re-confirmed, then attempts to isolate the compound responsible for the immunosuppressant property of *A membranaceus* will be warranted.

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APPENDIX A:

Thin-Layer Chromatography

A sample of 1 gram of the milled herb was extracted by boiling in 10mL of methanol for 30 minutes. The mixture was filtered through a Whatman No.4 filter paper. Preparation of the reference solution was conducted in the same way. 10μL of the resultant liquid was applied as a band approx 10mm from the base of a silica C_{60} F_{264} TLC plate (Alltech). The plate was placed into an equilibrated tank containing acetic acid, formic acid, water and ethyl acetate as a solvent system in the ratio 11:11:27:100. The plate was allowed to run until the solvent front was approximately 9 cm up the plate, when it was removed and air dried. The plate was then sprayed with 1% w/v methanolic diphenylboric acid followed by 5% ethanolic polyethylene glycol and viewed under UV light at 365nm.

APPENDIX B: Reagents

Coupling buffer:

Na_2CO_3	1.18g
NaH CO_3	3.47g

Dissolve in 1L of distilled water and adjust to pH 9.6. Store at 4°C and use within a fortnight.

Phosphate Buffered Saline

NaCl	8.0g
Na_2HPO_4	1.1375g
KCl	0.2g
KH_2PO_4	0.2g

Dissolve in 975mL of distilled water and adjust pH to 7.2 with phosphoric acid. Make to a final volume of one litre and prepare fresh weekly.

Tissue Culture Media

(i) Without fetal bovine serum (FBS)

To Dulbecco's modified eagle media (Gibco, Aukland NZ) add 25mN HEPES, 12μM 2-mercaptoethanol and 100 units/mL penicillin-streptomycin

(ii) With 10% FBS

Add 10% fetal bovine serum to the media in (I).

Wash buffer

To 1L PBS add 0.5mL Tween 20 (Sigma, St. Louis).

APPENDIX C: Mouse Interferon-γ Assay procedure:

1. Prepare all reagents, samples and standards.
2. Remove excess microplate strips from the plate frame.
3. Add 50 microloitres of Assay diluent RD1-21 to each well.
4. Add 50 microlitres of standard, control or sample to the centre of each well. Mix by gently tapping the plate frame for 1 minute. Cover the plate and incubate for 2 hours at room temperature.
5. Aspirate and wash each well 5 times.
6. Add 100 microlitres of mouse IFN-γ conjugate to each well. Cover and incubate for 2 hours at room temperature.
7. Wash as in step 5.
8. Add 100 microlitres of substrate solution to each well. Gently tap the plate to ensure thorough mixing. Incubate for 30 minutes at room temperature protected from light.
9. Add 100 microlitres of stop solution to each well. Gently taps to ensure mixing.
10. Determine the optical density of each well within 30 minutes using a microplate reader set to 450nm.

APPENDIX D: Raw Data and Statistical Analysis

D.1 Total antibody titres in mice following oral gavage with *Astragalus membranaceus*

Group	Titre	Group	Titre
PBS	10.7	Ext	7.1
PBS	5.4	Ext	0
PBS	1.8	Ext	0
PBS	1.9	Ext	13.1
PBS	0	Ext	6.3
PBS	1.4	Ext	17.5
PBS	1.1	PBS Vac	2.6
Ext Vac	11.7	PBS Vac	136.8
Ext Vac	0	PBS Vac	155.7
Ext Vac	8.2	PBS Vac	26.96
Ext Vac	5.3	PBS Vac	152.6
Ext Vac	13.7	PBS Vac	160.9
Ext Vac	19.3		
Ext Vac	13.6		

Analysis of Variance Report
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Database C:\Program Files\NCSS97\Report\total antibody.S0
Response C2

Tests of Assumptions Section

Assumption	Test Value	Prob Level	Decision (0.05)
Skewness Normality of Residuals	-2.9689	0.002988	Reject
Kurtosis Normality of Residuals	2.8996	0.003736	Reject
Omnibus Normality of Residuals	17.2224	0.000182	Reject
Modified-Levene Equal-Variance Test	3.7577	0.025587	Reject

Expected Mean Squares Section

Source Term	DF	Term Fixed?	Denominator Term	Expected Mean Square
A: C1	3	Yes	S(A)	S+sA
S(A)	22	No		S(A)

Note: Expected Mean Squares are for the balanced cell-frequency case.

Analysis of Variance Table

Source Term	DF	Sum of Squares	Mean Square	F-Ratio	Prob Level	Power (Alpha=0.05)

A: C1	3	45431.78	15143.93	12.76	0.000048*
	0.998995				
S(A)	22	26111.97	1186.908		
Total (Adjusted)	25	71543.74			
Total	26				

* Term significant at alpha = 0.05

Kruskal-Wallis One-Way ANOVA on Ranks
Hypotheses
Ho: All medians are equal.
Ha: At least two medians are different.

Test Results

Method	**DF**	**Chi-Square (H)**	**Prob Level**	**Decision(0.05)**
Not Corrected for Ties	3	11.11762	0.011107	Reject Ho
Corrected for Ties	3	11.15576	0.010913	Reject Ho

Number Sets of Ties	1
Multiplicity Factor	60

Group Detail

Group	**Count**	**Sum of Ranks**	**Mean Rank**	**Z-Value**	**Median**
Oral Extract vaccinated	7	100.50	14.36	0.3468	11.7
Oral PBS non-vaccinated	6	129.00	21.50	2.9212	144.7
Oral PBS vaccinated	7	54.50	7.79	-2.3123	1.8
Oral extract non-vaccinated	6	67.00	11.17	-0.8520	6.7

Means and Effects Section

Term	**Count**	**Mean**	**Standard Error**	**Effect**
All	26	29.75615	31.67571	
A: C1				
Oral Extract vaccinated	7	10.25714	13.02145	-21.41857
Oral PBS non-vaccinated	6	105.9267	14.06478	74.25095
Oral PBS vaccinated	7	3.185714	13.02145	-28.49
Oral extract non-vaccinated	6	7.333333	14.06478	-24.34238

D.2 Total antibody titres in vaccinated versus non-vaccinated mice administered *Astragalus membranaceus* extract by intraperitoneal route

Group	Titre
PBS Vac	157.8
PBS Vac	153.4
PBS Vac	18.2
PBS Vac	18.2
PBS Vac	17
PBS Vac	14.1
PBS Vac	21.9
PBS Vac	24.8
Ext Vac	21.9
Ext Vac	21.5
Ext Vac	12.6
Ext Vac	8.8
Ext Vac	15.4
Ext Vac	14.9
Ext Vac	15.5
Ext Vac	14.8
Ext	15.6
Ext	13
Ext	4.5
Ext	3.4
Ext	0
Ext	8
Ext	19.6

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Response C2

Tests of Assumptions Section

Assumption	Test Value	Prob Level	Decision (0.05)
Skewness Normality of Residuals	3.5393	0.000401	Reject
Kurtosis Normality of Residuals	2.8289	0.004670	Reject
Omnibus Normality of Residuals	20.5296	0.000035	Reject
Modified-Levene Equal-Variance Test	2.0405	0.156159	Accept

Expected Mean Squares Section

Source Term	DF	Term Fixed?	Denominator Term	Expected Mean Square
A: C1	2	Yes	S(A)	S+sA
S(A)	20	No		S(A)

Note: Expected Mean Squares are for the balanced cell-frequency case.

Analysis of Variance Table

Source	Sum of	Mean		Prob	Power

Term	DF	Squares	Square	F-Ratio	Level (Alpha=0.05)
A: C1	2	8734.045	4367.022	3.07	0.068991
S(A)	20	28492.93	1424.646		
Total (Adjusted)	22	37226.97			
Total	23				

* Term significant at alpha = 0.05

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Kruskal-Wallis One-Way ANOVA on Ranks

Hypotheses

Ho: All medians are equal.
Ha: At least two medians are different.

Test Results

Method	DF	Chi-Square (H)	Prob Level	Decision(0.05)
Not Corrected for Ties	2	9.242042	0.009843	Reject Ho
Corrected for Ties	2	9.251184	0.009798	Reject Ho

Number Sets of Ties	2
Multiplicity Factor	12

Group Detail

Group	Count	Sum of Ranks	Mean Rank	Z-Value	Median
IP Extract non-vaccinated	8	138.50	17.31	2.7434	20.05
IP Extract vaccinated	8	90.50	11.31	-0.3550	15.15
IP PBS vaccinated	7	47.00	6.71	-2.4722	8

Means and Effects Section

Term	Count	Mean	Standard Error	Effect
All	23	26.73478	26.00238	
A: C1				
IP Extract non-vaccinated	8	53.175	13.34469	27.17262
IP Extract vaccinated	8	15.675	13.34469	-10.32738
IP PBS vaccinated	7	9.157143	14.26608	-16.84524

D.3 Determination of Interferon-γ levels in vaccinated versus non-vaccinated mice administered *Astragalus membranaceus* extract via the oral route.

Group	Concentration
PBS Vac	151
PBS Vac	386
PBS Vac	279
PBS Vac	231
PBS Vac	138
PBS Vac	222
PBS Vac	397
PBS Vac	409
Ext Vac	69
Ext Vac	228
Ext Vac	365
Ext Vac	409
Ext Vac	409
Ext Vac	409
Ext Vac	373
Ext Vac	395
Ext Vac	396
Ext	135
Ext	310
Ext	376
Ext	400
Ext	328
Ext	275
Ext	336
Ext	96
Ext	222
PBS	19
PBS	83
PBS	136
PBS	69
PBS	46
PBS	13
PBS	44
PBS	93
PBS	29

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Tests of Assumptions Section

Assumption	**Test Value**	**Prob Level**	**Decision (0.05)**

Skewness Normality of Residuals	-2.4449	0.014491	Reject
Kurtosis Normality of Residuals	1.4562	0.145325	Accept
Omnibus Normality of Residuals	8.0980	0.017440	Reject
Modified-Levene Equal-Variance Test	2.5830	0.071113	Accept

Expected Mean Squares Section

Source Term	DF	Term Fixed?	Denominator Term	Expected Mean Square
A: C1	3	Yes	S(A)	S+sA
S(A)	31	No		S(A)

Note: Expected Mean Squares are for the balanced cell-frequency case.

Analysis of Variance Table

Source Term	DF	Sum of Squares	Mean Square	F-Ratio	Prob Level	Power (Alpha=0.05)
A: C1	3	43.47584	14.49195	21.90	0.000000*	1.000000
S(A)	31	20.51796	0.6618698			
Total (Adjusted)	34	63.9938				
Total	35					

* Term significant at alpha = 0.05

Analysis of Variance Report

Kruskal-Wallis One-Way ANOVA on Ranks

Hypotheses

Ho: All medians are equal.
Ha: At least two medians are different.

Test Results

Method	DF	Chi-Square (H)	Prob Level	Decision(0.05)
Not Corrected for Ties	3	21.12553	0.000099	Reject Ho
Corrected for Ties	3	21.15812	0.000098	Reject Ho

Number Sets of Ties	2
Multiplicity Factor	66

Group Detail

Group	Count	Sum of Ranks	Mean Rank	Z-Value	Median
Control - vaccinated	8	171.00	21.38	1.0607	2.186
Extract - non vaccinated	9	180.50	20.06	0.6982	2.649
Extract - vaccinated	9	233.50	25.94	2.6986	3.383
PBS Control - non vaccinated	9	45.00	5.00	-4.4159	-4.4159
	0.039				

Means and Effects Section

Term	Count	Mean	Standard Error	Effect
All	35	1.906857		
	1.919681			
A: C1				
Control - vaccinated	8	2.3685	0.2876347	
	0.4488195			

Extract - non vaccinated	9	2.356	0.2711846	0.4363194
Extract - vaccinated	9	2.903667	0.2711846	0.9839861
PBS Control - non vaccinated	9	5.055556E-02	0.2711846	-1.869125

D.4 Interferon-γ levels in vaccinated versus non-vaccinated mice administered *Astragalus membranaceus* extract by intraperitoneal route.

Group	Concentration
Ext	0
Ext	8
Ext	11
Ext	8
Ext	0
Ext	14
Ext	27
Ext	33
Ext	47
Ext Vac	41
Ext Vac	12
Ext Vac	30
Ext Vac	16
Ext Vac	7
Ext Vac	0
Ext Vac	34
Ext Vac	6
Ext Vac	23
PBS Vac	5
PBS Vac	5
PBS Vac	7
PBS Vac	25
PBS Vac	12
PBS Vac	9
PBS Vac	40
PBS Vac	40
PBS Vac	14

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Tests of Assumptions Section

Assumption	Test Value	Prob Level	Decision (0.05)
Skewness Normality of Residuals	1.4379	0.150458	Accept
Kurtosis Normality of Residuals	-1.3393	0.180462	Accept
Omnibus Normality of Residuals	3.8614	0.145045	Accept
Modified-Levene Equal-Variance Test	0.0271	0.973339	Accept

Expected Mean Squares Section

Source Term	DF	Term Fixed?	Denominator Term	Expected Mean Square
A: C1	2	Yes	S(A)	S+sA
S(A)	21	No		S(A)

Note: Expected Mean Squares are for the balanced cell-frequency case.

Analysis of Variance Table

Source Term	DF	Sum of Squares	Mean Square	F-Ratio	Prob Level	Power (Alpha=0.05)
A: C1	2	6.090278E-05	3.045139E-05	0.21	0.809891	0.078940
S(A)	21	3.002431E-03	1.429729E-04			
Total (Adjusted)	23	3.063333E-03				
Total	24					

* Term significant at alpha = 0.05

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Kruskal-Wallis One-Way ANOVA on Ranks

Hypotheses

Ho: All medians are equal.
Ha: At least two medians are different.

Test Results

Method	DF	Chi-Square (H)	Prob Level	Decision(0.05)
Not Corrected for Ties	2	0.8	0.670320	Accept Ho
Corrected for Ties	2	0.8020924	0.669619	Accept Ho

Number Sets of Ties	6
Multiplicity Factor	36

Group Detail

Group	Count	Sum of Ranks	Mean Rank	Z-Value	Median
IP Extract - non vaccinated	7	94.50	13.50	0.4446	0.012
IP PBS vaccinated	9	97.50	10.83	-0.8944	0.01
IP extract vaccinated	8	108.00	13.50	0.4899	0.017

Means and Effects Section

Term	Count	Mean	Standard Error	Effect
All	24	1.683333E-02	1.696759E-02	
A: C1				
IP Extract - non vaccinated	7	0.018	4.519369E-03	1.032407E-03
IP PBS vaccinated	9	1.477778E-02	3.985709E-03	-2.189815E-03
IP extract vaccinated	8	0.018125	4.227483E-03	1.157407E-03

D.5 Determination of serum interferon-γ levels in mice administered *Astragalus membranaceus* extract by the oral route.

Group	Concentration
Extract	30
Extract	235
Extract	47
Extract	10
Extract	10
Extract	38
Extract	24
Extract	29
Lipophil	18
Lipophil	19
Lipophil	21
Lipophil	154
Lipophil	18
Lipophil	21
Lipophil	141
Lipophil	125
Hydrophi	114
Hydrophi	43
Hydrophi	20
Hydrophi	104
Hydrophi	20
Hydrophi	53
Hydrophi	136
Hydrophi	137
Polysac	140
Polysac	124
Polysac	159
Polysac	131
Polysac	135
Polysac	34
Polysac	150
Polysac	18
PBS	21
PBS	112
PBS	136
PBS	212
PBS	106
PBS	117
PBS	159
PBS	73

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Tests of Assumptions Section

Assumption	Test Value	Prob Level	Decision (0.05)
Skewness Normality of Residuals	2.3227	0.020197	Reject
Kurtosis Normality of Residuals	1.7880	0.073783	Accept
Omnibus Normality of Residuals	8.5916	0.013626	Reject
Modified-Levene Equal-Variance Test	0.1659	0.954282	Accept

Expected Mean Squares Section

Source Term	DF	Term Fixed?	Denominator Term	Expected Mean Square
A: C1	4	Yes	S(A)	S+sA
S(A)	35	No		S(A)

Note: Expected Mean Squares are for the balanced cell-frequency case.

Analysis of Variance Table

Source Term	DF	Sum of Squares	Mean Square	F-Ratio	Prob Level	Power (Alpha=0.05)
A: C1	4	0.7172086	0.1793022	0.97	0.436429	0.274307
S(A)	35	6.471808	0.1849088			
Total (Adjusted)	39	7.189017				
Total	40					

* Term significant at alpha = 0.05

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Response C3

Kruskal-Wallis One-Way ANOVA on Ranks
Hypotheses
Ho: All medians are equal.
Ha: At least two medians are different.

Test Results

Method	DF	Chi-Square (H)	Prob Level	Decision(0.05)
Not Corrected for Ties	4	4.916616	0.295961	Accept Ho
Corrected for Ties	4	4.918	0.295815	Accept Ho

Number Sets of Ties	3
Multiplicity Factor	18

Group Detail

Group	Count	Sum of Ranks	Mean Rank	Z-Value	Median
Crude extract	8	118.50	14.81	-1.5385	0.14

Extract - Lipophilic fraction	8		203.00	25.38	1.3187
0.889					
Extract - hydrophilic fraction	8		148.00	18.50	-0.5410
0.525					
Extract - polysaccharide fraction	8		200.50	25.06	1.2342
0.7675					
PBS control	8	150.00	18.75	-0.4734	0.293

Means and Effects Section

Term	**Count**	**Mean**	**Standard Error**	**Effect**
All	40	0.61085		0.61085
A: C1				
Crude extract	8	0.432125	0.1520316	-
0.178725				
Extract - Lipophilic fraction	8	0.745625	0.1520316	
0.134775				
Extract - hydrophilic fraction	8	0.5245	0.1520316	-
0.08635				
Extract - polysaccharide fraction	8	0.784625	0.1520316	
0.173775				
PBS control	8	0.567375	0.1520316	-
0.043475				

D.6 Interferon-γ levels in mice administered *Astragalus membranaceus* extract or fractions derived from *Astragalus membranaceus* by the oral route

Group	Concentration
Extract	109
Extract	119
Extract	16
Extract	21
Extract	153
Extract	123
Extract	118
Extract	138
Extract	116
Lipophil	187
Lipophil	23
Lipophil	96
Lipophil	145
Lipophil	85
Lipophil	33
Hydrophi	195
Hydrophi	119
Hydrophi	99
Hydrophi	12
Hydrophi	167
Hydrophi	156
Hydrophi	41
Hydrophi	21
Hydrophi	22
Polysac	11
Polysac	13
Polysac	141
Polysac	132
Polysac	144
Polysac	139
Polysac	74
Polysac	12
Polysac	40
PBS	24
PBS	21
PBS	26
PBS	19
PBS	52
PBS	147
PBS	53
PBS	24
PBS	127

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Response C4

Tests of Assumptions Section

Assumption	Test Value	Prob Level	Decision (0.05)
Skewness Normality of Residuals	0.3059	0.759708	Accept
Kurtosis Normality of Residuals	-2.9073	0.003646	Reject
Omnibus Normality of Residuals	8.5458	0.013941	Reject
Modified-Levene Equal-Variance Test	1.1698	0.339873	Accept

Expected Mean Squares Section

Source Term	DF	Term Fixed?	Denominator Term	Expected Mean Square
A: C1	4	Yes	S(A)	S+sA
S(A)	37	No		S(A)

Note: Expected Mean Squares are for the balanced cell-frequency case.

Analysis of Variance Table

Source Term	DF	Sum of Squares	Mean Square	F-Ratio	Prob Level	Power (Alpha=0.05)
A: C1	4	0.5401716	0.1350429	0.87	0.489229	0.250405
S(A)	37	5.722229	0.1546548			
Total (Adjusted)	41	6.262401				
Total	42					

* Term significant at alpha = 0.05

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Kruskal-Wallis One-Way ANOVA on Ranks
Hypotheses
Ho: All medians are equal.
Ha: At least two medians are different.

Test Results

Method	DF	Chi-Square (H)	Prob Level	Decision(0.05)
Not Corrected for Ties	4	2.034699	0.729377	Accept Ho
Corrected for Ties	4	2.034864	0.729346	Accept Ho

Number Sets of Ties	1
Multiplicity Factor	6

Group Detail

Group	Count	Sum of Ranks	Mean Rank	Z-Value	Median
Crude Extract	6	146.00	24.33	0.6111	0.6085

Extract - hydrophilic fraction	9		209.00	23.22	0.4751
0.665					
Extract - lipophilic fraction	9		177.00	19.67	-0.5058
0.499					
Extract - polysaccharide fraction	9		156.50	17.39	-1.1342
0.171					
PBS control	9	214.50	23.83	0.6437	0.79

Means and Effects Section

Term	Count	Mean	Standard Error	Effect
All	42	0.5604762		
0.5654333				
A: C1				
Crude Extract	6	0.6348333	0.1605484	0.0694
Extract - hydrophilic fraction	9	0.6191111	0.1310873	
5.367778E-02				
Extract - lipophilic fraction	9	0.5266666	0.1310873	-
3.876667E-02				
Extract - polysaccharide fraction	9	0.3667778	0.1310873	-
0.1986556				
PBS control	9	0.6797778	0.1310873	
0.1143444				

D.7 Serum Interleukin-12 levels in mice following oral administration of *Astragalus membranaceus* extract or fractions derived from *Astragalus membranaceus*.

Group	Concentration
Extract	6646
Extract	6581
Extract	2127
Extract	4204
Extract	4447
Extract	2081
Extract	3722
Extract	3784
Lipophil	1680
Lipophil	2810
Lipophil	2799
Lipophil	6359
Lipophil	3452
Lipophil	2001
Lipophil	5187
Lipophil	3984
Hydrophi	1709
Hydrophi	3824
Hydrophi	3932
Hydrophi	2254
Hydrophi	2945
Hydrophi	3078
Hydrophi	3211
Hydrophi	3344
Polysac	3477
Polysac	3610
Polysac	3743
Polysac	3876
Polysac	3141
Polysac	3344
Polysac	1849
Polysac	2561
PBS	1500
PBS	3657
PBS	2185
PBS	2157
PBS	4137
PBS	3304
PBS	2237
PBS	2964

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Response C5

Tests of Assumptions Section

Assumption	Test Value	Prob Level	Decision (0.05)
Skewness Normality of Residuals	1.2810	0.200194	Accept
Kurtosis Normality of Residuals	0.2719	0.785664	Accept
Omnibus Normality of Residuals	1.7149	0.424239	Accept
Modified-Levene Equal-Variance Test	1.2082	0.324758	Accept

Expected Mean Squares Section

Source Term	DF	Term Fixed?	Denominator Term	Expected Mean Square
A: C1	4	Yes	S(A)	S+sA
S(A)	35	No		S(A)

Note: Expected Mean Squares are for the balanced cell-frequency case.

Analysis of Variance Table

Source Term	DF	Sum of Squares	Mean Square	F-Ratio	Prob Level	Power (Alpha=0.05)
A: C1	4	3.204224	0.8010559	1.84	0.143082	0.501807
S(A)	35	15.23278	0.4352223			
Total (Adjusted)	39	18.437				
Total	40					

* Term significant at alpha = 0.05

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Response C5

Kruskal-Wallis One-Way ANOVA on Ranks
Hypotheses
Ho: All medians are equal.
Ha: At least two medians are different.

Test Results

Method	DF	Chi-Square (H)	Prob Level	Decision(0.05)
Not Corrected for Ties	4	4.207317	0.378675	Accept Ho
Corrected for Ties	4	4.207317	0.378675	Accept Ho

Number Sets of Ties	0
Multiplicity Factor	0

Group Detail

Group	Count	Sum of Ranks	Mean Rank	Z-Value	Median
Crude Extract	8	171.00	21.38	0.2367	1.649

Extract - hydrophilic fraction	8		163.00	20.38	-0.0338
1.586					
Extract - lipophilic fraction	8		135.00	16.88	-0.9806
1.469					
Extract - polysaccharide fraction	8		134.00	16.75	-1.0144
1.3695					
PBS Control	8	217.00	27.13	1.7921	2.1035

Means and Effects Section

Term	Count	Mean	Standard Error	Effect
All	40	1.723925		
1.723925				
A: C1				
Crude Extract	8	1.86125	0.233244	
0.137325				
Extract - hydrophilic fraction	8	1.611375	0.233244	-
0.11255				
Extract - lipophilic fraction	8	1.478	0.233244	-
0.245925				
Extract - polysaccharide fraction	8	1.457625	0.233244	-0.2663
PBS Control	8	2.211375	0.233244	0.48745