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Adaptive immune response-modifying and antimicrobial properties of *Andrographis paniculata* and andrographolide

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For the award of Doctorate of Philosophy

February 2009

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ABSTRACT

Andrographis paniculata (AP) is a traditional herbal medicine which is widely used for the treatment of many diseases in Asia. Recently, various biological activities of AP extract or andrographolide (AND), such as immunostimulatory activity, anti-inflammatory effect, cytokine induction or deduction, a potential cancer therapeutic agent and T cell activation suppression, have been reported. However the potential of AP extract or AND to stimulate the specific or adaptive immune response using microbial vaccines has not been determined. In this project, AND has been purified from AP and its identity confirmed by the melting point, colour test, TLC, UV absorption spectrum, ESI-MS and ^{1}H-NMR. An aqueous and two ethanolic extracts of *Andrographis paniculata* and AND, an active principle of *Andrographis paniculata*, were investigated for their antimicrobial activity against nine bacterial species *in vitro* using the disc diffusion method. It was discovered that neither the aqueous extract nor AND were bacteriostatic or bactericidal against *S. typhimurium*, *E. coli, S. sonnei, S. aureus, P. aeruginosa*, *S. pneumoniae, S. pyogenes, L. pneumophila* or *B. pertussis* but the two ethanolic extracts of AP were bacteriostatic against *L. pneumophila* and *B. pertussis*. It was also observed that the ethanol extract of AP and AND stimulated both antibody and cell-mediated immunity (CMI) responses to a killed *S. typhimurium* vaccine. Mice were vaccinated with either one dose or two doses of killed *S. typhimurium* vaccine. They were fed two different quantities of an ethanol extract of AP or AND for 14 days in mice immunised with one dose of the vaccine, and for 28 days in mice immunised with two doses of vaccine, respectively. Both the extract and AND significantly increased the IgG antibody titres against *S. typhimurium*, with the increase in antibody titres being statistically significant in the two dose vaccine group. Although not statistically significant, there was also a substantial increase in the IgG antibody titres in the one dose vaccine group. Splenocyte cultures from mice from both the immunisation groups treated with the extract or AND stimulated with the *S. typhimurium* lysate showed a significant increase in the production of IFN-γ in both 14 and 28 day AP extract or AND treatment groups. The increase indicates the induction of a cell-mediated immune response. To confirm the immunomodulatory potential of AP extract and determine the immunomodulatory potential of AND, experiments were conducted using mouse salmonellosis as a model system. Mice were vaccinated with two doses of killed *S. typhimurium* vaccine by intraperitoneal (i/p) route and orally dosed with AP extract at 25 mg/kg bodyweight or AND at 4 mg/kg bodyweight for total 28 days, followed by oral challenge with virulent *S. typhimurium*. Both AP extract and AND substantially increased the survival rate by 50% after mice were challenged with 10 fold of a sublethal dose (1.5×10^{6} cfu) of virulent *S. typhimurium*. They also promoted clearance of *S. typhimurium* from challenged mice by days 8 or 12 post-challenge with 1.5×10^{5} cfu virulent *S. typhimurium* respectively. Sera IgG, IgA antibody titres against *S. typhimurium* and

IFN-γ or IL-2 were detected after the mice were challenged by the oral route with a sublethal dose (1.5x10 ^{5} cfu) of virulent *S. typhimurium* for 12 days. It was thus concluded that both *Andrographis paniculata* and AND not only elicited both humoral and cell-mediated immune responses in the mouse model, but also increased the protective efficacy against salmonellosis on mice vaccinated with inactivated *S. typhimurium*. Therefore the ability of *Andrographis paniculata* and AND to promote acquired immunity, particularly in inducing CMI may be important in protection against intracellular pathogen infection.

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, except where otherwise acknowledged.

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ACKNOWLEDGMENTS

I heartily thank my former principal supervisor A/Prof. T. K. Mukkur for his support, provision of research funding, his research direction of this project, help in the drafting of the manuscripts and correction of draft of the dissertation. I wish to acknowledge that without his research direction and help in the correction of my dissertation after his departure from the University of Southern Queensland, it would not have been possible to submit this thesis in its present state. I sincerely thank my current supervisors Dr. Ray Marshall and Dr. John Dearnaley for their advice, help and encouragements. I thank them for their encouragements for my carrying on my project after Prof Mukkur retired, and their help in my drafting dissertation and correction of my dissertation. I express my thanks to Dr. R. A. Barrow (Department of Chemistry, Faculty of Science, Australian National University, Canberra 0200, Australia) for his assistance in conduct the electrospray ionization mass spectrometry (ESI-MS) and ^{1}H- nuclear magnetic resonance spectroscopy (^{1}H-NMR). I thank Dr. Austen Chen, Dr. Scott Fry, Mrs. Adele Jones and Mrs. Morwenna Boddington for their help, support and friendships. Thanks to all others who are not mentioned here for their help and encouragement.

Finally, I would like to express my heartfelt thanks to my husband, Jiuyong, my daughter Cicy, my son Victor, my mother and my parents-in-law for their support and understanding during my studying.

PUBLICATIONS DURING MY PhD STUDY

1. **Youhong Xu**, Austen Chen, Scott Fry, Russell A. Barrow, Raymond L. Marshall and Trilochan K. S. Mukkur (2007) Modulation of immune response in mice immunised with an inactivated *Salmonella* vaccine and gavaged with *Andrographis paniculata* extract or andrographolide. Journal of International Immunopharmacology, 7, 515-523.
2. **Youhong Xu**, Raymond L. Marshall and Trilochan K. S. Mukkur (2006) An investigation on the antimicrobial activity of *Andrographis paniculata* extracts and andrographolide *in vitro*. Asian Journal of Plant Sciences, 5 (3), 527-530.
3. Renee Cornford-Nairn, T. Rosetti, Scott Kershaw, Scott Fry, **Youhong Xu**, Grant Daggard, Trilochan K. S. Mukkur (2005) A metabolite-deficient mutant of *Bordetella pertussis* that induces secretory IgA and cell-mediated immunity in mice vaccinated by the intranasal route. A poster on 2005 FOS conference, USA.

Appendix L contains copies of the above publications

ABBREVIATIONS

AG	average gain
AND	andrographolide
AP	*Andrographis paniculata*
APC	antigen-presenting cells
APE	*Andrographis paniculata* extract
BSA	bovine serum albumin
bw	body weight
CDMEM	complete Dulbecco's Modified Eagle medium
CFU	colony forming unit
CMI	cell-mediated immunity
CTL	cytotoxic T lymphocytes
DA	14-deoxyandrographolide
DDA	14-deoxy-11, 12-didehydroandrographolide
DCs	dendritic cells
DIDPHSP	Department of the infectious disease of the people's hospital of Shantou Prefecture
DMEM	Dulbecco's Modified Eagle medium
EDTA	ethylenediamine tetraacetic acid
ELISA	enzyme linked immunosorbent assay
ESI-MS	electrospray ionization mass spectrometry
EtOH	ethyl alcohol
FBS	fetal bovine serum
hr	hour
^{1}H-NMR	^{1}H- nuclear magnetic resonance spectroscopy
HPBLs	human peripheral blood lymphocytes
HRP	horseradish peroxidase
IFN-γ	interferon gamma
IgA	immunoglobulin A
IgG	immunoglobulin G
IL-2	interleukin-2
IL-4	interleukin-4
iNOS	inducible nitric oxide synthase
i/p	intraperitoneal
LB	Luria-Bertani
M cell	membraneous cell
MALT	mucosa-associated lymphoid tissue
2-ME	2-mercaptoethanol
MeOH	methanol
MHC	major histocompatibility complex
min	minute

OD	optical density
o/n	overnight
OPD	o-phenylenediamine dihydrochloride
PAMPs	pathogen-associated molecular patterns
PBS	phosphate buffered saline
PBST	PBS and Tween-20
PMSF	phenylmethylsulfonyl fluoride
PPRC	Pharmacopoeia of the People's Republic of China
rpm	revolution per min
RT	room temperature
s.c.	subcutaneously
SCHRI	Sichuan Chinese Herb Research Institute
sec	second
SE	standard error
S-IgA	secretory IgA
ST	standard
TCR	T cell receptors
Th	T-helper
TLC	thin layer chromatography
TNF	tumour necrosis factor
Tween 20	polyoxyethylenesorbitan monolaurate
WE	Water extract of *Andrographis paniculata*
WHO	World Health Organization

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Chapter 1 Literature Review

This Chapter provides the background literature underpinning the research embodied in this doctoral dissertation. It includes the immunological concepts relevant to the exploration of the immunobiological properties of *Andrographis paniculata* (AP) and one of its bioactive pharmaceuticals, andrographolide (AND). Also reviewed are the strategies used in the development of vaccines against infections caused by the intracellular pathogen, *Salmonella typhimurium*, the model used in this study to investigate the immunomodulatory properties of AP and AND.

1.1 The Immune system

There are two aims of the immune response that are involved in protection against foreign invaders including infectious agents. Host defences against foreign invaders can be innate and adaptive or acquired immunity. Innate immunity is constituted by intrinsic defence mechanisms that are always present ready to recognize infectious agents by recognition of structures (pathogen-associated molecular patterns, PAMPs). These structures are shared by different types macrobes (bacteria, viruses or fungi), but are not present on the host cells. The recognition of PAMPs is affected by pattern recognition receptors or PRRs on the host cells compusing the immune system. The latter are encoded in the germ line and not by somatic recombination of genes (Abbas et al., 2007). Because the results of this investigation embodied in this dissertation examined the immune-response modifying effect of AP extract and AND on adaptive or specific immunity, no discussion of the mechanisms of innate immunity in protection against foreign invaders has been presented in this review. However, excellent reviews on innate immunity have been published elsewhere (Beutler, 2003; Beutler and Rietschel, 2003; Janeway and Medzhitov, 2002; Medzhitov and Janeway, 2002; Medzhitov and Janeway, 2000). Adaptive immunity, in contrast to innate immunity, is the ability of the host to induce an acquired immune response specific to a given infectious antigen (Levine et al., 2004; Black, 2002; Janeway, 2001). Aspects relevant to research embodied in this thesis are presented below.

1.1.1 Adaptive or acquired immunity

In most instances, the innate immune system is unable to completely eliminate a foreign invader and requires the assistance of antigen-specific acquired immune effector mechanisms such as humoral and / or cell-mediated immunity (the two arms

of acquired immunity). Acquired immunity occurs after exposure to an antigen and is effective at preventing infection by activating antigen-specific arms of the immune response that results in improved response after repeated exposure and immunological memory (Playfair and Bancroft, 2006; McNeela and Mills, 2001). Acquired immunity is more specialized than innate immunity in that another cell type, lymphocyte, is brought into play and supplements the protection provided by innate immunity. And acquired immunity, that came into play relatively late in evolution, is present only in vertebrate species. Although the two arms of acquired immunity have different sets of participants and different sets of purposes, their aim is the same, which is to eliminate the antigen. Of these two arms of the acquired immune response, humoral immunity is mediated mainly by circulating antibodies that are produced by epitope-specific B cells, with or without interaction with CD4 T cells depending upon the thymus-dependence or –independence of stimulating epitope or antigen, following differentiation into plasma cells, not withstanding the participation of B cells as antigen-presenting cells (APCs), Whereas cellular or cell-mediated immunity, on the other hand, is mediated by T cells (Playfair and Bancroft, 2006).

1.1.2 Cell populations involved in acquired immunity

Apart from the antigen-presenting cells, the two types of lymphocyte populations required for the induction of acquired immunity are B and T cells. Both these types of cells are involved in the presentation of thymus-dependent antigens following phagocytosis. B cells are derived from bone marrow after fetal life (liver, in the fetal life), produce antibodies after differentiation to plasma cells and are mainly effective against extracellular infection. Two distinct populations of T-cells are recognized by the presence of two different types of co-receptor surface proteins (CD antigens), CD4 and CD8 lymphocytes (Gao et al., 2002; Konig, 2002; Nemazee, 2000). Cytotoxic T cells (CTLs) and Th1 cells produce cytokines leading to the activation of macrophages and proliferation of CTLs, are mainly effective against intracellular pathogens (Schluns and Lefrancois, 2003; Kaech, Wherry and Ahmed, 2002; Seder and Hill, 2000).

After binding of antigen/epitopes displayed by the major histocompatiblity complex molecules by the lymphocyte receptors, the cells are stimulated into a cell cycle, producing large numbers of identical cells. This clonal expansion or clonal proliferation is the fundamental basis of acquired immunity providing large numbers of effector cells (e.g. plasma cells producing antibodies or Th1 T-lymphocytes for activating macrophages or CD8 CTLs depending on the pathway of antigen presentation) to cope with infectious agents and also producing memory cells for subsequent encounters with the same antigen (Levine et al., 2004).

The effector T-cells fall into functional categories based on their interaction with peptide antigens derived from different pathogens processed via endogenous or exogenous pathways (Janeway et al., 2001). Peptides from the intracellular pathogens, which are processed via the endogenous pathway because they multiply in the cytoplasm, are carried to the cell surface by the MHC class I molecules and presented to CD8 T cells. These CD8 T cells differentiate into cytotoxic T cells that kill the infected cells (Jankovic et al., 2001; Harty et al., 2000; Abbas et al. 1996). On the other hand, peptides derived from ingested extracellular bacteria or soluble proteins, which are processed via the exogenous pathway, are carried to the cell surface via MHC class II molecules and presented to CD4 T cells (Levine et al., 2004). These helper T cells can formerly differentiate into two types of effector T cells, Th1 and Th2. Intracellular pathways, which are capable of surviving in large numbers within the macrophages and dendritic cells, tend to stimulate the differentiation of Th1 cells. In contrast, the extracellular pathways tend to stimulate the production of Th2 cells. Th1 cells are involved in the activation of microbiocidal properties of macrophages and differentiation of B cells for production of highly opsonic IgG isotype antibodies e.g. IgG 2a in the mouse (Goffman, 2006; Constant and Bottomly, 1997). Th2 cells on the other hand, initiate the humoral immune response by activating naive antigen-specific B-cells to produce IgM which undergo antibody class switching upon booster vaccination to produce IgG, IgA or IgE antibody classes (Goffman, 2006; Janeway et al., 2001). Regardless of the antigen presented to the antigen-presenting cells, cytokines play a crucial role in determining the type of effector T cell stimulated and also the regulation of the Th1 and Th2 subset responses (McGuirk and Mills, 2002; Jankovic et al., 2001; Constant and Bottomly, 1997; Abbas et al., 1996). More recently, Th17 cells have been described as differentiating from Th1 or Th2 cells as the third types of effector T cells. These cells may be effective protection against certain extracellular pathogens, but also play a role in the amplification of autoimmune disorders (Leceta et al., 2007). Cytokines also play an important role in determining and regulating this type of effector T cells (Bettelli et al., 2007; Steinman, 2007; Romagnani, 2006; Harrington et al., 2005).

1.1.2.1 Production of effector T cells

In order to be activated, a naive T cell must recognize and interact with foreign peptides bound in the MHC class I and class II pocket of the APCs (dendritic cells, macrophages and B cells) and bind with high affinity to each through cell to cell interaction mediated by cell-adhesion molecules particularly LFA-1, CD2 and ICAM-3 on the T cell and ICAM-1, ICAM-2, IFA-3 and DC-SIGN (c-type pectin which binds to ICAM-3 with high affinity) (Gao et al., 2002; Konig, 2002; Tybulewicz, 2002; Butcher et al., 1999; Petty and Todd, 1996), and the simultaneous delivery of a co-stimulatory signal via the B7 molecules, expression of which is enhanced by

interaction of CD40 ligand (T cell)-CD 40L (APC) and CD28 (CTLA-4)-B7 interactions (Coico et al., 2003; Janeway et al., 2001).

1.1.2.2 Activation of CD4 T cells and their functions--biological perspectives

As described above, two signals are required for activation of naive CD4 T cells in addition to cell-cell interactions via the adhesion molecules. One signal is the recognition of antigen bound to MHC class II on the APC (including B cells) by the TCR on the CD4 T cell surface whereas the other is the ligation for B7 co-stimulatory molecules on the APC to the molecules on the CD4 T cell (Dubey et al., 1995). Once CD4 T cells have been activated, they then progress to Th differentiation. The differentiation process is influenced by a number of factors including the type of APC, the concentration of antigen, the ligation of select co-stimulatory molecules and the local cytokine environment (Szabo et al., 2003). The APC can be dendritic cells, tissue macrophages, and/or B cells. Dendritic cells (found in almost all tissues) have potent antigen-processing capabilities and express abundant MHC class II molecules. Recent studies have suggested that dendritic cells may be the major or exclusive APC in the primary responses of CD4 T cells by acquiring the antigen at the site where it enters the body (Jenkins et al., 2001; Moser and Murphy, 2000; Pulendran et al., 1999; Rissoan et al., 1999). On the other hand, B cells act as the major APC in the responses of primed (memory) CD4 T cells; however, dendritic cells have been shown to present only a limited number of epitopes, whereas B cells can present a broader range of peptides (Gapin et al., 1998).

Another important factor that is considered to influence Th polarization is the nature and abundance of an antigen (Constant and Bottomly, 1997; Tao et al., 1997), although the evidence presented is conflicting. Several studies have demonstrated that small quantities or low affinity of antigen favors Th differentiation to Th2, while high affinity and abundance of antigen favors Th1 response (Chaturvedi et al., 1996; Guery et al., 1996; Hayglass et al., 1986; Pfeiffer et al., 1995). In contrast, other studies demonstrated the opposite dichotomy in response, in which high doses of antigen lead to Th2 response and low doses lead to Th1 response (Balamuth et al., 2001; Sarzotti et al., 1996; Bancroft et al., 1994). A key difference among these studies may be due to the type of antigen used. The studies in which low doses of antigen favored Th1 response used parasites as antigens, whereas those that showed low doses of antigen inducing Th2 used soluble proteins. This could indicate that the antigen itself influences Th differentiation.

The most important determinant of Th differentiation is considered to be cytokines. The cytokine IL-12 was initially characterized as a dominant cytokine promoting the induction of Th1 response; however, newly emerged evidence suggests that other

cytokines, such as IFN-γ and TNF-β play a significant role in Th1 development (Szabo et al., 2003; Jankovic et al., 2001; Abbas et al., 1996). IFN-γ has now been demonstrated to be the most important in this respect. IFN-γ is not only secreted by NK, CD8 and CD4 Th1 cells, but also by a number of other cells including macrophages, dendritic cells, naive CD4 cells and B cells (Grogan et al., 2001; Fukao et al., 2000; Ohteki et al., 1999; Yoshimoto et al., 1998). IFN-γ plays an important role in both the innate and acquired immune system. It has an essential role in the elimination of intracellular pathogens, via activation of macrophages, resulting in an increased phagocytosis, MHC class I and II expression, and the induction of IL-12, nitric oxide, and superoxide production (Boehm et al., 1997). Studies on *in vivo* Th differentiation have shown that the absence of IFN-γ (either IFN-γ deficient or by administrating anti-IFN-γ antibodies) inhibit Th1 development and promote the Th2 response (Reiner and Locksley, 1995; Wang et al., 1994; Scott, 1991). Other studies have shown that IFN-γ alone, independently of IL-12, was capable of inducing Th1 development (Bradley et al., 1996; Lu et al., 1998; Smeltz et al., 2002). Additional studies have shown that IL-12 may not be demanded to initiate Th1 response (Brombacher et al., 1999; Kaplan et al., 1998; Mullen et al., 2001). IL-12 may be more critical in the late stages of infections to induce optimal IFN-γ secretion by T cells (Park et al., 2000; Yap et al., 2000). IL-12 is secreted by activated APC such as dendritic cells, monocytes, and macrophages (Kato et al., 1996; Koch et al., 1996; Ma et al., 1996). IL-12 and IFN-γ together form a Th1-promoting loop: IL-12 induces production of IFN-γ by T cells, which in turn stimulates the production of IL-12. As for Th2 development, IL-4 was demonstrated to have the greatest influence in driving Th differentiation to Th2 (Le Gros et al., 1990; Swain et al., 1990). Basophils (Seder et al., 1991), Th2 cells or mast cells (Singh and Agrewala, 2006; Plaut et al., 1989) are the main sources of IL-4. Another cytokine IL-10 has also been reported to promote Th2 development; however, its major role may be more to do with suppressing Th1 development by suppressing IL-12 activity and thus IFN-γ production (Hsieh et al., 1993). Similarly, IL-12-induced IFN-γ also suppresses IL-4, thus promoting Th1 response by down regulating Th2 cytokines (Grogan et al., 2001). TGF-β1, IL-6, IL-1β, TNF-α and IL-23 were recently demonstrated to promote Th17 response (Liang et al., 2006; Harrington et al., 2005; Hofstetter et al., 2005; Park et al., 2005), the latter being considered to be responsible fordestructive tissue damage in autoimmune disorders and delayed-type hypersensitivity reactions . Th1 and Th2 cytokines, such as IFN-γ and IL-4 were reported to potently suppress naive precursor cells to Th17 cell development, whereas committed Th17 cells were resistant to suppression by Th1 or Th2 cytokines (Hofstetter et al., 2005).

Based on their patterns of cytokine production and their functional responses, the primed CD4 T cells are traditionally divided into two major subsets, Th1 and Th2 cells (London et al., 1998; Carter and Dutton, 1996; Mosmann and Sad, 1996). Th1 cells secrete IL-2, IFN-γ and tumor necrosis factor beta (TNF-β), which are mainly involved in the induction of cell-mediated immunity (CMI) and activation of CD8 T

cells, NK cells and macrophages. Th2 cells produce IL-4, IL-5, IL-10 and IL-13, which are essential for the induction of humoral immunity such as in the activation of B cells to produce antibodies and their class switching (Szabo et al., 2003). Prior to Th polarization towards a Th1 or Th2 phenotype, a subset called Th0 cells has been identified and is thought to be the precursor of Th1 and Th2 cells. Th0 cells produce IL-2, IFN-γ and IL-4 (Paliard et al., 1988; Firestein et al., 1989), but do not express receptors for IL-12 until activated (Igarashi et al., 1998). Finally after the course of response to antigen, the majority of the activated T cells die off by apoptotic mechanisms, and only a small portion of T cells become the long-lived antigen-specific CD4 T memory cells (Jenkins et al., 2001). Th17 cells have recently been identified as a third independent T cell subset that is distinct from Th1 and Th2. Th17 cells produce IL-17 and IL-22, which are CD4 T cell-derived cytokines and associated with autoimmune disorders and T cell-mediated tissue damage (Steinman, 2007; Liang et al., 2006; Harrington et al., 2005; Park etal., 2005). It is worth noting that though our understanding of Th cell biology over the past several years has increased substantially, the molecular mechanisms regulating the initiation of a Th1 or Th2 or Th17 response and their down-regulatory pathways are still not completely understood.

1.1.2.3 Activation of CD8 cells and their functions--biological perspectives

CD8 T cells, a major component of cell-mediated immunity, are normally referred to as CTL. CTL cells kill cells that have been infected by pathogens such as bacteria, viruses and protozoa, as well as tumor cells and transplanted foreign cells during graft rejection (Coico et al., 2003). Upon binding to antigen-MHC class I complex, and in the presence of cytokines by Th1 cells, CD8 cells are activated to proliferate and differentiate into cytotoxic T cells. As almost every cell in the body, except the immunologically privileged sites such as gametes, neurons, lens of the eye and trophoblast cells (Delves and Roitt, 2000a and 2000b), expresses MHC class I, CD8 cells are capable of killing off almost any cell in the body that is infected with a pathogen. MHC class I molecules are generally only associated with peptides that are generated in the cytosol by proteasome-mediated protein degradation. However, evidence has shown that CD8 cells can be induced by MHC class I molecules presenting exogenous antigens either in soluble (Staerz et al., 1987; Wraith et al., 1987) or particulate forms (Falo et al., 1995; Harding and Song, 1994), or by tumor cell-derived heat shock proteins (Udono and Srivastava, 1993). How these antigens, which are taken up by phagocytosis or macropinocytosis gain access to the cytosol is not yet known. This unusual pathway is found to be particularly prominent in macrophages and dendritic cells (Bevan, 1987). CD8 cells also attack cells harboring vacuolar pathogens such as *Mycobacteria* and *Salmonella* species (Kerksiek and Pamer, 1999). CD8 cells also secrete cytokines such as tumour necrosis factor (TNF)

and IFN-γ, which play important roles in target cell killing (Sad et al., 1995). In addition, pathogen-specific CD8 cells produce chemokines that act in concert with inflammatory mediators to attract inflammatory cells such as neutrophils, monocytes, and lymphocytes to sites of infection (Fahy et al., 2001).

1.1.2.4 Activation of B cells and antibody responses--biological perspectives

In the presence of cytokines produced by Th2 cells, specific recognition of foreign antigen by cell surface Ig induces B cells to proliferate and differentiate into antibody-producing plasma cells, as well as undergo antibody class switching (Born et al., 1999). The pattern of cytokines produced by activated T cells directs the antibody class switching. For example, if the T cell produces IL-4, then B cells will switch to making IgE and IgG1, whereas IFN-γ directs B cells to switch to making IgG2a (Constant and Bottomly, 1997). However, some antigens such as bacterial lipopolysaccharides and polysaccharides that have numerous repeating epitopes are capable of activating B cells to produce an antibody without T cell help (known as thymus-independent antigen), however, these B cells only make IgM and do not undergo class switching (Mond et al., 1995).

An antibody response usually occurs in two phases: the primary and secondary responses. When exposed to an immunogen for the first time (such as primary immunization), there is a lag of usually 10-12 days before the first antibody IgM becomes detectable in the serum (Figure 1-1). This lag phase in the primary response is necessary for T and B cells to interact with the antigen, to proliferate, and to differentiate. Then some of the B cells enter exponential production phase by differentiating into plasma cells and secrete IgM, while other B cells continue to proliferate and then undergo class switch to IgG. Some of these B cells then synthesize IgG exponentially while others become memory cells. After a few weeks, the antibody level declines. In some instances, IgM may be the only antibody made in the primary response (Benjamini et al., 2000). If at a later date the same antigen is encountered, there is a far more rapid immune response (secondary response) and IgG production is much greater and may remain detectable for months or years. On the other hand, IgM is produced at the similar level as it was in the primary response (because there are no memory cells for IgM) and IgM may disappear altogether after a short time. IgA and IgE may also appear in the secondary response (Benjamini et al., 2000).

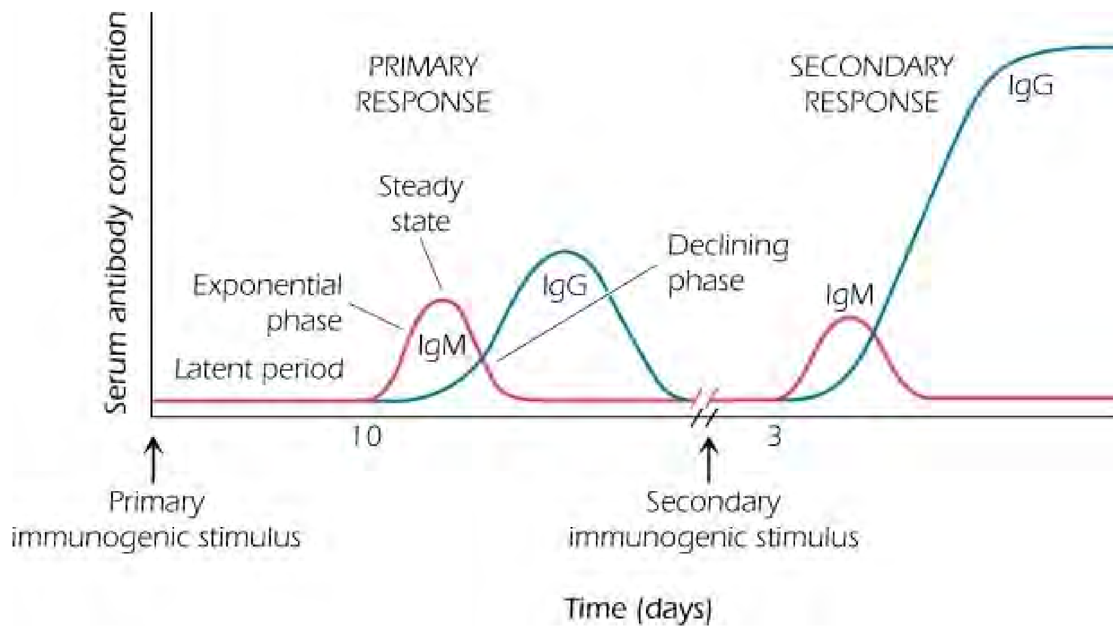


Figure 1-1 Kinetics of serum antibody responses (This Figure is adapted from Figure 4.11 of Benjamini et al., 2000)

The primary response is slow onset, low magnitude, short-lived and production of IgM and/or IgG. The secondary response is rapid onset, high magnitude, long-lived and greater production of IgG that may not be followed by production of other isotypes depending on a number of factors including the route of immunization (Coico et al., 2003)

1.1.3 Mucosal immunity

Skin and mucosa provide physical and chemical barriers to prevent entry of pathogens. Pathogens adjust to infect mucosae and express virulence factors. These factors allow them to adhere, colonize or invade the epithelium. Therefore, mucosal immunity is critical in prevention of mucosally acquired infectious diseases (Neutra et al., 1996b; Neutra, 1998). The major mucosal portals of entry for pathogens include the gastrointestinal tract, respiratory tract, and urogenital tract. Because of extensive exposure to the outside world, the majority of the body's lymphoid tissue is distributed along mucous membranes forming the MALT (Mucosa-associated lymphoid tissue) system (Neutra et al., 1996b). The MALT is found in the submucosal areas of the gastrointestinal, respiratory and urogenital tracts, in the lamina propria of the intestinal wall, and aggregates of densely packed lymphoid cells organized into Peyer's patches in the lower ileum (Hobson at al., 2003; Neutra et al., 1996b). Major mucosal inductive sites, where mucosal immune responses are

initiated, are found in both the intestinal and upper respiratory tract including Peyer's patches, nasal-associated lymphoid tissue in the oropharyngeal cavity, the tonsils, and adenoids (Frederik et al., 2000; Neutra et al., 1996b). Common features of these inductive sites are specialized epithelial cells termed M (Membraneous) cells, macrophages, dendritic cells, CD4 and CD8 cells and secretory IgA (S-IgA) producing B cells (Neutra et al., 1996a). M cells are thought to uptake, transport, process and possibly present antigens to subepithelial lymphocytes initiating immune responses (Allan et al., 1993; Neutra et al., 1996b; Neutra and Kraehenbuhl, 1993). Under the influence of local cytokines and accessory cell populations, B cells are activated and switched to IgA isotype (Weinstein et al., 1991; Defrance et al., 1992; Fujihashi et al., 1997). The activated lymphocytes then migrate via the regional lymph nodes and thoracic duct into the blood stream. These cells then selectively enter the spleen for clonal expansion before they enter via the blood into the mucosal effector sites such as lamina propria and probably other mucosal sites in the body (Russell et al., 1996). Hence both peripheral and mucosal immunity can be induced far from the site of original antigen stimulation. In addition, this broad immunity is both humoral and cellular. S-IgA secreted into the lumen is the principle antibody in mucosal immunity (Mazanec et al., 1993; Mestecky and Jackson, 1994). The major role of S-IgA is to prevent absorption of pathogens such as viruses, bacteria and toxins. This is achieved by blocking the adhesion of the pathogens while they are still on the external side of the epithelial barrier. Thus the pathogen can be washed out in the flow of secreted fluids and mucous which washes over the epithelial membranes. IgA has also been shown to be important in the intraepithelial neutralization of viruses. As IgA traverses the epithelial cell, it may encounter pathogen(s) intracellularly, where it has the potential to neutralize the pathogen (Mazanec et al., 1992; Neutra et al., 1996b).

Cellular immunity may also play an important role in combating mucosally infectious pathogens. These mucosally committed T cells may function either to prevent mucosal surface from injury by infectious pathogens or by exhibiting cellular cytotoxicity directed against intracellular pathogens (Musey et al., 1997; Wong and Pamer, 2003).

1.2 Potential role of Immunomodulators

The development of the immune system is to eliminate foreign (non-self) invaders to prevent the establishment of infectious disease. However in certain infectious or non-infectious insults, immune responses to self-antigens leads to the manifestation of autoimmune conditions (Coico et al., 2003). One reason for the development of type 1 diabetes, for example, is the formation of auto-antibodies against pancreatic beta-cells. Another example is rheumatic fever, a heart disease, which may occur following infection with *Streptococcus pyogenes* due to the formation of antibodies to bacterial antigens which share antigenic determinants with the heart muscle (Veasy and Hill,

1997). There also are autoimmune conditions in which cell-mediating effector arm of the immune response is involved in the pathological process, for example, rheumatoid arthritis in which the chronically inflamed synovium becomes densely crowded with lymphocytes resulting in the destruction of cartilage and bones (Joosten et al., 1999). Then there are secondary immunodeficiency disorders such as AIDS and conditions requiring chemo- or radiation therapy in which cells of the immune system suffer great damage and destruction (Plettenberg et al., 1997). Resolution of such conditions would be greatly assisted if immunomodulating compounds capable of either suppressing or stimulating the induction of cellular or humoral immune responses respectively were available.

Cellular and humoral immune responses are regulated by cytokines promoting the induction of distinct Th1 or Th2 subtypes of T cells (described in Sections 1.1.2.2 and 1.1.2.4). The discovery of immunomodulators with the potential of promoting the selective induction of these distinct populations of T cells could greatly improve immune responses thus adding value to immunotherapy (Dumont, 2002).

A diterpene lactone, andrographolide (AND), isolated from *Andrographis paniculata* (AP) has been reported to be immunostimulatory or immunosuppressive particularly in promotion or down-regulation of innate non-specific immunity as assessed by cytokine production and anti-carcinogenic activity (Burgos et al., 2005; Kumar et al., 2004; Rajagopal et al., 2003; Panossian et al., 2002; Shen et al., 2002; Madav et al., 1996). It has also been reported to either stimulate (Puri et al., 1993) or suppress adaptive immunity depending on the administration of AND (Iruretagoyena et al, 2006; 2005). However, essentially no information on the modulation of the cell-mediated effector arm in adaptive immunity against infectious disease agents by this medicinal plant is available, the theme underpinning the research embodied in this thesis.

Although AP is widely used in infections and infestations in traditional Chinese medicine, it has been suggested that its main clinical benefit could be due to a possible immune-enhancing effect (Mills and Bone, 2000). The immunomodulatory property of AP and the diterpene lactone AND will be reviewed in Section 1.3.5.6.

Adjuvants are substances that can be used in combination with specific antigens to produce immunity better than the antigens used alone (Schmidt et al., 2007). There are a number of adjuvants which have been proven to stimulate the production of antibodies and many are used in vaccines currently in the marketplace (Edelman, 2002; Hunter, 2002) and yet aluminum-based adjuvants such as aluminum hydroxide or phosphate are the only adjuvants licensed for use in humans (Schmidt et al., 2007) e.g., in acellular pertussis vaccine (Guy, 2007; HogenEsch, 2002). However, there are no universally accepted immunostimulants, which promote the induction of cell-mediated immunity. Whilst a wide variety of immunosuppressants are being used in certain conditions such as in cancer patients undergoing chemo- or radiotherapy, most

of them suppress both Th1 and Th2 responses rendering the patient susceptible to infections yielding with undesirable outcomes (Garay et al., 2007; Caras et al., 2004; Roila et al., 1998).

1.3 Andrographis paniculata

1.3.1 General description of *Andrographis paniculata*

Andrographis paniculata (Burm.f.) Nees, a member of the family of *Acanthaceae* and commonly known as Kalmegh, is an herb used in traditional medicine in China, India and Southeast Asia. It is known as king of bitters in English and Chuan Xin Lian in Chinese, but is more commonly referred to simply as andrographis. AP is an up to 1 meter tall annual shrub with lanceolate leaves and small white to pink, hairy flowers. It grows in moist deciduous forests and is also cultivated. The leaf is the main medicinal part, but the whole plant including the root has been used (WHO, 2002).

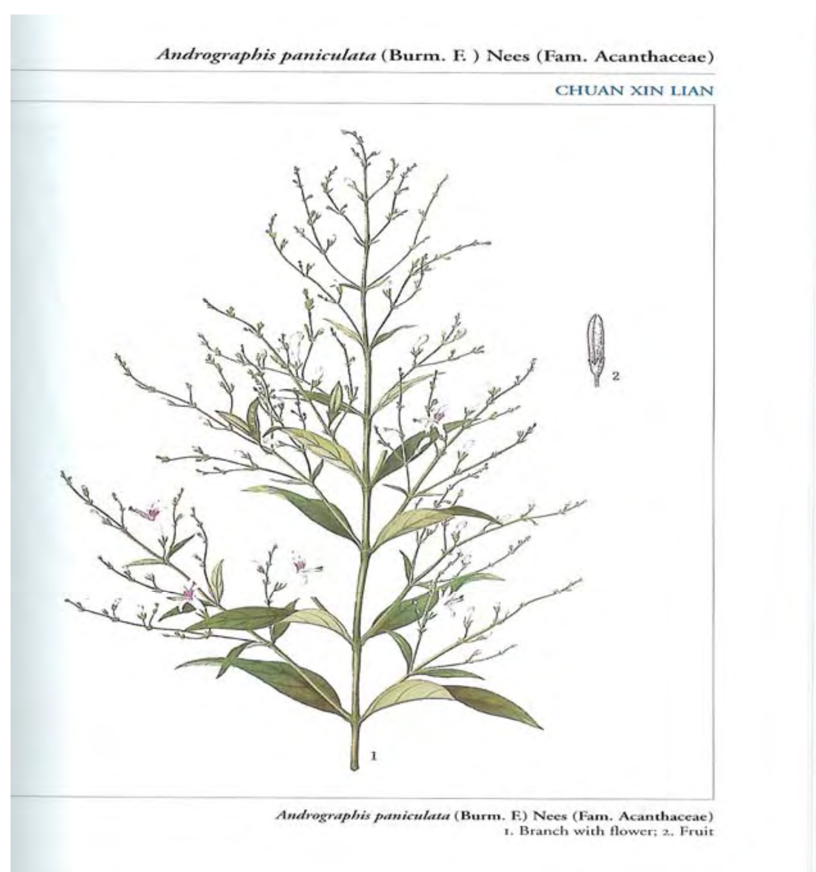


Figure 1-2 *Andrographis paniculata* (Burm.f.) Nees plant

(This Figure is adopted from Wu, 2005)

1.3.2 Chemical composition of AP

Boorsma first isolated AND from AP in 1896 and Corter identified AND as a lactone in 1911. It is a colourless or light yellow crystal compound with a very bitter taste (Tang and Eisenbrand, 1992; SCHRI, 1973).

Deng et al (1982) reported there are four lactones in *Andrographis paniculata*. They are (1) 14-deoxyandrographolide, which was also identified by Sangalungkarn et al (1990) and Garcia et al (1980), (2) AND, (3) neoandrographolide (a non-bitter, C_3 O-glucoside derivative of the major constituent andrographolide) and (4) 14-deoxy-11,12-didehydroandrographolide which were also identified by Dhamma-upakorn et al (1992). AND and total lactone are the common names used in clinics for the active ingredients (Deng et al., 1982). The other medicinal chemical principles are diterpenoids viz. 14-deoxyandrographolide, -19β-D-glucoside which has been isolated from the leaves (Techadamrongsin et al., 1999; Chem and Liang, 1982). Du et al (2003) separated AND and neoandrographolide from the leaves of AP using high-speed counter-current chromatography.

The content of the major component, AND depends on both growing region and the collection time. AP grows best in the tropical and subtropical areas of China and Southeast Asia. The leaves contain more than 2% andrographolide before the plant blooms and less than 0.5% afterwards. The stem contains 0.1-0.4% of andrographolide (Zhu et al., 1984). The best harvesting time is early autumn (Sharma et al., 1992). AP is sold commercially as a medicine in China, India, Thailand and Malaysia. Thin-layer chromatography, ultraviolet spectrophotometry, liquid chromatography and volumetric and colorimetric techniques are used in a variety of laboratory methodologies to ensure a standardized level of andrographolides (PPRC, 1997; Sharma et al., 1992).

The other active chemical constituents of AP which have been identified so far include diterpene dimers (Matsuda et al., 1994) and flavonoids (Zhu and Liu., 1984; Koteswara et al., 2004).

1.3.3 General characterization of Andrographolide

Andrographolide's chemical name is 3α, 14, 15, 18-tetrahydroxy-5β, 9βH, 10α-labda-8(20), 12-dien-16-oic acid γ-lactone. Its molecular formula and weight are $C_{20}H_{30}O_5$ and 350.4 (C 68.54%, H 8.63%, O 22.83%) respectively. A colourless plate from ethanol or methanol with a melting range from 205-235.3°C has been reported (Fujita et al., 1984; Rajani et al., 2000; Du et al., 2003). It is also reported that its UV absorption maxima in methanol or ethanol is 222-224 nm (Fujita et al.,

1984; Rajani et al., 2000; Du et al., 2003). Its structure was analysed by X-ray, ^{1}H, ^{13}C-NMR and ESI-MS (Fujita et al., 1984; Medforth et al., 1990; Rajani et al., 2000; Du et al., 2003; Cui et al., 2005). It does not mix well with water, but is soluble in acetone, chloroform, ether and hot ethanol. Crystalline AND was reported to be highly stable even at 70°C (75% relative humidity) over a period of 3 months (Lomlim et al., 2003).

Rajani et al. (2000) reported a simple and rapid method for isolation of AND from the leaf of AP. They extracted it from the leaf powder using 1:1 mixture of dichloromethane and methanol in cold condition and then isolated andrographolide directly from the extract by recrystallisation. They confirmed the compound through IR, UV, MS, melting point and co-chromatography with a reference standard on TLC. The purity of the compound was also checked by TLC, UV absorption spectrum, HPLC, LCMS and differential scanning calorimetry (DSC), the DSC gave the melting point of andrographolide as 235.3°C.

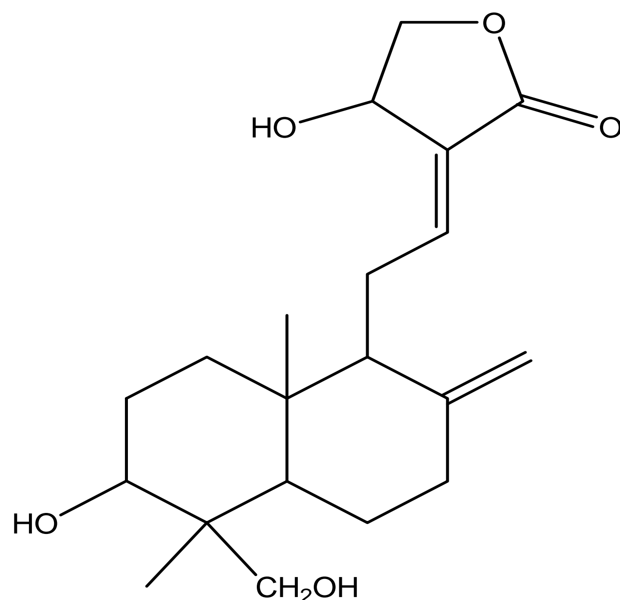


Figure 1-3 Structure of andrographolide (Tang and Eisenbrand, 1992)

1.3.4 Traditional uses of AP

AP, as a traditional medicinal plant, has been used for centuries to successfully treat respiratory, skin infections, fever, sore throat, herpes, dysentery, lower urinary tract infections and to reduce inflammation and stop diarrhoea (Techadamrongsin et al., 1999). Clinically, the use of this herb is reported in contemporary and ancient Chinese writings (WHO, 1978). In Traditional Chinese Medicine, AP is a bitter and ‘cold property’ herb. It is used in the treatment of ‘hot’ conditions such as acute infections

and fever, including throat infection, pneumonia, tonsillitis, dysentery, gastroenteritis and pyelonephritis (Mills and Bone, 2000; Bensky and Gamble., 1993; Deng et al., 1982). It is also prescribed for snakebite (Chang and But, 1986; Duke and Ayensu, 1985). It is used in Malaysian folk medicine for diabetes and hypertension (Ahmad and Asmawi, 1993; Zhang and Tan, 1996; Zhang and Tan, 2000). Yeung et al. (1987) reported that AP had pharmacological properties which include antibacterial, immunological, antivenomous and antithrombotic properties. More recently, AP has been used in the treatment of chronic bronchitis, administered via aquapuncture, i.e. the injection of an infusion into acupuncture points (Duke and Ayensu, 1985). In Ayurvedic medicine, it is used as a bitter tonic and stomachic, for diabetes, debility, hepatitis and as an anthelmintic (Mills and Bone, 2000).

AP extract has been used in different forms, such as tablet or injection. In China, in tablets form it has different names: “Kan Jang” tablets, “Chuanxinlian” tablets, “Xiaoyan Lidan” tablets and “Chuanxinlian antiphologistic” Pills (Zhao et al., 2002). The injection forms are “Yamdepieng” and “Chuanxinlian Ruangas” injection (Maunwongyathi, 1994). In Indian pharmacopoeia, at least 26 Ayurvedic formulas are widely used (Kumar et al., 2004).

The knowledge of the benefits of AP for human health has also led to its use in livestock production in Thailand. AP or mixtures of AP and other plants have been used to treat poultry in broiler production instead of antibiotics on several farms. Farmers believe that AP or the plant mixture containing AP reduce mortality especially that caused by digestive tract and respiratory tract diseases (Chaiwongkeart, 1997), but little systematic research has been reported.

1.3.5 Physiological and pharmacological properties of AP and AND

Andrographis paniculata has been widely studied in the last 50 years. Previous studies have focused on *Andrographis paniculata’s* pharmacological composition, efficacy, safety and mechanisms of action (Panossian et al., 2002; WHO, 2002; Mills and Bone, 2000).

1.3.5.1 Anti-diarrhoeal and intestinal effects

Diarrhoea diseases are one of the top ten causes of death worldwide, especially for children under the age of five in developing countries (Lopez et al., 2006). An inflammation of the intestinal tract can cause acute diarrhoea. The inflammation can be caused by pathogenic bacteria overgrowth or a viral or parasitic infections and irritations. Medications and certain foods are the sources of pathogenic growth.

Campylobacter, *Salmonella*, *Shigella* and *Escherichia coli* are common bacteria that cause diarrhoea. Although antibiotics are effective in treating bacterial infections, antibiotic-resistant strains of bacteria can be produced by the over use of antibiotics. Many drugs, such as kaolin-pectin, loperamide, bismuth, etc, are used to relieve the symptoms of diarrhoea, but they may cause undesirable side effects (Sindermsuk, 1993). An inexpensive and easily obtainable herbal remedy would benefit many people, especially those in developing countries. Experiments in animals demonstrate that AP can prevent diarrhoea. Extracts of AP have effectually been shown against the diarrhoea connected with *E. coli* infections (Duke and Ayensu, 1985). The AP components, AND and neoandrographolide showed comparable activity to loperamide (Imodium), the most common anti-diarrhoea drug. Gupta et al (1990) reported that the active ingredients against diarrhoea are AND and deoxyandrographolide.

In Thailand, an extract made by boiling AP stem with methanol was reported to be effective against *Proteus vulgaris* and AP stem and leaves blended powder can be effective against the *Shigella* bacteria but is not effective against cholera (Thanangkul and Chaichantipyuth, 1985). Patients, who were orally administrated 1 g blended powder every 12 hours for 2 days, were more effective than those given a dose of 500 mg every 6 hours for 2 days (Thanangkul and Chaichantipyuth, 1985). In China, this regimen was combined with rehydration (Yin and Guo, 1993). There were 66 cures of 80 treated patients (82.5% cure rate). Seven patients (8.8%) responded favourably to the treatment. This was indicated by reduced nausea and vomiting. Only seven patients (8.8%) did not respond to the treatment. Laboratory tests of stool samples confirmed the effectiveness of the treatment (Yin and Guo, 1993). A comparison experiment was conducted in a pharmacological research institute in Shanghai, China in 1973. 165 patients were given AP tablets equal to the amount of 15.6 g crude powder per day. Twenty-eight patients were given Fluroxone, a common drug used to treat dysentery. The result showed the effective rate of AP was 75.2% and the effective rate of Fluroxone was 71.4% (SCHRI, 1973).

AP was believed to be effective against bacterial dysentery and diarrhoea because it has antibacterial activities. Pleumjai and Sithisomwonges (1990) found *in vitro* that AP extracts with 70% and 80% ethanol could kill bacteria that cause diarrhoea such as *E. coli* and *V. cholera*, but Sindermsuk (1993) could not confirm this effect. The antibacterial activity of aqueous, ethanol or methanol extracts of *Andrographis paniculata* has been tested *in vitro* against *Escherichia coli*, *Staphylococcus aureus*, *Proteus vulgaris, Salmonella typhi*, *Shigella* species and *Pseudomonas aeruginosa* (George & Pandalai, 1949; Nakanishi et al., 1965; Bunyapraphatsara, 2000; Prajjal et al., 2003). However, no antibacterial activity was observed with the aqueous extract tested against *E. coli*, *S. aureus*, *S. typhi* or *Shigella* species (Leelarasamee et al., 1990). So it is necessary to extend these investigations using a broader spectrum of microbial pathogens of relevance to human health.

In one study on mice it was found that 50% and 85% alcohol extracts of AP leaf powder were effective in reducing intestinal tract movements (Choudhury & Poddar, 1985). The researchers compared the effect of AND and AP extract on intestinal brush-border membrane-bound hydrolases. They suggested that both andrographolide and AP extract activated intestinal disaccharidases; the latter accelerated intestinal digestion and absorption of carbohydrate.

1.3.5.2 Effect on upper respiratory infections

One of the most common causes of childhood illness and adult discomfort is upper respiratory infection. Upper respiratory infection includes any infection in the nose, throat, sinuses, and ears. The common cold is the most common upper respiratory infection. Viruses and bacteria in the surrounding environment may cause upper respiratory infection (Ozkan and Dweik, 2004; Black, 2002). Crude aqueous or alcohol extractions of AP and one principle of AP, AND have been reported to be effective in the treatment of upper respiratory infections (Hancke et al., 1995; Caceres, 1997; Melchior et al., 1997; SCHRI, 1973; Thamlikitkul et al.,1991; Subjareun, 1996). Sixtyone adult patients were used to measure the effectiveness of AP extract compared to a placebo, to reduce the symptoms associated with the common cold. The effects of AP were measured on days 0, 2 and 4 of treatment. On day 2, the group taking AP extract displayed alleviation of several of the associated symptoms. On day 4, the group receiving 1200 mg extract daily showed a significant reduction ($p<0.0001$) in clinical symptoms such as sore throat, tiredness, muscular ache and malaise, as compared with the placebo group (Hancke et al., 1995).

One clinical trial has investigated the efficacy of a standardized AP extract to prevent the common cold by Caceres (1997). 107 healthy students in a rural school had daily taken either placebo or a dose of 200 mg (minimum 5.8% AND) of Kan Jang (a formulation of AP provided by the Swedish Herbal Institute) for three months. The number of colds occurring over a three month period was observed. After 1 month no significant difference was found. However, the difference was statistically significant in the second and third month. The placebo group was 2.1 times more likely to catch a cold than the Kan Jang group. The incidence of the common cold was 30% in the AP group, whereas the incidence was 62% in the placebo group.

In a study conducted by Melchior et al (1997), fifty adult patients received either 1020 mg extract or a placebo daily for 5 days to evaluate the efficacy of AP extract on the initial symptoms of the common cold and uncomplicated sinusitis. The results demonstrated that the AP group took fewer sick leave days than the placebo group. Furthermore, 68% of treated patients felt totally recovered, but only 36% of the placebo group recovered.

In China, clinical studies of bacterial and viral respiratory infections demonstrated favourable effects after patients were orally taken AP or andrographolides. Investigations from the Sichuan Traditional Medicine Research Institution found that AP reduced body temperature in the treatment of infectious diseases associated with cold symptoms: 70 out of 84 treated patients achieved normal body temperature within 48 hours (Melchior et al., 1997). In 1972, researchers from the Chinese Herb Research Institute used deoxyandrographolide and neoandrographolide to treat 24 cases of upper respiratory tract infection, acute tonsillitis and bronchitis. The average recovery time was about 4 to 7 days compare to the placebo which was 14 days (SCHRI, 1973). The antibiotic rifampin is usually used to treat tuberculosis. 10 % of patients still die when rifampin is used alone (Aquinas and Sanatorium (1977). In a study, an injectable solution of 2.5% andrographolide (50-80 mg/kg body weight per day) was used for two months. The therapeutic results were improved in seventy cases of tubercular meningitis. 30% of patients were cured with a fatality rate of 8.6%. The combination of AND plus rifampin resulted in a 2.6 fold decrease in fatality rates (DIDPHSP, 1977).

AP was used to treat cough and sore throat by Thamlikitkul et al (1991). They compared AP leaf powder with Paracetamol and found that 6 g AP powder per day after 3 days reduced fever and sore throat better than Paracetamol. However, after 7 days there was no difference between them. In 1996, AP was compared with penicillin in its capacity to heal sore throat. The results showed that there was no significant difference in recovery rate between the two groups of patients. More than 75% of patients were cured with an additional 15% showing improvement (Subjareun, 1996).

More recently, AP extracts in fixed combination with Kan-jang, *A. senticosus*, *Eleutherococcus senticosus* Maxim, *Schizandra chinensis* Bail and *Glycyrrhiza glabra* L. extracts were also found to show significant improvement in the verum group as compared with the placebo in the treatment of uncomplicated upper-respiratory tract infection (Melchior et al., 2000; Poolsup et al., 2004; Coon and Ernst, 2004; Amaryan et al., 2003; Gabrielian et al., 2002; Spasov et al., 2004).

1.3.5.3 Effect on inflammation and fever

AP is used as a folk medicine for reducing inflammation. Three ingredients: deoxyandrographolide, AND and neoandrographolide are effective in reducing inflammation (Dutta & Sukul., 1982). In other studies it was found that AP extracted with alcohol (Sawasdimongkol et al., 1990; Chantasutra & Limpapanichkul, 1989), AP extracted with water (Sawasdimongkol et al., 1990) and AP extracted with chloroform (Chantasutra & Limpapanichkul, 1989) reduced inflammation. Madav et al (1996) found that AND significantly inhibited carrageenin-, kaolin-and nystatin-

induced paw oedema (different models of inflammation) in rats after oral administration at doses of 30, 100 and 300 mg/kg bodyweight. In China, it has been reported that AND has some beneficial effects as an anti-inflammatory agent (Chiou et al., 2000; Shen et al., 2000), whereas Deng et al (1982), who studied rats and mice concluded that four lactones from AP have anti-inflammatory and anti-pyretic effects. Deng et al (1982) also suggested AP might exert its anti-inflammatory effect through stimulation of the adrenal gland since the herb showed no effect when the adrenal gland of the animals were totally removed. Habtemariam (1998) suggested that AND inhibited tumour necrosis factor-α to induce endothelial monocyte adhesion, which is part of the inflammatory process. Other possible mechanisms involved in AND anti-inflammatory effects were also reported (Amroyan et al., 1999; Shen et al., 2002; Xia et al., 2004; Hidalgo et al., 2005). Shen et al. (2002) reported that the possible mechanism involved in its anti-inflammatory effect might be due to AND preventing oxygen radical production by human neutrophils. Xia et al. (2004) and Hidalgo et al. (2005) suggested its anti-inflammatory effect involved AND inhibiting a nuclear factor kappa B (NF-kappaB) binding to DNA in endothelial cells or HL-60-derived neutrophilic cells, and thus reducing the expression of proinflammatory proteins. In a study from Thailand, rats were given injections with carrageenan (an agent for stimulating inflammation) to study the anti-inflammatory effect of AP water extract ranging from 500, 1250, 2500 mg / body weight. The result showed that water extract of AP effectively reduced the paw volume in rats treated with AP whereas the control group did not (DMPRD, 1990).

The ability of AP to lower fever has been demonstrated in several laboratories (Huang, 1993; Madav et al., 1995; DMPRD, 1990). Rat studies have shown that fever produced by different fever-inducing agents, such as *Pneumococcus spp*., hemolytic *Streptococcus*, *P. multocida* and the chemical 2, 4-dinitrophenol can be lowered by AND, neoandrographolide and dehydroandrographolide in China (Huang, 1993). Indian researchers (Madav et al., 1995) tested AP to determine whether it could reduce fever in rats. They found that there was a reduction in rectal body temperature for 30, 100 and 300 mg of AND /kg bodyweight. The antipyretic activity of 300 mg of AND / kg bodyweight was comparable to that of 300 mg aspirin. In Thailand, studies on rabbits showed that the antipyretic effect of 2.5 g 85% ethanol extract of AP / kg bodyweight was as effective as 300 mg/kg of aspirin (DMPRD, 1990).

1.3.5.4 Cardiovascular activity

Amroyan et al (1999) found that AND from AP did not affect the biosythesis of eicosanoids, but inhibited the platelet-activating factor (PAF)-induced human blood platelet aggregation (eicosanoids and PAF are two of the most important inflammatory mediators. Inhibition of the biosythesis of eicosanoids is characteristic for non-steroidal anti-inflammatory drugs, while PAF antagonists are used as

potential agents in inflammation). They indicated that the mechanism of action may differ from that of non-steroidal anti-inflammatory drugs. This mechanism is most likely combined with the cardiovascular and antithrombotic activity of AP. Zhang and Tan (1996) found a hypotensive activity of aqueous extract of AP in rats and they suggested that the aqueous extract of AP lowers the systolic blood pressure of spontaneously hypertensive rats possibly by reducing circulating angiotensin-converting enzyme in the plasma as well as by reducing free radical levels in the kidneys. A hypotensive activity of AND in rats was also reported by Yu et al (2003). In further studies by Zhang & Tan (1997, 1998) on cardiovascular activity of 14-deoxyandrographolide (DA) and 14-deoxy-11, 12-didehydroandrographolide (DDA) in rats, they reported that DA and DDA significantly decreased the mean arterial pressure and heart rate of anaesthetized rats. Both DA and DDA activated nitric oxide production in endothelial cells; the latter caused relaxation of the isolated rat thoracic aortae. Chiou et al. (1998) reported that andrographolide inhibits nitrite synthesis by suppressing expression of inducible nitric oxide synthase (iNOS) protein *in vitro*, and the inhibition of iNOS synthesis may be good at haemodynamic effects of AND in endotoxic shock. Zhao and Fang (1991) demonstrated that AP decreased the damage that occurred to the heart muscle when given to dogs that had one hour myocardial infection. Such damage occurs after the blood supply is restored to the muscle. A sudden influx of oxygen (which produces free radicals that damage tissue) and abnormally high amounts of calcium cause the damage. In the same article, abnormal changes in heart were prevented by pretreatment with AP through electrocardiography. Clumping of platelets was also inhibited and clotting that could cause infarction was not induced (Zhao and Fang, 1991). The effect of AP was that it activated fibrionolysis, which dissolves clots in the body (Huang, 1993). Hsu et al. (2004) reported that AND increased the radioactive glucose uptake in cultured myoblast C2C12 cells and the uptake may reduce glucose in blood flow.

1.3.5.5 Hepatoprotective and choleretic activity

AP has also shown to protect liver. In Ayurvedic medicine, there are 26 different formulations containing AP used to treat liver disorders. AP is hepatoprotective in mice treated with carbon tetrachloride or tert-butylhydroperoxide (Kapil et al., 1993). These chemicals are highly toxic compounds which damage the liver by causing lipid peroxidation (Choudhury and Poddar, 1984). They produced free radicals and the later destroyed cellular membranes around liver cells. Significant liver protection occurred when the AP compounds were given to animals three days earlier than when the toxic chemicals were given. This protection was assigned to the antioxidant ability of the AP compounds and the hepatoprotective effect could compare with the known hepatoprotective agent silymarin by biochemical parameter (Rana and Avadhoot, 1991). Handa and Sharma (1990a) reported that AND was the major active antihepatotoxic principle of AP against carbontetrachloride. AP was also reported to

be better than silymarin in protecting the liver against paracetamol toxicity (Visen et al., 1993) and against paracetamol and galactosamine (Handa and Sharma, 1990b). Hepatoprotective effect of AND was studied in rats. An acute hepatitis was induced by a single dose of galactosamine (800 mg/kg, ip) and paracetamol (3 g/kg, po) in rats. The livers of experimental rats were used to monitor hepatoprotective activity by determining the serum transaminases (GOT and GPT), alkaline phosphatase and bilirubin in serum, hepatic triglycerides and histopathological changes (Choudhury et al., 1987). The *in vivo* hepatoprotective effect of AND against galactosamine or paracetamol-induced hepatotoxicity in rats was confirmed by the results (Visen et al., 1993; Handa & Sharma, 1990b).

Infective hepatitis is an acute liver inflammation (Zender et al., 2003). In India, twenty patients of infective hepatitis (hepatitis A) were treated with 200 mg of AP extract twice times per day for over twenty-four days. The conjunctiva of the eyes and the urine changed colour from yellow to normal. It was reported that 80% of the patients were cured and 20% improved based on symptoms changes and biochemical tests. 90% of patients had regained appetites and 83% were relieved from general depression (Chturvedi et al., 1983). In another similar mode study in China, 83% of 112 cases of hepatitis were recovered (Deng, 1978).

Andrographolide showed choleretic activity in rat and guinea pig increasing bile volume as well as the amount of bile salts and bile acids (Shukla et al., 1992). The bile flow of the animals pretreated with AND was increased when paracetamol, which usually decreases bile production, was given to the animals. In this case, AND was more powerful as compared to silymarin. Andrographolide also increases the amount of bile that acetaminophen toxicity decreases (Holt & Comac, 1998).

1.3.5.6 Immunomodulatory activity

Intra-gastric administration of ethanol extracts of the stems and leaves (25 mg/kg bodyweight) or purified andrographolides (1 mg/kg bodyweight) to mice was reported to stimulate antibody production and the delayed-type hypersensitivity response to sheep red blood cells (Puri et al., 1993). The extract and purified andrographolide was also reported to stimulate an innate immune response in mice, measured by macrophage migration index, phagocytosis of [^{14}C] leucine-labelled *E.coli*, and proliferation of splenic lymphocytes stimulated with *Andrographis paniculata* extract (Puri et al., 1993). However, the mechanism of the immunostimulation of andrographolide was not investigated.

Andrographolide has been reported to have both immuno-stimulant and suppressant activities. The immunomodulatory property of a diterpene lactone andrographolide was reported to be associated with enhancement of proliferation of human peripheral blood lymphocytes as well as the production of key cytokines and the expression of

immune activation markers (such as INF-γ, neopterin and β-2-microglobulin) in whole blood cells in culture *in vitro* (Panossian et al., 2002). Rajagopal et al (2003) and Kumar et al. (2004) reported the immunostimulatory activity of andrographolide *in vitro* in PHA stimulated HPBLs (human peripheral blood lymphocytes) by increased proliferation of lymphocytes and production of IL-2. However, Iruretagoyena et al. (2005) reported that andrographolide could interrupt T cell activation both *in vitro* and *in vivo*. *In vitro*, this molecule could interrupt T cell proliferation and cytokine release in response to allogenic stimulation. T cell activation by antigen-pulsed dendritic cells (dendritic cells (DCs), one of antigen-presenting cells) was suppressed by andrographolide in B3Z/0T4H T cell hybridomas (DCs-T cell co-culture). The authors suggested that andrographolide could interrupt maturation of DCs and their ability to present antigens to T cells. *In vivo* immune responses such as antibody response to a thymus-dependent antigen (NP17-BSA) and delayed-type hypersensitivity were extremely lessened in mice treated with andrographolide. In addition, Iruretagoyena et al. (2006) reported that andrographolide enhanced the tolergenic properties of immature DCs in Experimental Autoimmune Encephalomyelitis (EAE) by inhibiting NF-kappaB activation in murine DCs. Andrographolide was also reported to reduce IFN-γ and IL-2 production in murine T-cells stimulated with concanavaline A (Con A) *in vitro* (Burgos et al., 2005). Moreover, Qin et al. (2006) reported that andrographolide inhibited the production of TNF-α and IL-12 in macrophages stimulated by lipopolysaccharide. Hence, the molecular and cellular mechanisms responsible for the immunomodulatory properties of andrographolide are still unclear.

1.3.5.7 Other activities of AP

AP was demonstrated to have anti-cancer activity. Matsuda et al. (1994) found that methanol extract of AP had potent cell differentiation-inducing activity on mouse leukaemia (M1) cells *in vitro*. Trivedi and Rawal (1998) also reported that an aqueous extract of AP had prolonged effects in inhibiting the tumorous condition of mouse liver, induced by hexachloro cyclohexane (hexachloro cyclohexane is an agent used for inducing liver damage to an extent of tumorous condition). A methanol extract of AP has also reported by Kumar (2004) to inhibit the proliferation of HT-29 (human colon cancer). Andrographolide has shown cytotoxic activity against KB (human epidermoid carcinoma), P388 (lymphocytic leukaemia), PC-3 (prostate cancer) and myeloid leukemic HL-60 cells (Kim et al., 2005; Cheung et al., 2005; Siripong et al., 1992). Furthermore, Indian researchers reported that andrographolide not only inhibited the *in vitro* proliferation of different tumor cell lines, representing various types of cancers (Satyanarayana et al., 2004; Kumar et al., 2004; Nanduri et al., 2004; Rajagopal et al., 2003), but it also exerts direct anticancer activity on cancer cells by cell-cycle arrest at G0/G1 phase through induction of cell-cycle inhibitory protein p27 and decreased expression of cyclin-dependent kinase 4(CDK4) (Rajagopal et al.,

2003). Other suggestions of andrographolide-induced cell death were also reported either through the apoptotic pathway, via action of the caspase cascade (Kim et al., 2005) or regulation of expression of pre-apototic markers (Zhou et al., 2006; Cheung et al., 2005).

AP was also reported to show anti-virus activity. Aqueous extracts of AP leaves were reported to inhibit human immunodeficiency virus (HIV)-1 replication in the lymphoid cell line MOLT-4 (Yao et al., 1992). A hot aqueous extract of the aerial parts reduced (greater than 3 times of standard deviation below the mean) in the percentage of HIV antigen-positive H9 cells (Chang & Yeung, 1988). Syncytia formation in co-cultures of uninfected and HIV-1 infected MOLT cells was suppressed by a methanol extract of leaves at 70μg/ml (Otake et al., 1995). Dehydroandrographolide not only inhibited HIV-1 (UCD123) infection of H9 cells at 1.6μg/ml and 50μg/ml, respectively, but also inhibited HIV-1 infection of human lymphocytes at 50μg/ml (Chang et al., 1991). Andrographolide was reported to inhibit HIV-1 by increasing plasma CD4 lymphocyte level in HIV-1 infected patients (Calabrese et al., 2000). Andrographolide, neoandrographolide and 14-deoxy-11, 12-didehydroandrographolide showed anti-HSV-1 (herpes simplex virus 1) activity in human foreshin fibroblast and green ape kidney cell lines at tested concentrations (Wiart et al., 2005).

1.3.6 Pharmacokinetics and safety of AP

AND is highly bioavailable in human and animals. Panossian et al (2000) used validated analytical methods (HPLC, capillary electrophoresis and GC-MS) to determine the amount of AND in the blood plasma of rats and human volunteers orally administrated of AP extract. They found that AND was quickly and almost entirely absorbed into the blood. AND reached a peak plasma value in 1.5-2 hours and had a mean plasma residence time of 10 hours when the rats were orally administration of AP extract at a dose of 20 mg/kg bodyweight. The C_{max}, AUC in rat at 2 hours were 1.3 μg/ml, 7.1 μg h/ml after oral administration of 20 mg/kg APE. They were 3.0 μg/ml, 15.1 μg h/ml after oral administration of 200 mg/kg. After radiolabelled AND was given oral dose to mice, AND accumulated in organs throughout the viscera. In one study, after 28 hours, 20.9% of labelled AND was accumulated in the brain; 14.9% in the spleen; 11.1% in the heart; 10.9% in the lung; 8.6% in the rectum; 7.9% in the kidney; 5.6% in the liver; 5.1% in the uterus; 5.1% in the ovary and 3.2% in the intestine (Zheng et al., 1982). Absorption and excretion is fast. The kidney (urine) and the gastrointestinal tract remove almost 80% AND within 8 hours. Ninety percent is taken away within 48 hours.

Human studies have not found that AP associates with any side effects, although animal studies raise concerns about its effects on fertility such as male reproductive

system toxicity. However, according to Hancke et al. (2006), no toxicity was observed when humans had been treated in adose of 1200 mg daily for 4 days.

Burgos et al. (1997) used male rats to evaluate the possible testicular toxicity of AP dried extract for 60 days. No toxicity was found after the rats were treated with 20, 200 and 1000 mg/kg bodyweight for 60 days by evaluation of reproductive organ weight, testicular histology, ultrastructural analysis of Leydig cells and testosterone level. They suggested that AP extract had no subchronic testicular toxicity effect in male rats. Another subchronic toxicity of AP was given at doses of 0, 0.12, 1.2 and 2.4 g/kg bodyweight / day with doses equal to 1, 10 and 20 times used for human doses (6g/day/person), to 24 rats for 6 months. Rat growth rate and body weight were normal. Also no abnormalities were found in blood serum, inner organs, testis and ovary (DMPRD, 1990).

None of the mice died when mice received oral extract of AP (10 g/kg bodyweight) per day for seven days (Huang, 1978). This high amount did decrease activity and caused general lethargy, but the mouse heart, kidney, liver and spleen were found to be normal. Also extract of 20 g leaves with 600 ml water administered at the rate of 10 ml/kg bodyweight did not produce abnormalities in rats and rabbits (Chantasutra & Limpapanichkul, 1989). No abnormal cardiovascular responses were found when rabbits were given intravenous andrographolide (10 mg/kg bodyweight). Liver enzyme tests and heart, liver, kidney and spleen were normal in these animals (Guo et al., 1988). In another study for toxicity, rats or rabbits received 1 g/kg bodyweight of andrographolide or neoandrographolide orally for 7 days; there was no effect on their body weight, blood counts, liver or kidney function (Yin and Guo., 1993).

AP has been reported to have anti-fertility effects in experimental animals. Akbarsha et al. (2000; 1990) found that AP leaves at a dose of 20 mg per day for 60 days had an antispermatogenic effect in male rats, but Panossian et al (1999) found that a high therapeutic dose of AP extract (at 2g/kg bw) could not induce progesterone-mediated termination of pregnancy in pregnant rats. Male rats were also reported becoming infertile at intakes of 20 mg/ day or at high doses (Akbarsha et al., 1990; Zoha et al., 1989). Other side effects are that high doses may cause gastric discomfort, anorexia and emesis but few side effects and no toxicity have found (Chang and But, 1986). However, in general, evidence to date indicates that AP is a natural product with low toxicity when used appropriately.

1.4 Mouse salmonellosis as a model system

1.4.1 Salmonellosis

Salmonellosis is a serious infectious disease of both animals and humans. Over 2000 Salmonella serovars have been identified, most of which can colonize virtually all animals including food-producing animals (Mandell et al., 2005). Salmonellosis may manifest, depending on the serovar, as:

(1) Enteric fever, also called typhoid fever, caused by *S. typhi* or paratyphoid fever caused by *S. paratyphi*.
(2) Entertitis, characterized by fever, nausea, vomiting and bloody or nonbloody diarrhoea generally with abdominal cramps, which in immunocompletent human is generally a self-limiting disease.
(3) Bacteraemia commonly encountered with infection caused by *S. typhi*, *S. paratyphi*, *S choleraesuis* and *S. enteritidis*.

While enteric fever and bacteraemia warrant immediate treatment with an effective antibiotic, selected by *in vitro* susceptibility tests, which may include fluoroquinolones, trimethoprim/sulfamethoxazole or a broad-spectrum cephalothin, antitriches are not recommended for treatment of enteritis, the latter being self-limiting. Both these clinical manifestations that involve *S. typhi* as the etiological agent are best handled using a prevention or prophylactie strategy involving vaccination.

1.4.2 Salmonella vaccines and mechanisms of protection

Traditionally, vaccines have been prepared in two ways, either live vaccines comprising live weakened or attenuated pathogens that have reduced pathogenicity but retained immunogenicity, or dead (non-living) vaccines such as killed or inactivated whole pathogens, and soluble pathogen proteins or protein subunits (Lee and Bishop, 1997). Live attenuated Salmonella vaccines may have come very close to meeting the criteria for an ideal vaccine that replicate similarly to the natural microorganism *in vivo*, and elicit both humoral and cellular immunity similar to that elicited by the natural infection (Viret et al., 1999; Harrison et al., 1997; Mastroeni et al., 1993; Mukkur et al., 1991a, 1991b; Mukkur et al., 1987). However, they may pose some risk such as reversion to virulence, and may also be dangerous to immunocompromised hosts including the state of pregnancy (Black, 2002; Lee & Bishop, 1997). On the other hand, killed or nonviable vaccines may be safer than live vaccines, but they generally cannot efficiently enter the MHC class I pathway, and thus are unable to elicit cell-mediated immunity, a pre-requisite for protection against intracellular pathogens including *Salmonella* speices. In adoptive transfer experiments carried out by Mastroeni et al., (1993) in *Salmonella typhimurium*-susceptible mice,

they suggested that protection against an oral challenge of virulent *Salmonella typhimurium* required both immune T cells and sera. Killed vaccines generally produce a less broad immune response than live, attenuated vaccines (Babu et al., 2004; Babu et al., 2003; Harrison et al., 1997; Thatte et al., 1993). They are more likely to elicit an antibody response other than cell-mediated immunity although there are some reports that killed *Salmonella enteritidis* vaccine also induced cell-mediated response in chickens in an age-dependent manner (Okamura et al., 2004; 2003).

The efficacy of live vaccines in the mouse model is because of their ability in not only to elicit an antibody response but also induce in cell-mediated immunity, which killed vaccines fail to do (Harrison et al., 1997; Mastroeni et al., 1993; Mukkur et al., 1991a, 1991b; Mukkur et al., 1987). Orally ingested bacteria reach the spleen and liver to cause systemic infection by penetrating the intestinal mucosa and travelling via the lymph nodes (Finaly et al., 1997; Jones et al., 1996). During bacterial infection, macrophages serve as professional phagocytes and key effectors of the immune responses. However, *Salmonella typhimurium* uses the macrophage's phagocytic nature to its advantage, as it resides intracellularly within macrophages. This enables it to replicate in the macrophage's specialised vacuoles (Richter-Dahlfors et al., 1997; Leung and Finlay, 1991), protected from its host's killings when this occurs. The antimicrobial actions of infected macrophages are vital in determining the outcome of disease (Kaufman, 1993). IFN-γ (an indicator of CMI) is reported as an effective stimulator of macrophage gene expression and is therefore necessary when *Salmonella typhimurium* or other intracellular bacteria need to be cleared (Rosenberger et al., 2000; VanCott et al., 1998; Nauciel et al., 1992; Muotiala et al., 1990). In the *in vivo* mouse model of human typhoid fever, IFN-γ is released by NK and T cells 2-3 days following *Salmonella typhimurium* infection (Rosenberger et al., 2000).

Salmonelloses in food-producing animals and humans are continuing problem that warrants application of prophylactic strategies. The initial strategy used evaluating the protective efficancy of killed vaccines in the mouse model. Initial studies in mice, using killed *Salmonella* vaccines claimed those vaccines to be protective (Angerman and Eisenstein, 1980), but were later reported to be due to the innate resistance of some mouse strains to salmonellosis (Kaufmann et al., 2001). The most direct demonstration of the lack of relevance of anti-*Salmonella* antibodies to protection against salmonellosis was reported by Mukkur et al. (1991a) when mice immunized with the formalin-killed *S. bovis-morbificans* vaccine. High antibody titres did not protect mice against experimental challenge infection, whereas mice vaccinated with live attenuated *S. bovis-morbificans* were significally protection. The high level of protective efficacy recorded for the live attenuated *S. typhimurium* (*aroA*) vaccines in sheep and cattle (Villarreal-Ramos et al., 1998; Begg et al., 1990; Mukkur et al., 1987) clearly valuates the use of such vaccines against salmonellosis in food producing animals. Another attenuated *S. typhimurium* vaccine strain in which the catabohte receptor protein (crp) and adenylate cyclare (cya) genes were inactivated

(Δcrp, Δcya) has also been reported to impart protection in mice, chickens and humans (Levine et al., 2001; Hassan et al., 1993; Curtiss and Kelly, 1987). It is beyond the scope of this review of literature to provide the history leading to the development of aromatic-dependent (aroA/ aroD) and Δcrp, Δcya *S. typhi* vaccines. However, an excellent review on this aspect is available elsewhere of Levine et al. (2004).

Two new typhoid vaccines have been licensed and widely used in humans during the past 15 years. One is the Vi polysaccharide from *S. typhi* for parenteral (subcutaneous or intramuscular) and the other is a live attenuated strain of *S. typhi* Ty21a for oral application. The *S. typhi* Ty21a strain, which is derived by chemical mutagenesis, becomes completely deficient of the activities of the gal E gene product. Not only its ability to produce complete lipopolysaccharide is restricted, but Ty21a also has several nutritional auxotropies, approximately half the growth rate of the parent strain Ty2, does not produce H_2S, and lacks the Vi antigen (capsular acidic polysaccharide which is present on almost all virulent S. typhi strains). It takes galactose to facilitate production of lipopolysaacharide (which leads to immunogenicity) when Ty21a is grown in the low concentrations of galactose. However, an oversupply of galactose in growing Ty21a has been supposed to cause an accumulation of toxic metabolites within the bacterial cells, which leads to bacterial lysis. The attenuation and safety of the Ty21a strain has always been attributed to the combination of two factors, the gal E mutation and the lack of Vi antigen. This view has been shown to be at least partially incorrect by the observation that a similar mutant (Vi negative, *galE* deletion mutant) of *S. typhi*, constructed by recombinant DNA techniques, is virulent. Futhermore, galactose induced lysis of Ty21a is subdued in vitro in the prescence of glucose. Consequently, neither the safety of Ty21a nor the failure to recover vaccine organisms from people ingesting the usual dose could be completely explained by the combination of *galE* and Vi mutations. Hence, the mechanism of Ty21a attenuation is not completely understood.

These two vaccines have widely replaced the old heat-phenol inactivated whole-cell vaccine in many countries. Another acetone-killed whole-cell human typhoid vaccine given subcutaneously (s.c.) has also been in use for many years (http://www.who.int/vaccines/en/typhoid.shtml). Inactivation with acetone was originally designed to retain the protective Vi capsular antigen of *S. typhi*, thereby making the vaccine more immunogenic and protective than the older heat-phenol-killed vaccine that lacked the Vi antigen. However, it was discovered to elicit poor cell-mediated immunity, the latter considered to be important confering significant albeit limited protection (Tacket et al., 2000; Harrison et al., 1997; Mastroeni et al., 1993). The current formulations at least of the Vi- and Ty21a vaccines are only 50%-70% efficient in children of fewer than five years and young adults. Furthermore, neither the Vi-based polysaccharide vaccine nor the Ty21a vaccine is permitted to give children under two years old (WHO, 2003).

1.5 Immune response modifiers/adjuvants

Immune response modifiers or adjuvants are compounds or molecules that enhance or suppress one or both the effector arms of immune response (Akdis et al., Singh and Srivastava, 2003). This definition is based on the assumption that adjuvants enhance the delivery of the antigen to the lymph node followed by incompletely understood different mechanisms (O'Hagan, 2004). Description and discussion of different type of adjuvants or delivery systems used so far, some of which may also induce and enhance CMI, are beyond the scope of this literarure review. However, it is clear that the potential mechanism of action of andrographolide may have to involve consideration of the action of metabolites generated *in vivo* after oral administration of this immuno-stimulatery compound, an area of a research apportunity for the future.

1.6 Goals and objectives

There are many ways to protect ourselves against infectious diseases. For example: antibiotics and other antimicrobial agents may directly inhibit or kill the pathogens; vaccines activate the immune system to respond to an infectious agent by inducing antibodies or CMI. An adjuvant (such as alumium hydroxide, calcium phosphate gel) used in combination with specific antigen that produces more immunity than the antigen alone, has been reported (Edelman, 2002; HogenEsch, 2002; Hunter, 2002; Kenney et al., 2002; O'Hagan, 2000). It is worth to think other adjuvants to select for modulating humoral or CMI. Plants and plant derived substances have become new sources in the investigation of antimicrobials. The use of herbal extracts either as an alternative or complimentary medicine to the conventional chemotherapy for treatment of diseases has a long history or is well documented in China and India (Hoareau et al., 1999). Therefore, they have been perceived as sources for new antimicrobial research. It would be ideal if an immunodulating compound, capable inducing CMI in the target species (mice in this study), when administered at the same time as the non-CMI inducing vaccine such as the killed vaccine, was discovered as vaccines against salmonellosis capable of inducing both antibodies and CMI are protective both in humans and animals.

AP, as a traditional Ayurvedic/Chinese herbal medicine has been claimed to possess many therapeutic properties including immunostimulation. Clinically, AP extract has been used to treat patients with the common cold, urinary infections, dysentery and infectious hepatitis. More recently, various biological activities of AP extract or AND have been reported such as immunostimulatory activity (Puri et al., 1993), anti-inflammatory effect (Chiou et al., 2000; Shen et al., 2000; Madav et al., 1996; Sawasdimongkol et al., 1990; Chantasutra & Limpapanichkul, 1989; Dutta & Sukul., 1982), anti-human immunodeficiency virus (HIV) activity (Otake et al., 1995; Yao et

al., 1992; Chang and Yeung, 1988), cytokine induction (Kumar et al., 2004; Rajagopal et al., 2003; Panossian et al., 2002), cytokine deduction (Qin et al., 2006; Burgos et al., 2005), a potential cancer therapeutic agent (Zhou et al., 2006; Kumar et al., 2004; Rajagopal et al., 2003) and T cell activation suppression when given systemically (Iruretagoyena et al., 2006; 2005). These studies, taken together, suggested that one of the pharmacological effects of AP might be mediated via its immunomodulatory property (ies) (Mills & Bone, 2000). As a major chemical constituent of AP along with the above activities, AND might be the active principle. However the potential of AP extract or AND to modulate the specific or adaptive immune response, particularly to the infectious diseases caused by intracellular pathogens (such as *Salmonella typhimurium*) has not been determined. The purposes of this project were to determine the immunomodulatory and protective potentials of the ethanol extract of AP and one active principle of AP, AND, with the aim of assessing their cell-mediated immunity (CMI)-inducing and their protective potential particularly against infectious diseases caused by intracellular pathogen, *Salmonella typhimurium*. As described above, cytokine production involved in the induction of CMI or humoral immunity. In this project, IL-4, in addition to the measurement of antibody response was used as an indicator of humoral immunity, whereas induction of IL-2 and specially, interferon-γ were used as indicators of CMI.

The goals of the research embodied in this thesis were to (1) test the direct anti-microbical potential of AP *in vitro* using a broader spectrum of microbial pathogens of relevance to human health (2) to determine the immunomodulatory of AP using mouse salmonellosis caused by *Salmonella typhimurium* in mice as a model system (3) and to immunologically characterize the bioactive principle, andrographolide thought to be responsible for immunomodulatory activities.

These aims were achieved by:

1. Purification and chemically characterisation of purifed AND from AP.
2. Evaluation, *in vitro*, of the antibacterial activity of AND or AP extracts with different solvents, using the disk agar diffusion method--antibacterial susceptibility test.
3. Evaluation of the immune response-modifying potential of ethanol extract of AP (APE) and purified AND against intracellular microbial infections in mice immunized with killed *Salmonella typhimurium* vaccine, the latter stimulating only the humoral arm of immunity.
4. Determination of the protection status of mice, treated with APE and AND, following oral challenge with virulent *Salmonella typhimurium*.

Chapter 2 Purification and identification of andrographolide from *Andrographis paniculata*

2.1 Introduction

Andrographis paniculata, as a traditional Ayurvedic/Chinese herbal medicine has been claimed to possess many therapeutic properties (mentioned in Sections 1.3.4 and 1.3.5). One of the ways of administration of this medicine is via herbal extract prepared using aqueous or alcoholic solvents. Such extracts contain a mixture of bioactive and inactive chemical ingredients. In addition, the chemical composition varies depending on growing region and the collection time. Hence, it is desirable to identify the bioactive chemical ingredient (s) from the traditionally-claimed therapeutic herbs with the aim of (1) standardising the herbal extract or (2) evaluating the therapeutic potential of the purified bioactive principles for use in the treatment of the traditionally-claimed disease conditions. The objective of this study was to purify one of these major components (andrographolide) from *Andrographis paniculata* and to analyse its quality.

2.2 Materials and methods

2.2.1 Plant materials

AP whole plant coarse powder was purchased from Woods plus Woods Co. Ltd. (Sydney, Australia). The whole dried plants had been crushed and ground into powder prior to purchase. Preliminary tests and confirmatory tests were conducted to confirm the presence of AND in the purchased AP. Commercial AND (purchased from Sigma-Aldrich, 98%, St Louis, MO,) was used as standard.

2.2.2 Preliminary test (colour test)

1 g of AP powder with 20 ml of ethanol was boiled in a water bath for 5 mins. 300 mg of activated charcoal was added, stirred and then filtered. The filtrate was used for the following two tests. The first test involved 2 drops of a 2% w/v solution of 3,5-dinitrobenzoic acid in ethanol and 2 drops of a 5.7% w/v solution of

potassium hydroxide in ethanol being added to 0.5 ml of filtrate. A colour change to a purplish red indicated that the sample had active compounds (MPRI, 1999). The second test involved 3-5 drops of the 5.7% w/v solution of potassium hydroxide in ethanol being added to 0.5 ml of filtrate until a red colour appeared. The filtrate was set aside for 10 to 15 mins. The red colour changed to yellow. These two tests indicate that the sample contained diterpene lactones as active constituents (MPRI, 1999).

2.2.3 Confirmatory test

Thin layer chromatography (TLC) analysis was carried out following the colour test to confirm that AND is contained in AP powder. The filtrate was obtained as above (Section 2.2.2). The filtrate was evaporated under reduced pressure until fully dried. The filtrate powder and 1 mg of standard andrographolide were dissolved in 0.5 ml of warm ethanol separately. A precoated plate of silica gel 60F254 (Merck, Kilsyth, VIC) and mobile phase (chloroform: methanol: ethyl acetate 8: 1.5: 1) were used. 5 microliters was used for each spot. TLC of the isolated samples was firstly detected by UV radiation (Electronic UV Transilluminator, Quantum Scientific) and then confirmed by spraying with 2% w/v solution of 3,5-dinitrobenzoic acid in ethanol and an excess of 5.7% w/v of potassium hydroxide in ethanol.

2.2.4 Purification of AND

After confirmation that AP contained AND, a modification of the method reported by Rajani et al. (2000) was used to isolate AND from AP. Briefly, 200g whole dried crushed AP was extracted twice with a 1:1 mixture of dichloromethane and methanol by cold maceration (one with 1 liter and then 0.2 liter of the maceration mixture) (1×1 liter and 1×0.2 liter). Each extraction was conducted for 6 hours with constant stirring. The extract was filtered and the filtrate was concentrated *in vacuo* at a temperature below 70°C to produce a green mass (4.3g). The green mass was washed with toluene several times until most of coloured matter was removed from the residue. The toluene was completely removed from the residue by evaporation. The residue was dissolved in 60-70°C hot ethanol and filtered while hot. The filtrate was cooled in a refrigerator for crystallisation. This process was repeated several times until small yellow plates of constant melting point, range of 220-228°C (2.5g) were obtained (a melting range from 224-230°C is used in China in laboratories of government offices to test and control the quality of AND, http://www.plantpharm.net/new_page_25.htm). The isolation of AND was confirmed by UV absorption spectrum, TLC, ESI-MS and ^{1}H-NMR (see following Section).

2.2.5 Identification of purified AND

2.2.5.1 Melting point

Melting point was determined using a melting point apparatus (FSE, England).

2.2.5.2 Colour test

The colour test was performed by the following two methods (http://www.plantpharm.net/new_page_25.htm). The first method involved adding 1ml 1% w/v solution of 3,5-dinitrobenzoic acid in ethanol and 2 drops of 5.7% w/v of potassium hydroxide in ethanol to 1mg AND in 3ml ethanol. A colour change to purple indicated that the sample had active compounds such as AND present. The second method involved adding 2-3 drops of 5.7% w/v of potassium hydroxide in ethanol to 1 ml 0.1 mg/ml purified AND in ethanol until the solution showed a red colour. After sitting for 10-15 minutes, a colour change to yellow indicated that the sample had active compounds such as AND present.

2.2.5.3 UV maximum absorption spectrum (UV: λ max nm)

UV spectrophotometry was used to determine the maximum absorption spectra within the range from 200 to 270 nm (CE2021, 2000 SERIES; Cecil, UK) (http://www.plantpharm.net/new_page_25.htm). The concentration of purified AND and commercial AND (purchased from Sigma-Aldrich, 98%) was 25 µg/ml in ethanol.

2.2.5.4 Thin layer chromatography (TLC)

1 mg of commercial AND and purified AND was dissolved in 0.5 ml of warm ethanol (2mg/ml) separately. A precoated plate of silica gel 60F254 (Merck, Kilsyth, VIC) and mobile phase (chloroform: methanol: ethyl acetate 8: 1.5: 1) were used. 5 microliters were used for each spot. TLC of the isolated samples was firstly detected by UV radiation (Electronic UV Transilluminator, Quantum Scientific) and then by spraying with 2% w/v solution of 3,5-dinitrobenzoic acid in ethanol and excess 5.7% w/v potassium hydroxide in ethanol.

2.2.5.5 ^{1}H-NMR (^{1}H- nuclear magnetic resonance spectroscopy)

^{1}H-NMR was determined by Dr. Russell Barrow from the Australian National University (ANU). ^{1}H-NMR spectra were recorded at 298 K on a Varian Gemini 300 instrument with 300 MHz and referenced to the central peak from residual methanol at 3.30 ppm.

2.2.5.6 ESI-MS (electrospray ionization mass spectrometry)

ESI-MS was determined by Dr. Russell Barrow from the ANU. ESI-MS was recorded on a Bruker Apex 3 (Karlsruhe, Germany).

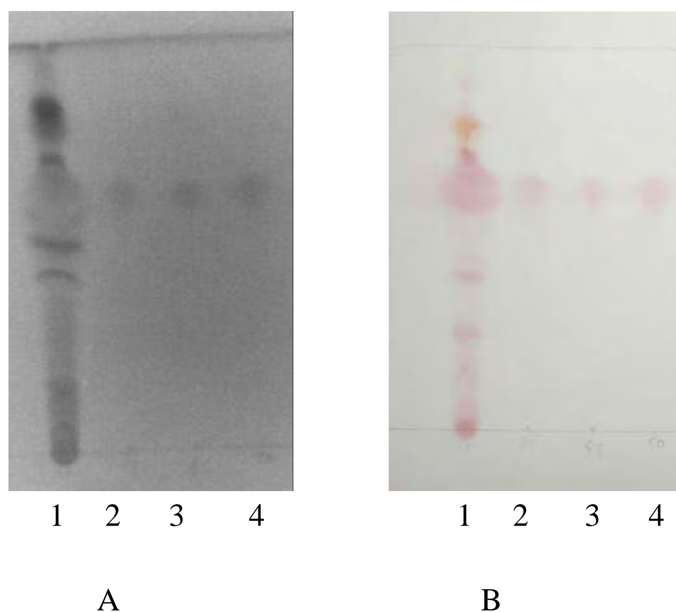
2.3 Results

2.3.1 Preliminary test

The result of preliminary test was that the colour of AP filtrate changed to purplish red after ethanolic 3,5-dinitrobenzoic acid and potassium hydroxide being added in the first test. The filtrate first appeared red in colour after ethanolic potassium hydroxide was added, and then the red colour changed to yellow 10 mins later in the second test.

2.3.2 Confirmatory test

The result of the TLC is shown in Figure 2-1. For the AP sample, six spots appeared (Figure 2-1B) and the Rf value of each spot is 0.05, 0.14, 0.24, 0.39, 0.62 and 0.79 respectively. A large spot with Rf value equal to 0.62 (Figure 2-1 A & B) shows the same Rf value as the standard AND (0.62) in the same precoated plate of silica gel 60F254 after development.



A: Photo of UV radiation B: Photo of spraying with reagents

Figure 2-1 Thin layer chromatography of AP extract and standard AND after development, lane 1: AP solvent sample, lanes 2, 3 and 4: standard AND

2.3.3 Purification of AND

2.5 g of pale yellow crystalline plates was obtained by the modification of the method reported by Rajani et al. (2000, Figure 2-2).

Figure 2-2 Andrographolide crystalline plates in bottles

2.3.4 Identification of purified AND

2.3.4.1 Melting point

The melting point of the purified sample was 220-228°C and the commercial sample (purchased from Sigma-Aldrich, 98%) was 222-228°C.

2.3.4.2 Colour test

In the colour test, the purified AND solution changed to purplish red after chemical solvents were added according to method 1. The solution first changed to red after the chemical was added by method 2, 10-15 minutes later it changed to yellow.

2.3.4.3 UV (EtOH): λmax nm

UV λmax = 224 nm in ethanol was obtained (Figure 2-3).

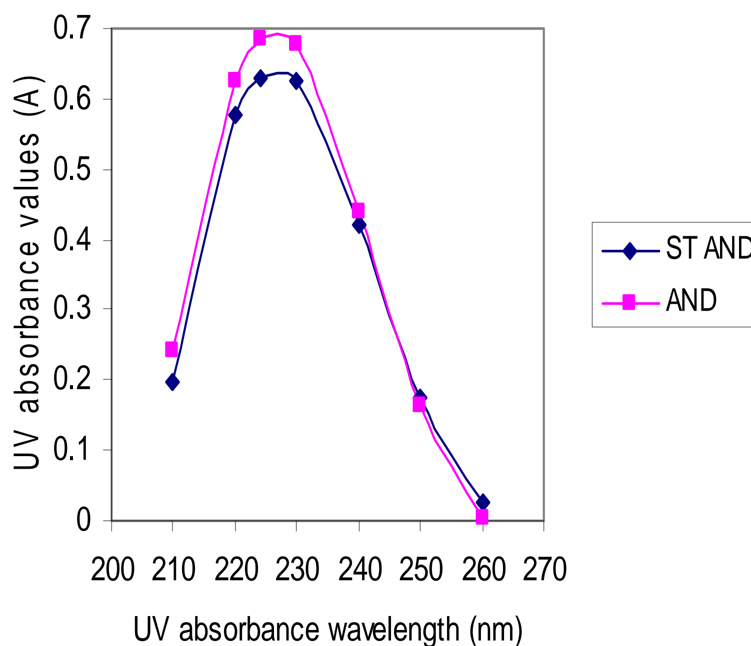
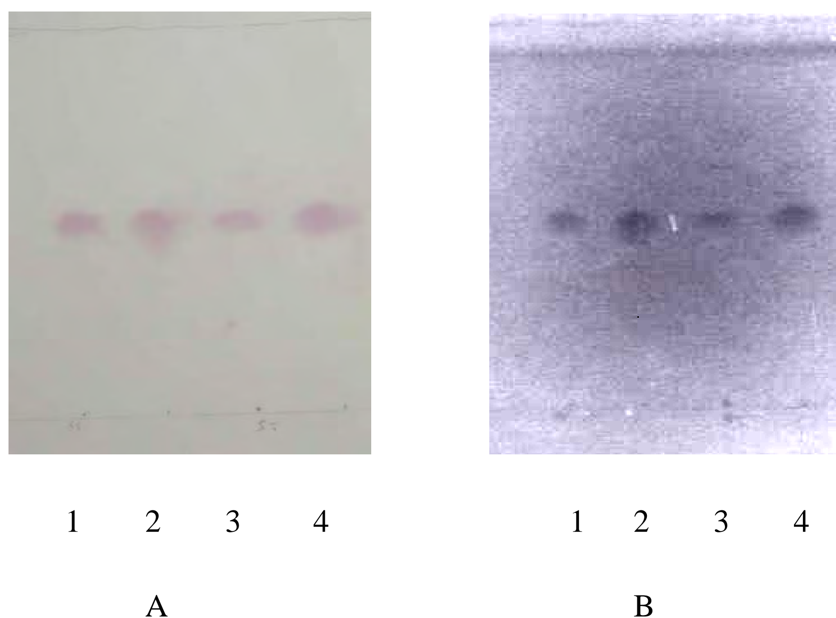


Figure 2-3 Absorption spectra of AND isolated (square) and reference standard (ST, diamond), in the UV ranges from 210 to 270 nm.

2.3.4.4 Thin layer chromatography (TLC)

The result of TLC is that only one purple/red spot appeared for each sample after development with the colour reaction reagents (Figure 2-4A) and one spot was detected by UV radiation (Figure 2-4B).
Rf (lane 1) =0.48; Rf (lane 3) =0.48;
Rf (lane 2) =0.48; Rf (lane 4) =0.48.



A: Photo after spraying with reagentsB: Photo of UV radiation

Figure 2-4 Thin layer chromatography of purified AND and standard AND after development, lanes 1 & 3: the standard AND, lanes 2 & 4: the purified AND.

2.3.4.5 ^{1}H-NMR

^{1}H-NMR was carried out by Dr. Russell Barrow of ANU (Figure 2-6 and Table 2-1). ^{1}H-NMR spectra were recorded at 298 K on a Varian Gemini 300 instrument with 300 MHz and referenced to the central peak from residual methanol at 3.30 ppm. ^{1}H-NMR spectroscopy (300MHz, CD_3OD) was entirely consistent with published data for AND and revealed the sample to be pure to the limits of detection (Medforth et al., 1990; Cui et al., 2005). δ at which signals are shown for purified AND and reference data is given in the below table (Table 2-1).

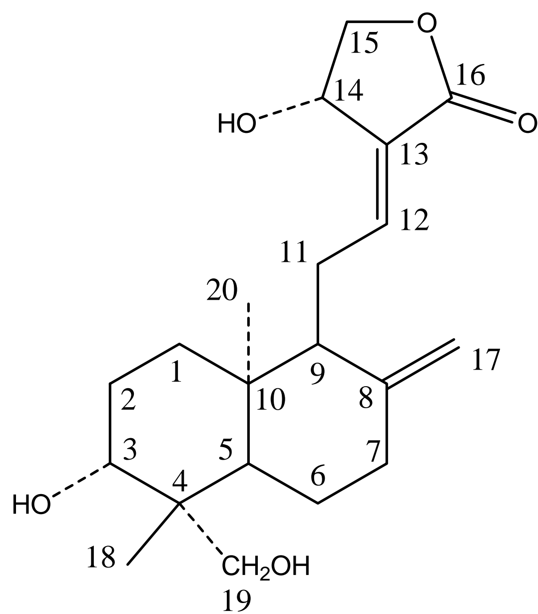
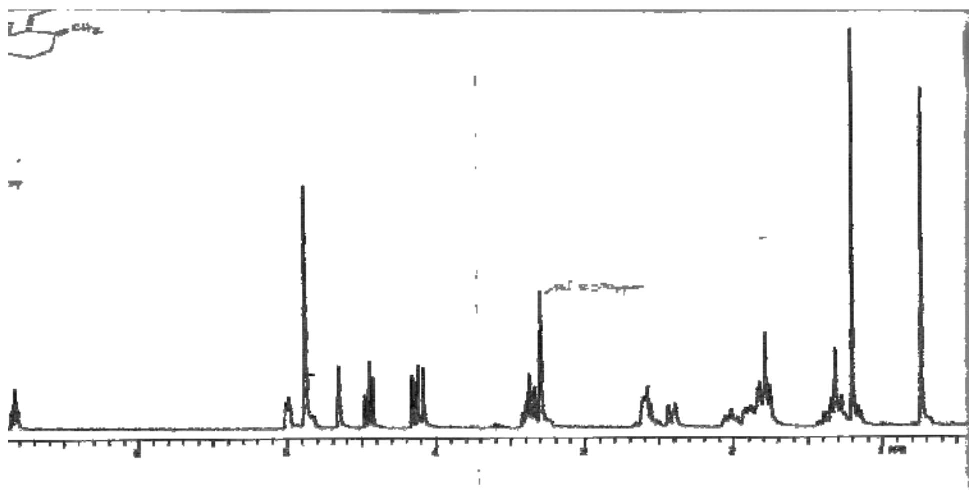


Figure 2-5 The structure of andrographolide

(a)



(b)

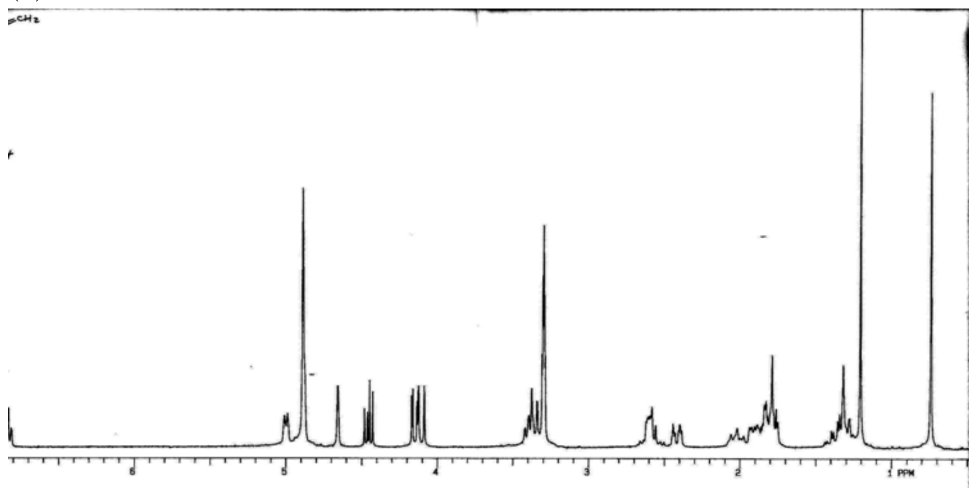


Figure 2-6 ^{1}H-NMR of purified AND (a) and standard AND (b)

Table 2-1 Proton chemical shift assignments of AND

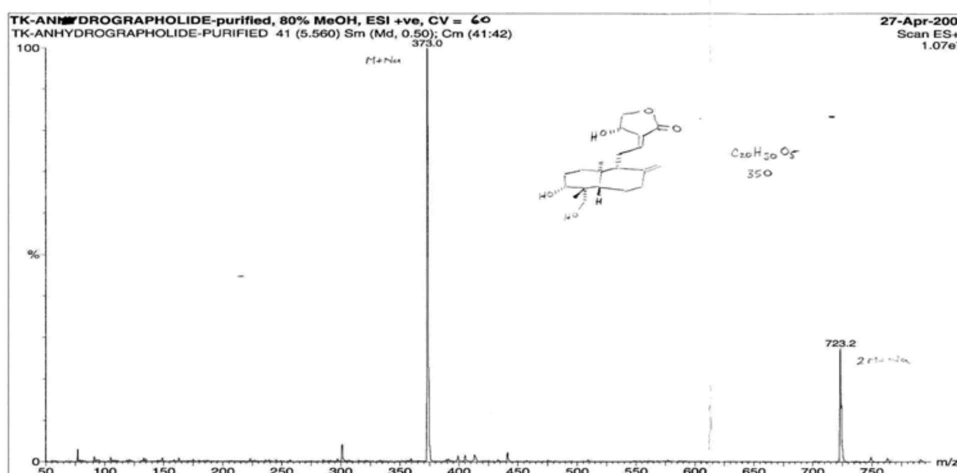
(ppm measured in CD_3OD and 300 MHz) relative to residual methanol (MeOH) at 3.30 ppm

Proton	δ of purified AND (ppm)	δ of reference data (ppm) (Medforth et al., 1990)	δ of reference data (ppm) (Cui et al., 2005)
H(1)ax	1.28	1.30	1.28
H(1)eq	1.80	1.80	1.83
H(2)ax	1.80	1.80	1.79
H(2)eq	1.78	1.80	1.76
H(3) ax	3.41	3.41	3.40
H(3)eq	-	-	-
H(5)ax	1.32	1.32	1.32
H(6)ax	1.38	1.38	1.36
H(6)eq	1.85	1.86	1.87
H(7)ax	2.03	2.04	2.03
H(7)eq	2.43	2.43	2.41
H(9)ax	1.91	1.92	1.91
H(11a)	2.61	2.63	2.60
H(11b)	2.59	2.58	2.60
H(12a)	6.85	6.85	6.84
H(12b)	-	-	-
H(14)	5.01	5.01	5.00
H(15a)	4.45	4.45	4.45
H(15b)	4.16	4.16	4.14
Me(16)	0.75	0.76	0.74
H(17a)	4.89	4.89	4.87
H(17b)	4.66	4.67	4.65
Me(18)	1.20	1.22	1.20
H(19a)	4.09	4.12	4.10
H(19b)	3.37	3.37	3.36

2.3.4.6 ESI-MS

ESI-MS was determined by Dr. Russell Barrow (ANU). ESI-MS was recorded on a Bruker Apex 3 (Karlsruhe, Germany). The data are given as follows (from Figure 2-7). ESI-MS (+ve) m/z: (1) purified AND: 373.0 $[M+ Na]^+$, 723.2 $[2M+ Na]^+$; (2) standard AND: 373.1 $[M+ Na]^+$, 723.0 $[2M+ Na]^+$. Hence, the molecular weight of purified AND is 350.

(a)



(b)

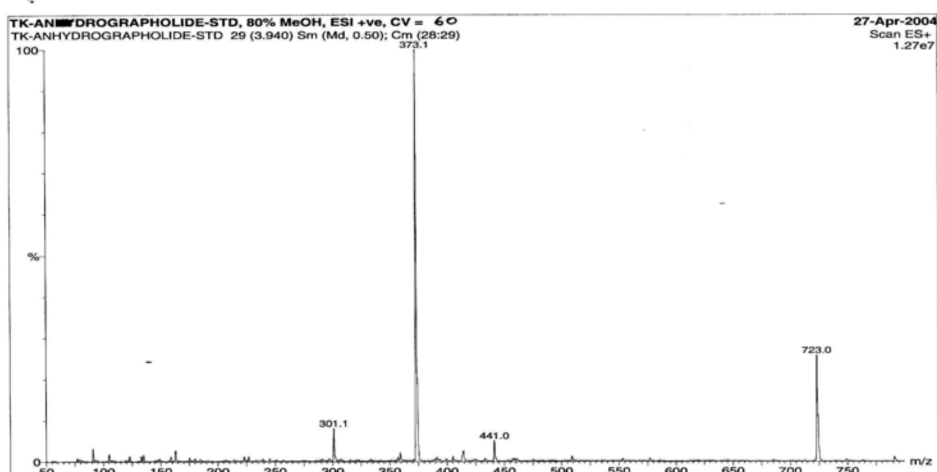


Figure 2-7 ESI-MS of purified AND (a) and standard AND (b)

(80% MeOH, ESI +ve, CV=60)

2.4 Discussion

The preliminary colour test used here is the official test used in the Thai laboratories to test and control the quality of medicinal plants (MPRI, 1999). This test is easy, inexpensive and quick. However, as Tipakorn (2002) mentioned, this is a primary test which only indicates the presence of compounds which react chemically like the active compounds of AP. Further identity tests in the laboratory are needed to confirm the presence of AND.

The confirmatory test showed that the AP plant sample appeared as a large spot and the spot had the same Rf value (0.62) as the AND standard which was used for comparison in this test (Figure 2-1). For the AP solvent sample, there were some additional spots between photos A and B. This is because some compounds which can be detected by UV are not shown by colour test and vice versa.

AND was purified from AP using a slightly modified method reported by Rajani et al. (2000). AND is a white or pale yellow plate crystal which does not mix well with water, but is soluble in chloroform, acetone, ether, methanol and hot ethanol. A 1:1 mixture of dichloromethane and methanol was added to the AP powder to extract AND from AP. After removing most of the colour from the extract mass, hot ethanol was used to dissolve AND and then allowed to crystallize in a refrigerator. Hot ethanol was substituted for methanol in the original literature method (Rajani. et al., 2000) because methanol remaining in AND may be injurious when the AND is dosed to mice.

The purified AND was identified by melting point, colour test, UV λmax, TLC, ESI-MS and ^{1}H-NMR and compared with commercial AND and the data from references (http://www.plantpharm.net/new_page_25.htm; Medforth et al., 1990; Cui et al., 2005). The melting point (224-230°C), colour tests (purple, red and yellow), UV λmax (224 nm in ethanol), TLC (single spot) are normally used in China in laboratories of government offices to test and control the quality of AND (http://www.plantpharm.net/new_page_25.htm). The data obtained here concur with these. The melting point of purified AND is slightly lower than the 235.3°C reported by Rajani et al. (2000). This may be due to the author using DSC, not a normal melting point apparatus to record melting point. The isolation of AND was further confirmed by mass spectrometry and ^{1}H-NMR spectroscopy. Electrospray ionisation mass spectrometry (ESI-MS) showed an intense pseudomolecular ion at *m/z* 373, consistent with a sodiated molecular ion (M+Na) for AND. High resolution mass measurement under electrospray conditions confirmed a molecular formula for the ion at *m/z* 373 as $C_{20}H_{30}NaO_5$. From ESI-MS data, the molecular weight of purified AND is 350 which is the same as the AND standard (Sigma-Aldrich, 98%). ^{1}H-NMR spectroscopy (300MHz, CD_3OD) was entirely

consistent with published data for AND and revealed the sample to be pure to the limits of detection (Medforth et al., 1990; Cui et al., 2005).

In order to further assure quality of the purified AND, it was necessary to have methods which are selective and sensitive for detection. TLC is the simplest and cheapest method of detecting plant constituents because the method is easy to run, reproducible and requires little equipment. HPLC coupled to mass spectrometry (LC/MS) is a newer technique. Mass spectrometry is one of the most sensitive methods of molecular analysis and yields information on the molecular weight as well as on the structure of the sample. Electrospray, one of three LC/MS interfaces, covers the ionization ion (ESI-MS) and has been used to identify the purified AND molecular weight as well as analysing its structure. NMR spectroscopy is by far the most powerful spectroscopic technique for obtaining structural information about organic compounds in solution (Hostettmann, 1999). The purified AND was completely confirmed by TLC, ESI-MS and ^{1}H-NMR.

2.5 Conclusions

Confirmatory tests indicated that the whole plant material contained the active compound of AP: andrographolide. All data from melting point, TLC, UV λmax, ESI-MS and ^{1}H-NMR indicate that AND was successfully purified from AP.

Chapter 3 An investigation on the antimicrobial activity of *Andrographis paniculata* extracts and andrographolide *in vitro*

3.1 Introduction

The development of bacterial resistance to currently available antibiotics has made it necessary to search for new antibacterial agents. New sources, especially natural products from plants, are being investigated because medicinal plants have been widely used for treatment of many types of acute and chronic diseases in Asia, and many plants with antimicrobial activity have been reported (Cowan, 1999; Al-Howiriny, 2002; Dulger and Gonuz, 2004). Crude aqueous or alcohol extractions of AP and one principle of AP viz., AND have been reported to be effective in the treatment of dysentery, diarrhoea and upper respiratory tract infection (Duke and Ayensu, 1985; Thanangkul and Chaichantipyuth, 1985; Gupta et al., 1990; Yin & Guo, 1993; Chturvedi et al., 1983; Chang and But., 1986; Hancke et al., 1995; Caceres, 1997; Melchior et al., 1997; Thamlikitkui et al., 1991; Subjareun, 1996; Melchior et al., 2000; Poolsup et al., 2004 and Coon & Ernst, 2004; Amaryan et al., 2003; Gabrielian et al., 2002; Spasov et al., 2004). The antibacterial activity of aqueous, ethanol or methanol extracts of *Andrographis paniculata* has been tested *in vitro* against *Escherichia coli*, *Staphylococcus aureus*, *Proteus vulgaris, Salmonella typhi*, *Shigella* species and *Pseudomonas aeruginosa in vitro* (George & Pandalai, 1949; Nakanishi *et al.*, 1965; Pleumjai and Sithisomwonges, 1990; Sindermsuk, 1993; Bunyapraphatsara, 2000; Prajjal *et al.*, 2003) with no antibacterial activity observed with the aqueous extract tested against *E. coli*, *S. aureus*, *S. typhi* or *Shigella* species (Leelarasamee *et al.*, 1990). Therefore, it is desirable to extend these investigations using a broader spectrum of microbial pathogens of relevance to human health. The objective of this chapter was to evaluate *in vitro* the antibacterial activity of andrographolide or *Andrographis paniculata* extracts with different solvents.

3.2 Materials and methods

3.2.1 Plant materials

AP whole plant coarse powder was purchased from Woods plus Woods Co. Ltd. (Sydney, Australia). The major active compound, AND was purified and identified as outlined in Chapter 2.

3.2.2 Preparation of extracts

Based on the methods used for chemical analysis described by Cowan (1999), AP powder was extracted in a series of three different solvents: distilled water, 80% and 100% food grade ethanol (recorded as water extract, 80%APE, 100%APE respectively).

AP powder (100g) was weighed and placed in flasks, and then soaked and stirred in distilled water, 80% or concentrated ethanol (1:10 w/v) overnight (o/n). After filtration through cotton wool, the filtrates were centrifuged at 10,000×g for 20 mins (Beckman Avanti J251 centrifuge, Beckman Instruments, Palo Alto, CA) at room temperature (Cowan, 1999; Puri et al., 1993). The extract solutions were evaporated in a rotary evaporator (Labrota 4001, Heidolph) at 60°C until dry. Dried extracts were weighed and stored in labelled sterile screw capped bottles at -20°C.

3.2.3 Microorganisms

The bacteria used included: *Salmonella typhimurium* (bovine, 12313), *Salmonella typhimurium* (*aroA* SL3261), *Salmonella bovis-morbificans*, *Escherichia coli* (ATCC 25922), *Escherichia coli* (Q 358), *Shigella sonnei*, *Staphylococcus aureus* (ATCC 49476), *Pseudomonas aeruginosa* (ATCC 27853), *Streptococcus pneumoniae, Streptococcus pyogenes, Legionella pneumophila,* and *Bordetella pertussis* (Tohama I), (obtained from stocks in the Department of Biological and Physical Sciences, University of Southern Queensland).

3.2.4 Analysis of the AP extracts by TLC

Thin layer chromatography analysis was used to authenticate the AP extracts as judged by the detection of AND. The AP extracts were dissolved as per their preparation details above (Section 3.2.2) and 5 micro liters (µl) were used for each spot. The AND was dissolved in hot ethanol (5 mg/ml) and 5 µl were used for each spot. A precoated plate of silica gel 60F254 (Merck, Kilsyth, VIC) and mobile phase (chloroform: methanol: ethyl acetate 8: 1.5: 1) were used. The

extracts fractionated by TLC were detected by UV radiation (Electronic UV Transilluminator, Quantun-Scientific). The Rf (retardation factor) of AND was 0.58.

3.2.5 Screening for antimicrobial activity

3.2.5.1 Preparation of discs with AP extracts or AND

The dry extracts prepared as described in Section 3.2.2, were dissolved as per their preparation details above (Section 3.2.4) and diluted with distilled water to obtain concentrations at 100 mg/ml, 10 mg/ml or 1 mg/ml (w/v) to determine which of these concentrations had an inhibitory effect on bacterial growth. AND was firstly dissolved with ethanol and then diluted with distilled water to obtain concentrations of 10 mg/ml, 1 mg/ml or 0.1 mg/ml (w/v). Fifty µl of each prepared dilution was added to each 6 mm diameter sterilized disc (Whatman, England). After adding the dilutions, the discs that had the equivalent to 5 mg, 0.5 mg or 50 µg of extracts, and 0.5 mg, 50µg or 5 µg of AND per disc, were placed in an oven and dried at 55°C overnight. The discs with 50 µl of 80% ethanol were also prepared in the same way as a control to account for any inhibition caused by any residual ethanol remaining after drying of the disc overnight.

3.2.5.2 Preparation of bacterial culture media

3.2.5.2.1 Culture media for *E. coli, S. typhimurium, S. bovis-morbificans, S. sonnei, S. aureus* and *P. aeruginosa*

E. coli, S. typhimurium, *S. bovis-morbificans*, *S. sonnei*, *S. aureus* and *P. aeruginosa* were grown on LB agar and broth. LB broth and agar were prepared according to the manufacturer’s instructions (Difco, Detroit, MI).

3.2.5.2.2 Culture media for *S. pneumoniae and S. pyogenes*

S. pneumoniae, S. pyogenes were grown on horse blood agar. 100ml of LB agar was prepared according to the manufacturers’ instruction (Difco, Detroit, MI). After sterilization at 121°C for 15 mins, the molten agar was allowed to cool to 50°C in a water-bath. 10 ml of sterile defibrinated horse blood was then added and mixed gently to avoid bubbles, and the agar mix was then poured into petri dishes.

3.2.5.2.3 Culture media for *L. pneumophila*

L. pneumophila was grown on Legionella CYE agar with BCYE growth supplement (Oxoid, Adelaide, SA). 2.5 g Legionella CYE agar base in 90 ml of distilled water was sterilized at 121°C for 15 mins. The sterile agar base was allowed to cool to 55°C and the contents of one vial (in 10 ml sterile distilled water) of Legionella BCYE growth supplement was added aseptically. The agar base and the supplement were gently mixed and then poured into sterile petri dishes.

3.2.5.2.4 Culture media for *B. pertussis*

B. pertussis was grown on charcoal agar. The charcoal agar was prepared according to the manufacturer's instruction (Difco, Detroit, MI). Briefly, 6.25 g charcoal agar base in 100 ml of distilled water was mixed thoroughly and boiled, with frequent agitation, for 1 min to completely dissolve the powder. After sterilizing at 121°C for 15 mins, the sterile agar base was mixed thoroughly and then poured into sterile petri dishes.

3.2.5.3 Preparation of bacteria

The bacteria were grown in petri dishes with appropriate agar. *S. pneumoniae* and *S. pyogenes* were grown on blood agar plates (see Section 3.2.5.2.2). *L. pneumophilia* was grown on legionella cye agar with BCYE supplement (see Section 3.2.5.2.3). *B. pertussis* was grown on charcoal agar (see Section 3.2.5.2.4). All other micro-organisms were grown on LB agar (see Section 3.2.5.2.1). The inoculum suspensions were prepared in 0.9% saline to an equivalent 1 McFarland Standard. Alternatively the suspensions were adjusted to an absorbance of 0.26 using a spectrophotometer set at wavelength of 640 nm (OD_{640} = 0.26). 0.1 ml of bacterial suspension was spread over the entire agar surface using a sterile swab. The plate was allowed to dry for 5 mins before placing the tested discs on the surface.

3.2.5.4 Determination of antimicrobial activity

The antibacterial activity of AND and each AP solvent extract was analyzed by disc diffusion susceptibility methods as described elsewhere by Navarro et al. (1996) or Dulger and Gonus (2004). The discs with extracts or AND were placed on the agar plates on which the bacteria were spread, followed by incubation at 37°C for 18 hours (the plates of *L. pneumophilia,* and *B. pertussis* were incubated for 3 days in a humid atmosphere). The results were interpreted using zone diameter of inhibition for susceptible strains. The diameters of inhibition zones

(clear zones) around the disc were measured in mm. Select reference antibiotic discs were used as positive controls, depending on the test micro-organisms. 80% ethanol soaked and dried discs were also used as a control.

The results were measured for medicinal plants as described by Dulger and Gonus (2004).

Resistant:	=< 10 mm	(-)
Moderately susceptible:	10-13 mm	(+)
Susceptible:	> 14 mm	(++)

3.3 Results

3.3.1 The recoveries of AP extract

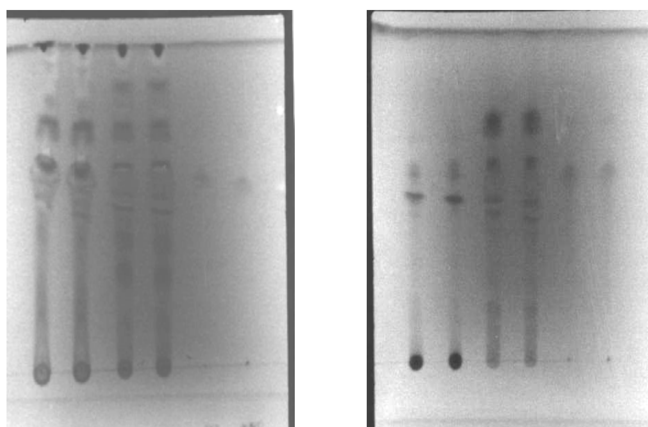
The following AP extracts recoveries were obtained (Table 3-1).

Table 3-1 AP extracts and their recoveries

Extract	Recovery
Absolute ethanol AP extract (100% APE)	9.8 %
80% ethanol extract (80% APE)	6.0 %
Water extract (WE)	5.7 %

3.3.2 Detection of the AP extracts constituents

The thin layer chromatographs of the AP ethanol and aqueous extracts are shown in Figure 3-1. The water extract showed two major spots in a lane whereas all the ethanol extracts showed more than 3 spots in a lane. The spot representing AND (Rf =0.58) was present in all the three AP extracts. The colour associated with the APE extracts was removed by absorption with activated charcoal (Sigma, St Louis, MO).



Lane 1 2 3 4 5 6 1 2 3 4 5 6

A B

Figure 3-1 Thin layer chromatography of AP extracts

From left to right, A: lanes 1 & 2: 80% APE; lanes 3 & 4: 100% APE; lanes 5 & 6: AND. B: lanes 1 & 2: water extract; lanes 3 & 4: APE; lanes 5 & 6: AND

3.3.3 Antibacterial susceptibility profiles

None of the concentrations at 5 mg/disc, 0.5 mg/disc or 50μg/disc of AP extracts showed any antibacterial activity against *S. typhimurium* (bovine, 12313), *E. coli* (ATCC 25922), *E. coli* (Q 358), *S. typhimurium* (*area* SL3261), *S. bovis-morbificans* (sheep, liver), *S. sonnei*, *S. aureus, P. aeruginosa, S. pneumoniae* or *S. pyogenes*. However, the concentrations of 5 mg/disc, 0.5 mg/disc and 50μg/disc of 80% APE and 100% APE showed antibacterial activity against both *L. pneumophila* and *B. pertussis* (Table 3-2). The tested concentrations of AND showed no antibacterial activity towards any of the pathogens mentioned above. Antimicrobial activities of select antibiotics used as positive controls are shown in Table 3-3.

Table 3-2 Antimicrobial activity of AND or different AP extracts

(inhibition zone: mm diameter)

Micro-organisms	AND	Aqueous extract	80% ethanol extract	100% ethanol extract	Overnight dry of 80% ethanol
1. *S. typhimurium* (*aroA*, SL3261)	-	-	-	-	-
2. *S. typhimurium* (bovine,12313)	-	-	-	-	-
3. *E. coli* (ATCC 25922)	-	-	-	-	-
4. *E. coli* (Q 358)	-	-	-	-	-
5. *S. bovis-morbificans*	-	-	-	-	-
6. *Shigella sonnei*	-	-	-	-	-
7. *S. aureus*	-	-	-	-	-
8. *P. aeruginosa*	-	-	-	-	-
9. *S .pneumoniae*	-	-	-	-	-
10. *S. pyogenes*	-	-	-	-	-
11. *L. pneumophila*	-	-	++22 (5mg/disc) ++22(0.5mg/disc) ++22(50µg/disc)	++16 (5mg/disc) ++16(0.5mg/disc) ++16(50µg/disc)	-
12. *B. pertussis*	-	-	++18(5 mg/disc) +14(0.5mg/disc) +12(50µg/disc)	++12(5 mg/disc) +12(0.5mg/disc) -10(50µg/disc)	-

Note: For medicinal plants, Resistant = < 10 mm diameter zone of inhibition.

Table 3-3 Antimicrobial activity of select antibiotics against common intestinal and respiratory tract pathogens

Micro-organisms	ST	P10	VA30	E15	FOX30	SXT	AM10	GM10
1. *S. typhimurium* (*aroA* SL3261)	-	13	-	-	14	18	19	17
2. *S. typhimurium* (bovine,12313)	18	15	-	-	19	22	20	15
3. *E. coli* (ATCC 25922)	20	-	-	-	21	24	18	17
4. *E. coli* (Q 358)	-	-	-	9	24	29	25	16
5. *S. bovis-morbificans*	16	14	-	-	19	21	19	17
6. *Shigella sonnei*	10	-	-	-	-	20	-	15
7. *S. aureus*	10	28	9	18	14	-	22	9
8. *P. aeruginosa*	7	-	-	-	-	-	-	16
9. *S. pneumoniae*		34	18		16	15	22	
10. *S. pyogenes*		24	13	18	15	-		
11.*L.pneumophilia*				40	31	27	12	20
12. *B. pertussis*				30	22		26	22

Note: ST: Streptomycin (100μg), P10: Penicillin G (10IU), VA30: Vancomycin (30μg), E15: Erythromycin (15μg), FOX30: Cefoxitin (30μg), SXT: sulfamethoxazole/trimethoprim (23.75:1.25μg), AM10: Ampicillin (10μg), GM10: Gentamicin (10μg)

3.4 Discussion

Resistant bacteria are emerging world wide as a threat to the outcome of common infections in community and hospital settings. Resistance of *Salmonella sp* to streptomycin, tetracycline, ampicillin, chloramphenicol, sulfamethoxazole, ceftiofur, gentamicin, nalidixic acid, penicillin and cephalosporin have been reported (Zhao et al., 2005; White et al., 2003; Chaudhary and Aggarwal, 2004). Resistance to penicillin, macrolide and cephalosporin is also detected among the leading bacterial pathogens that cause respiratory tract infections, such as *S. pneumoniae, H. influenzae* (Dunbar, 2003; Lorenz, 2002). In the antibiotic control test, *S. typhimurium* (*aroA* SL3261), *S. typhimurium* (bovine, 12313) and *S. bovis-morbificans* were observed to resist to 5 tested antibiotics (ST, P10, VA30, E15 and GM10) as judged by the diameter zone of inhibition less than 18 mm. Resistance to 2 tested antibiotics (FOX30 and SXT) was also observed in strain *S. pneumoniae*. *P. aeruginosa* was shown to resist almost all 8 tested antibiotics. Bacteria become drug resistant in several different ways. Pathogens often become

resistant simply by preventing entrance of the drug (Prescott et al., 2002). Many gram-negative bacteria are unaffected by penicillin G because it can not penetrate the envelope's outer membrane (Prescott et al., 2002). Some pathogens, such *E. coli*, *P. aeruginosa* and *S. aureus*, have plasma membrane translocases that pump the drug out of the cells after it has entered (Prescott et al., 2002). Many pathogens produce β-lactamase to hydrolyse the β-lactam ring of many types of penicillin (Jain et al., 2003; Chaudhary and Aggarwal, 2004). β-lactamase has been found in a variety of *Enterobacteriaceae species*, including *Klebsiella* spp., *Escherichia coli*, *Enterobacter* spp., *Salmonella* spp., *Pseudomonas aeruginosa* and *Proteus mirabilis* etc. (Nathisuwan et al., 2001; Chaudhary and Aggarwal, 2004).

In the experiment, the direct antibacterial activities of AP extracts and one of its active compounds, andrographolide were determined using the disc diffusion test. The advantages of the disc diffusion are cost effectiveness and flexibility. It is the least expensive method and is very flexible because the selection of the tested discs is done by the user. Another benefit is that the method is quick to determine anti-microbial properties. In the disc diffusion susceptibility test, discs containing known amount of an antimicrobial agent are placed on the surface of an agar plate which has been inoculated with a standardized suspension of a strain of bacteria. The agent diffuses from paper into the agar, thereby preventing the growth of the organism in a zone around the disc. The zone diameter gives an indication of the sensitivity of the organism to the agent being tested (Prescott et al., 2002). However, this method has also a few sources of error. An important source of error in susceptibility testing is that certain bacterial species may not grow well in a particular culture medium. Therefore, the appropriate culture media were used for bacterial growth instead of using Muller-Hinton Agar. Another commonly used screen to determine antimicrobial susceptibility is the broth dilution assay (Hess et al., 1995). This method is difficult because the compound be tested must be diluted several times for each sample, so it is time-consuming and is more expensive than the disc diffusion test. Therefore, the disc diffusion test was chosen in the antibacterial activity test.

AP is an herbal medicine that has been used for therapy of respiratory tract infection as well as acute diarrhoea with a reported efficacy of 75-100% in Thailand (Leelarasamee et al., 1990). To investigate whether anti-bacterial activity was responsible for the reported therapeutic successes of AP, direct anti-bacterial activity of AP extracts and AND was determined using disc diffusion tests. Direct anti-bacterial activity of two ethanolic *Andrographis paniculata* extracts against *Legionella pneumophila,* and *Bordetella pertussis* was observed. However, no direct anti-microbial activity of the aqueous extract of AP and AND against the other microorganisms was observed, including *S. typhimurium*, *E. coli*,

S. sonnei, *S. aureus, P. aeruginosa*, *S .pneumoniae, S. pyogenes, L. pneumophila,* and *B. pertussis*.

The results obtained with the aqueous extract of AP did not support the findings reported by Prajjal et al. (2003) with respect to *S. aureus*, *E. coli* or *P. aeruginosa*. Although it is possible that the lack of antimicrobial activity of the aqueous extract in our study may be attributable to a lesser concentration of the aqueous extract used per disc than that used by Prajjal et al. (2003) viz., 5mg/disc instead of 10mg/disc, this is unlikely because (a) no zone of inhibition against the pathogens tested was observed in this study and (b) Leelarasamee et al. (1990) also did not observe any antimicrobial activity of the aqueous extract either *in vitro* against *Salmonella*, *E. coli*, *Shigella*, *S. aureus* even at concentrations as high as 25 grams/litre of AP powder suspended in water.

Although APE or AND did not show any direct antibacterial activity against intestinal pathogens tested in this study, extracts of AP have been claimed to have significant effects against the diarrhoea associated with *E. coli* infections using rabbit as the model system (Gupta et al., 1990). Yin & Guo (1993) found that a dose of 500 mg per day for six days of AND was effective in controlling or treating acute bacterial diarrhoea in human patients. In another study, AP was used to treat 1611 cases of bacterial dysentery and 955 cases of diarrhoea with overall effectiveness of 91.3% (Chaturvedi et al., 1983). However, we discovered that AP had no direct bacteriostatic or potential bactericidal reaction against *S. typhimurium* (bovine, 12313), *E. coli* (ATCC 25922), *E. coli* (Q 358), *S. typhimurium* (*aroA* SL3261), *S. bovis-morbificans* or *S. sonnei*.

Andrographis paniculata has also been used clinically for symptomatic treatment of the common cold and uncomplicated sinusitis, pharyngotonsillitis, pneumonia and bronchitis (Chang and But, 1986; Hancke et al., 1995; Caceres, 1997; Melchior et al., 1997; Thamlikitkui et al., 1991; Melchior et al., 2000; Poolsup et al., 2004; Amaryan et al., 2003; Gabrielian et al., 2002; Spasov et al., 2004 and Coon & Ernst, 2004). Chinese clinical studies involving oral administration of AP or AND to patients suffering from bacterial and viral respiratory infections reported positive effects. Details were described in Section 1.3.5.2. We found that ethanoic AP extracts, but not AND, were inhibitory *in vitro* only for those respiratory pathogens that were associated with bronchitis or pneumonia viz., *L pneumophila* and *B. pertussis* respectively. It is thus clear that a component/principle other than AND may be responsible for the previously reported antimicrobial activity. Research aimed at identifying the component(s) responsible for antimicrobial activity against a common intestinal pathogen tested in this investigation is reported in the following two chapters. The hypothesis is that the antimicrobial activity claimed in the use of AP against microbial infections *in vivo* may be due to the immunomodulatory effects of an AP extract and one of its active compounds including AND. As it has been suggested that the

main clinical benefit of AP could be due to a possible immune-enhancing effect (Mills & Bone, 2000).

The constituents of AP extracts were detected using TLC with UV radiation. From figure 3-1, AND is contained in all tested extracts of AP, no matter whether water or ethanol was used as extract solvents. TLC is the simplest and cheapest method of detecting plant constituents because the method is easy to run, reproducible and requires little equipment. Although two spots from the water extract and more than 3 spots from other extracts of AP were detected in TLC, there may be other constituents which could not be detected under UV radiation in the extracts.

3.5 Conclusions

Neither the aqueous extract nor AND were bacteriostatic or bactericidal against *Salmonella typhimurium*, *E. coli, Shigella sonnei, S. aureus, P. aeruginosa*, *S. pneumoniae, S. pyogenes, L. pneumophila* or *B. pertussis*, but the two ethanolic extracts of AP were bacteriostatic against *L. pneumophila* and *B. pertussis*. Although active constituents in AP have the capability to reduce the symptoms of upper respiratory tract infection as well as acute diarrhoea in human (Chapter 1, Sections 1.3.5.1 and 1.3.5.2), the effects seem to be more then a direct antibacterial effect. It has been suggested that the main clinical benefit of AP could be due to a possible immune-enhancing effect (see Chapter 1, Section 1.3.5.6). In the next two chapters, investigations of the immunomodulatory effects of an AP extract and one of its active compounds, AND using an immunized mouse model are reported.

Chapter 4 Evaluations of immunomodulatory properties of ethanol extract of *Andrographis paniculata* or andrographolide

4.1 Introduction

Traditional herbal medicines have been widely used for the treatment of many kinds of acute and chronic diseases in Asia. Mills and Bone (2000) suggested that the clinical efficacy of many herbal traditional medicines could be partially attributable to their immunoregulatory properties. In recent years, more and more researchers have become interested in discoveries of potent immunostimulant or immunosuppressive compounds isolated from herbal medicines. Most of these investigations have focussed on herbal products stimulating or suppressing innate immunity as assessed by cytokine induction by naïve lymphocytes *in vitro* (Tega et al., 2005; Ko et al., 2004a; Ko et al., 2004b; Nakada et al., 2002; Huang et al., 2001; Chen et al., 2005; Kang et al., 2005; Kang et al., 2004; Huang et al., 2004). Extracts of whole plants of *Andrographis paniculata* have been reported to have anti-cancer, anti-inflammatory, anti-allergic, immunostimulatory, antithrombotic, hypoglycaemic and hepatoprotective activities (Gupta *et al*., 1998; Habtemariam S, 1998; Handa & Sharma, 1990; Matsuda *et al*., 1994; Puri *et al*., 1993; Trivedi & Rawal, 1998; Zhao & Fang, 1991; Zhang & Tan, 1996 & 2000). Andrographolide has been reported to be anti-inflammatory, to stimulate or suppress innate immunity as assessed by cytokine induction or deduction and anti-carcinogenic activity (Kumar et al., 2004; Madav et al., 1996; Panossian et al., 2002; Burgos et al., 2005; Rajagopal et al., 2003; Shen et al., 2002). It has also been reported to either stimulate (Puri et al., 1993) or suppress adaptive immunity (Iruretagoyena et al, 2005; 2006). This experiment was designed to investigate the effects of APE and AND on both humoral and cell mediated immunity in mice vaccinated with inactivated *Salmonella typhimurium*. Mice immunised with an inactivated *Salmonella typhimurium* vaccine have been previously reported to induce only humoral (antibody) response but little or no cell-mediated immune response or immunity (CMI) in the mouse model (Mukkur et al., 1987; 1991a; 1991b; Harrison et al., 1997), although in chickens, a killed *Salmonella enteritidis* vaccine was reported to induce splenocyte lymphocyte proliferation following stimulation with ConA (a T–cell mitogen) apparently in an age-dependent manner (Okamura et al., 2004; 2003). The mouse model was chosen for this investigation

because of the demonstrated inability of the killed salmonella vaccines to induce CMI in this model.

4.2 Materials and Methods

4.2.1 Preparation of an ethanol extract of *Andrographis paniculata*

200g of whole dried plant coarse powder (purchased from Woods plus Woods Co. Ltd., Sydney, Australia) was percolated with 100% food grade ethanol twice (1×700ml and 1x200 ml) and each time soaked and stirred for 6 hours at room temperature. After filtration through cotton wool, the filtrates were centrifuged at 10000×g for 20 mins at room temperature (Beckman Avanti J251 centrifuge, Beckman Instruments, Palo Alto, CA). The extract was evaporated in a rotary evaporator (Labrota 4001, Heidolph) at 60°C until dry. A stock solution for the AP extract (APE) was prepared by suspending APE in food grade ethanol at 50 mg/ml, which was then serially diluted in PBS immediately prior to experiments.

4.2.2 Isolation of andrographolide

A modification of the method reported by Rajani et al. (2000) was used to isolate AND from AP (as described in Chapter 2, Section 2.2.4). The isolation of AND was further confirmed by UV absorption spectrum, TLC, LC-MS and ^{1}H-NMR (as described in Chapter 2, Section 2.3.4).

4.2.3 Animals

All experiments with laboratory animals were carried out to comply with the Animal Care and Protection Act 2001 and approved by the Animal Ethics Committee of the University of Southern Queensland (approved number: 04STU235). Six week old female Balb/c mice were purchased from the Animal Resource Centre, (Perth, Australia). A one-week acclimatization period was allowed for all mice prior to any experimentation. The animals were maintained in specific pathogen free conditions in the PC2 Animal House at the University of Southern Queensland and housed with 12 hours of light and 12 hours of darkness in a controlled temperature (23±1°C). Two mice groups were used. One group was administered one dose of vaccine and treated with APE or AND for 14 days. Another group was given two doses of vaccines and treated with APE or AND for 28 days (as described in Sections 4.2.4 and 4.2.10 below).

4.2.4 APE and AND treatment

The treatment started 1 week before the (primary) immunization and continued through all the experiment (for treatment periods see Section 4.2.3). Mice were orally given a daily dose equal to 25 mg/kg bodyweight (bw) and 50 mg/kg bw of APE in 250 µl PBS or 1 mg/kg bw and 4 mg/kg bw of AND in PBS using a flexible plastic gavage (plastic sheath of 24 G3/4, Insyte*TM* Catheter, BD). The first day of APE or AND treatment was recorded as day 1. At the doses used, APE and AND were well tolerated by mice, and no apparent evidence of toxicity was observed. As controls, age-matched female mice were immunized with killed *S. typhimurium* alone or were provided with PBS alone. Therefore, for each mouse group, there were the following six sub-groups (each sub-group containing 5 mice):

(1) PBS group: Mice in this group were provided with PBS alone.
(2) Vaccine group: Mice were immunized with 1 or 2 dose(s) of whole-cell killed *S. typhimurium* alone on day 7 or days 7 and 21.
(3) 25APE + Vaccine group: Mice were treated with 25 mg/kg bw of APE and immunized with 1 or 2 dose(s) of whole-cell killed *S. typhimurium*.
(4) 50APE + Vaccine group: Mice were treated with 50 mg/kg bw of APE and immunized with 1 or 2 dose(s) of whole-cell killed *S. typhimurium*.
(5) 1AND + Vaccine group: Mice were treated with 1 mg/kg bw of AND and immunized with 1 or 2 dose(s) of whole-cell killed *S. typhimurium*.
(6) 4AND + Vaccine group: Mice were treated with 4 mg/kg bw of AND and immunized with 1 or 2 dose(s) of whole-cell killed *S. typhimurium*.

4.2.5 Reagents and culture media

A stock solution for AND was prepared by dissolving AND in food grade ethanol at 5 mg/ml, which was then serially diluted in PBS immediately prior to experiments. Goat anti-mouse IgA (α-) HRP conjugate, goat anti-mouse IgG (Fc) HRP conjugate, o-phenylenediamine dihydrochloride (OPD) peroxidase substrate and concanavalin A (ConA) were purchased from Sigma (St Louis, MO). Defibrinated horse blood was sourced from Progen Pty Ltd (Brisbane). LB (Luria-Bertani) Miller and LB agar Miller were purchased from Difco Pty Ltd (Detroit, MI). Dulbecco's Modified Eagle Medium (DMEM), penicillin-streptomycin, fetal bovine serum and HEPES buffer solution (1M) were purchased from Gibco (Invitrogen Corporation, Auckland, NZ). Polymyxin B was from Sigma (St Louis, MO) and 2-mercaptoethanol was from BDH (Merck Pty Ltd, Kilsyth, VIC). Details regarding the sourcing of chemicals are provided in appendix K. Enzyme-linked immunosorbent assay (ELISA) kits were used for IL-4, IL-2 and IFN-γ (R & D Systems, USA) determination (as described in Section 4.2.15).

4.2.6 Bacteria

The avirulent *S. typhimurium* (*aroA* SL3261) (obtained from stock in the Department of Biological and Physical Sciences, University of Southern Queensland) was used in these experiments.

4.2.7 Preparation of SL3261 (whole-cell killed) vaccine for immunization

Avirulent *S. typhimurium aro*A strain SL3261 was used to prepare the inactivated whole-cell vaccine. A single colony was picked up from an LB agar plate grown with *S. typhimurium* and inoculated in 10 ml LB broth, then incubated with agitation overnight (o/n) at 37°C at 200rpm (Bioline Shaker Incubator, Edwards Instrument Co, Australia).1 ml o/n culture was transferred to 200ml LB broth and incubated with agitation at 37°C at 200rpm until an optical density at 600 nm reached 1.2 (OD_{600}=1.2).

A series of dilutions was prepared in LB broth, and dilutions 10^{-4} to 10^{-8} were plated out on LB agars for o/n incubation at 37°C to count the number of colony forming units (cfu) per ml. Formalin (37%) to a final concentration of 1% was added to the culture broth, which was re-incubated with agitation for 4 hours at 37°C at 200rpm. 100 µl of the broth culture was then plated out on LB agar to check for sterility. The remaining broth was centrifuge at 8500xg for 15 mins at room temperature (Biofuge Primo-R, Heraeus, supplied by Radiometer Pacific) and the pellets were washed three times with sterile phosphate buffered saline (PBS, pH 7.2). The final pellets were resuspended in 50ml sterile PBS equivalent to a bacterial count of 1.0×10^{10} cfu/ml, and stored at -20°C and prior to use diluted in PBS.

4.2.8 Preparation of *Salmonella* antigen for ELISA

Avirulent *S. typhimurium aro*A strain SL3261 was used to prepare the antigen for ELISA. *S. typhimurium* strain SL3261 was grown and processed as mentioned in Section 4.2.7 above. After washing three times with sterile PBS, the final pellet was resuspended in 10 ml of sterile borate saline coating buffer (see Appendix D for preparation) instead of in 50 ml sterile PBS and then sonicated on ice six times and each time had 20 second bursts at 60 duty-cycle, output 7 with 20 second pauses (Branson Sonifier 450, Branson Ultrasonics Corporation, Danbury, CT). The cell lysate was further diluted with sterile borate saline coating buffer (120mM NaCl, 50mM H_3BO_4 & 24 mM NaOH, pH 9.0) to OD_{600}=0.4, and stored at 4°C until used.

4.2.9 Preparation of SL3261 lysate for splenocyte stimulation

Avirulent *S. typhimurium aro*A strain SL3261 was used to prepare lysate for splenocyte stimulation. Formalin (37%) to a final concentration of 1% was added to an o/n, stationary 200 ml LB culture of SL3261, which was re-incubated with agitation for 4 hours at 37°C at 200rpm (Bioline Shaker Incubator, Edwards Instrument Co, Australia). 100 µl of the broth culture, was then plated out on LB agar to check for sterility. The remaining broth culture was pelleted at 8500×g for 15 mins (Biofuge Primo-R, Heraeus, supplied by Radiometer Pacific). The pellet was washed once in 40ml PBS containing 5 mM EDTA (ethylene diamine tetraacetic acid) and twice with PBS separately. The pellets were resuspended in 10 ml PBS and sonicated on ice six times and each time had 20 second bursts at 60 duty-cycle, output 7 with 20 second pauses (Branson Sonifier 450, Branson Ultrasonics Corporation, Danbury, CT). Cell disruption was checked by gram stain. The cell lysate was further diluted to one-third volume sterile PBS. The dilution was stored in a sterile tube at -20°C until used.

The protein concentration was determined using Coomassie® Plus *TM* Protein Assay reagent kit (Pierce, Rockford, USA, See Appendix B, Determination of protein concentration).

4.2.10 Immunization

One dose ($1x10^7$ cfu) of whole-cell killed *S. typhimurium* was administered on day 7, whereas, two doses ($1x10^7$ cfu and $5x10^7$ cfu) of whole-cell killed *S. typhimurium* were administered on days 7 and 21 (vaccine preparation see Section 4.2.7 above). Animals received bacteria in 250 µl PBS by the intraperitoneal [i/p] route.

4.2.11 Sampling of mice

Mice were anaesthetized intraperitoneally with a mixture of 40µl ketamine (100mg/ml, Troy Laboratories P/L, Smithfield, NSW) and 40µl xylazine (20mg/ml, Troy Laboratories P/L, Smithfield, NSW). A cardiac puncture (23G ¾ needle, Becton-Dickinson, Eight Mile Plains, QLD) was then performed to obtain the blood sample, followed by cervical cordotomy to ensure rapid and painless euthanasia before collection of the organs.

4.2.11.1 Serum collection

Blood was collected from anaesthetized mice via cardiac puncture in a 23G3/4 needle attached to 1 ml syringe (Becton-Dickinson, Eight Mile Plains, QLD). These were then added to serum collection tubes (Sarstedt, Australian Pty Ltd, Ingle Farm, SA) and clotted on ice for at least 30 mins. The blood was centrifuged at 6100g for 2 mins at room temperature (Mikro 12-24, Hettich Zentrifugen, Tuttlingen). Serum was transferred to a microfuge tube and stored at -20°C.

4.2.11.2 Collection of spleens

After cardiac puncture and cervical cordotomy, the spleen was aseptically removed by cutting through the peritoneal membrane to expose the lower abdominal organs (livers, intestines and spleen). The spleen was removed and placed into a 50ml tube with 5 ml of Dulbecco's Modified Eagle Medium (DMEM) without Fetal Bovine Serum (FBS) (See Appendix C (a)). The tube was left on ice for further preparation. The spleens from each group were placed in the same tube.

4.2.11.3 Collection of small intestines

Small intestine was aseptically removed, finely cut and put into a tube containing 3 ml of PBS with 1mM PMSF and 50mM EDTA solution on ice for preparation of small intestinal washes (see Section 4.2.12).

4.2.12 Preparation of small intestinal washes for determination of anti-*S. typhimurium* IgG and IgA by ELISA

Small intestinal washes were prepared as described by Mukkur et al (1995). Briefly, the finely cut small intestine was put into a tube containing 3 ml of PBS with 1mM PMSF and 50mM EDTA solution on ice. After being vortexed for 2 minutes, the solution with small intestine was centrifuged at 4500g for 10 mins at 4°C (Biofuge Primo-R, Heraeus, supplied by Radiometer Pacific). The supernatant was collected and stored at -20°C until ELISA for IgG and IgA (described in Section 4.2.13).

4.2.13 Measurement of antibody responses by ELISA

The measurement of antibody responses in serum and small intestinal washes by ELISA was mentioned as the following. 100 μl of killed and sonicated *S.*

typhimurium (OD_{600}=0.4) was used to coat wells of a 96-well microtiter plate (Sarstedt, Australian Pty Ltd, Ingle Farm, SA). The plates were incubated o/n at 4°C and washed with PBS-T (PBS containing 0.05% Tween 20) three times. 200 µl PBS-T containing 2.5% skim milk powder block buffer was added and the plates blocked for 1 hour at RT and then they were washed with PBS-T three times. A series of twofold dilutions of mouse sera or small intestinal washes in PBS-T containing 2.5% skim milk powder were added, in 50 µl. A positive control anti- *S. typhimurium* high-titre serum was included on each plate as an internal standard. Plates were incubated for 1 hour at 37°C and washed 4 times with PBST. Antibody isotypes were detected using 50 µl /well diluted horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody, diluted 1/1000 for Ig A and 1/2000 for Ig G in PBS-T containing 2.5% skim milk powder. The plates were incubated for 1 hr at 37°C and washed. The plates were developed with 100µl 0.4 mg/ml OPD substrate in the dark at room temperature for 30 mins. Plates were read at 450 nm on the BIO-Rad 550 microplate reader (Hercules, CA). Antigen-specific titres were calculated as follows. A curve extrapolated by plotting absorbance due to specific binding (raw absorbance minus absorbance of antigen control) against dilution factor. The antibody titre was extrapolated by bisecting the curve with a cut-off point, determined by the maximum absorbance for the non-immunized and non-treated mouse serum. The point at which the curve bisected the cut-off line was extended to the x-axis to obtain a dilution factor titre, the reciprocal of which was considered to be the antibody titre of the serum sample. Titres were then normalized to the internal standard positive control serum titre included in each assay to control for plate-to-plate variations as described elsewhere (Mukkur et al., 1995).

4.2.14 T-cell assay

Spleens were removed in aseptic conditions, placed into 5 ml DMEM without FBS media (See Appendix C (a)) and mashed through a sieve with a syringe plunger. After rinsing thoroughly through a sieve 3 times using 1ml DMEM (without FBS) media each time, the spleen extract was centrifuged at 1000rpm for 10 mins at room temperature (Biofuge Primo-R, Heraeus, supplied by Radiometer Pacific). Supernatants were discarded and the cell pellets were washed once again in 10 ml DMEM without FBS, and resuspended to 1×10^7 cells/ml in DMEM with 10% FBS (see Appendix C (b)). The method for white cell counting was described in Appendix A. 1 ml of cells was then added to each well of a 24 well plate containing 1 ml of culture medium (DMEM media with 10% FBS, see Appendix C (b)). Cells were plated in duplicate (5×10^6 cells/well) and then were incubated with the relevant concentration of antigen [2 µg/ml killed and sonicated *S. typhimurium*; 2 µg /ml ConA (Sigma)] in 5% CO_2, 37°C (Sanyo MCO-20AIC,

Japan). The supernatants were harvested at 72 hours and stored at -20°C prior to use.

4.2.15 Measurement of cytokine production

Interleukin-2 (IL-2), IL-4 or interferon-γ (IFN-γ) were measured by Quantikine^{R} Mouse IL-2, IL-4 and IFN-γ Immunoassay kits (R & D Systems Inc, USA) following the manufacturer's manuals. Briefly, standards were prepared using a series of dilutions of mouse IFN-γ, or IL-2 or IL-4 as per instructions. All samples, standards and controls were aliquoted into the pre-coated plate as per instructions. The plate was allowed to incubate for 2 hours at room temperature, followed by 5 washes using the wash buffer provided by the manufacturer. Mouse IFN- γ, or IL-2 or IL-4 conjugate provided was added to the appropriate plate and then the plate was incubated for 2 hours at room temperature, followed by 5 washes using the wash buffer provided. The plate was then incubated with substrate solution provided by the manufacturer for 30 mins in the dark. The stop solution was added and the plate was read at 450 nm on the BIORad 550 microplate reader (Hercules, CA). A standard curve was plotted from the standard data, and the concentration for samples and standards extrapolated. Each sample was analyzed in duplicate.

4.3 Statistical Analysis

All statistical analysis was conducted using SPSS 12.0 for Windows™. The data was analysed using one-sided, independent samples t-tests, comparing each group to the vaccine group. A value of $p < 0.05$ was considered significant.

4.4 Results

4.4.1 Antigen specific antibody responses in mice treated with APE and AND for 14 days

4.4.1.1 Analysis of serum antibody responses by ELISA

Mice immunized with one dose of whole-cell killed *S. typhimurium* (mentioned in Section 4.2.10) were treated with or without APE at doses of 25 and 50mg/kg bw or AND at doses of 1 and 4 mg/kg bw for 14 days (see Section 4.2.4). The anti- *S. typhimurium* IgG and IgA titres in sera were determined and calculated (described in Materials and Methods in Section 4.2.13). The anti- *S. typhimurium* IgG levels of APE or AND treated mice were found to increase when compared with the

titres of untreated mice but the enhancement in the titres was not statistically significant (Figure 4-1 and Appendix E.1.2). IgA was also determined in pooled sera (n=5) for each group, but no IgA was detectable in all the groups tested.

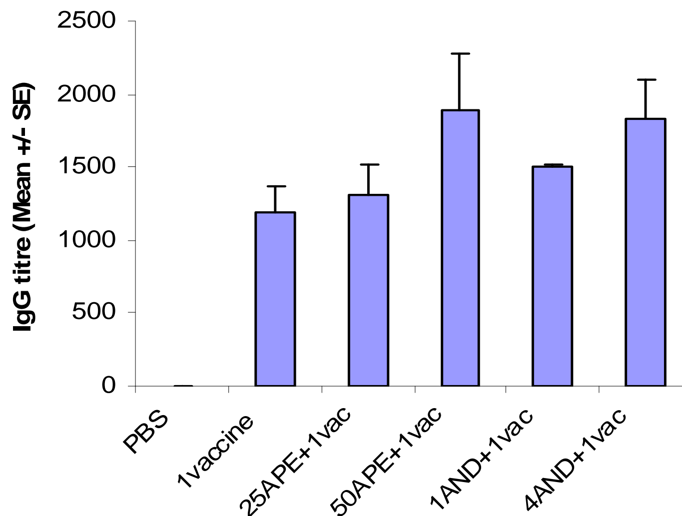


Figure 4-1 Anti-*S typhimurium* serum IgG titres of treated with APE or AND, and untreated mice immunized with one dose of killed *S. typhimurium* vaccine.
All values are means ± standard errors (SE) around the means. PBS: mice treated with PBS. 1vaccine: mice vaccinated with inactivated *S. typhimurium*. 25APE+1vac: mice treated with 25 mg/kg bw of APE and immunized with killed *S. typhimurium*. 50APE+1vac: mice treated with 50 mg/kg bw of APE and immunized with killed *S. typhimurium*. 1AND+1vac: mice treated with 1 mg/kg bw of AND and immunized with killed *S. typhimurium*. 4AND+1vac: mice treated with 4 mg/kg bw of AND and immunized with killed *S. typhimurium*.

4.4.1.2 Analysis of antibody responses in pooled small intestinal washes by ELISA

No antibodies (IgG and IgA) against *S. typhimurium* were detected in pooled small intestinal washes of any of the groups (data not shown).

4.4.2 Antigen specific antibody responses in mice treated with APE and AND for 28 days

4.4.2.1 Analysis of serum antibody responses by ELISA

Mice immunized with 2 doses of whole-cell killed *S. typhimurium* (mentioned in Section 4.2.10) were treated with or without APE at doses of 25 and 50 mg/kg bw

or AND at doses of 1 and 4 mg/kg bw in 250 µl for 28 days (see section 4.2.4). The IgG and IgA titres in sera were determined and calculated (described in Materials and Methods in Section 4.2.13). APE or AND treatment of vaccinated mice, at all given doses elicited significantly higher levels of anti- *S. typhimurium* Ig G than the levels elicited in untreated mice (Figure 4-2 and appendix E.3.2). However no serum IgA was detectable in either the treated or untreated vaccinated mice.

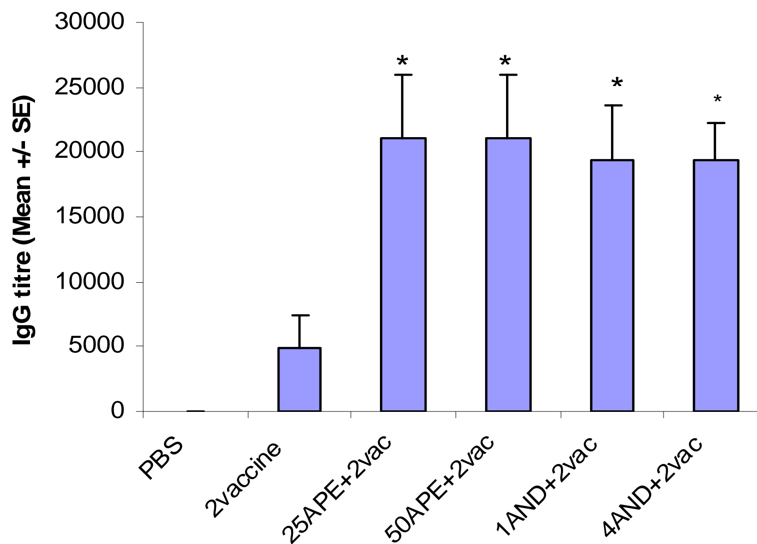


Figure 4-2 Anti- *S. typhimurium* serum IgG titres of treated with APE or AND, and untreated mice immunized with 2 doses of killed *S. typhimurium* vaccine.
Sera were taken from groups of five mice after vaccination with killed *S. typhimurium* by i/p route or treated orally with APE or AND at two different doses for 28 days. All values are means ± standard errors of the means. PBS: mice treated with PBS. 2vaccine: mice vaccinated with 2 doses of inactivated *S. typhimurium*. 25APE+2vac: mice treated with 25 mg/kg bw of APE and immunized with 2 doses of killed *S. typhimurium*. 50APE+2vac: mice treated with 50 mg/kg bw of APE and immunized with 2 doses of killed *S. typhimurium*. 1AND+2vac: mice treated with 1 mg/kg bw of AND and immunized with 2 doses of killed *S. typhimurium*. 4AND+2vac: mice treated with 4 mg/kg bw of AND and immunized with 2 doses of killed *S. typhimurium*. The asterisk * indicates significant difference of IgG titre ($P<0.05$) compared to 2vaccine group.

4.4.2.2 Analysis of antibody responses in pooled small intestinal washes by ELISA

Anti- *S. typhimurium* IgG was weakly detected in pooled small intestinal washes of mice vaccinated with *S. typhimurium* (Figure 4-3). No anti- *S. typhimurium* IgA was detected in pooled small intestinal washes of mice vaccinated with *S. typhimurium* (data not shown).

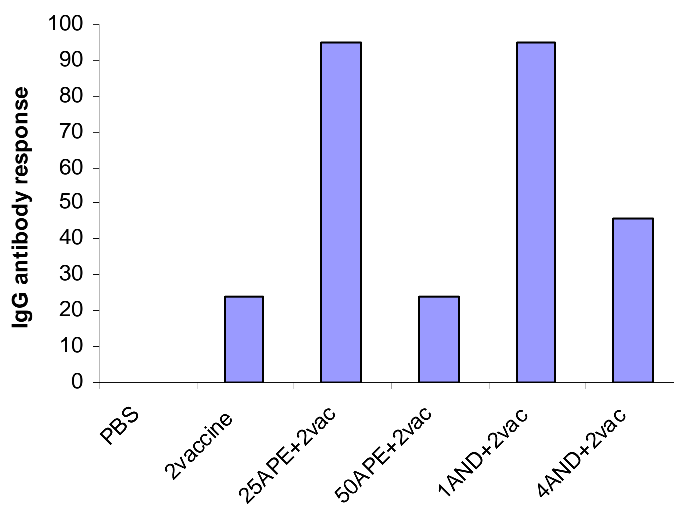


Figure 4-3 IgG response against *S. typhimurium* in pooled small intestinal washings of treated with APE or AND, and untreated mice immunized with 2 doses of killed *S. typhimurium* vaccine.
Pooled small intestinal washes were taken from groups of five mice after vaccination with killed *S. typhimurium* by i/p route or treated orally with APE or AND at two different doses for 28 days.

4.4.3 Antigen specific antibody responses in mice treated with APE for 14 and 28 days

An alternately represented histogram to compare the *Salmonella*-specific antibody response data from mice immunized with one or 2 doses of inactivated *S. typhimurium* vaccine and orally gavaged with APE for 14 or 28 days is shown in Figure 4-4.

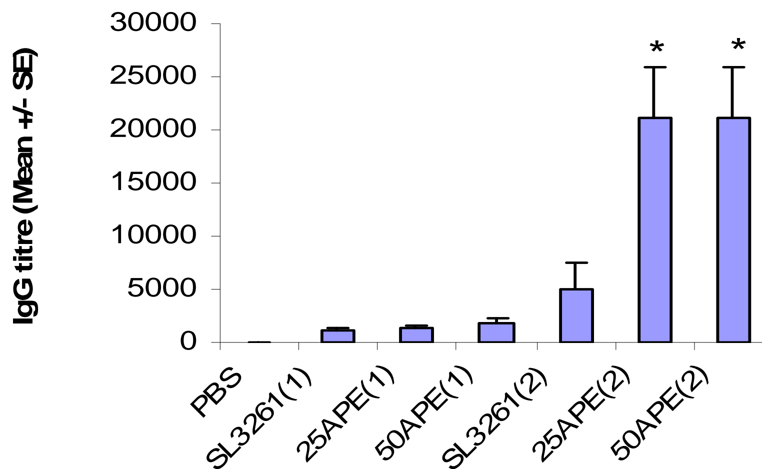


Figure 4-4 Comparison of the *anti-S. typhimurium* serum IgG titres of mice immunized with inactivated *S. typhimurium* and fed APE for 14 or 28 days.
Comparison of the *anti-S. typhimurium* serum IgG titres of mice immunized with one dose (marked as 1 in parenthesis on the X-axis) and 2 doses (marked as 2 in parenthesis on the X-axis) of the inactivated *S. typhimurium* and fed APE for 14 or 28 days respectively. All values are means ± standard errors (SE) around the means. The asterisk * indicates significant difference of IgG titre ($P<0.05$) compared to groups of 25APE (1) or 50 APE (1).

It can be seen that the anti-*S. typhimurium* serum IgG titres in the group of mice immunised with 2 doses of the inactivated *S. typhimurium* (SL3261) vaccine and treated with the APE for 28 days were significantly higher ($p< 0.05$) than in mice immunised with one dose of the inactivated *S. typhimurium* (SL3261) vaccine and orally gavaged with the APE for 14 days (Figure 4-4). However, the serum IgG antibody titres of untreated mice vaccinated with one dose of SL3261 were lower than that of mice vaccinated with 2 doses of the inactivated *S. typhimurium* vaccine although the difference was not statistically significant ($p=0.108$).

4.4.4 Antigen specific antibody responses in mice treated with AND for 14 and 28 days

A differently represented histogram to compare the *Salmonella*-specific antibody response data from mice immunised with one or 2 doses of inactivated *S. typhimurium* vaccine and orally gavaged with AND for 14 or 28 days is shown in Figure 4-5.

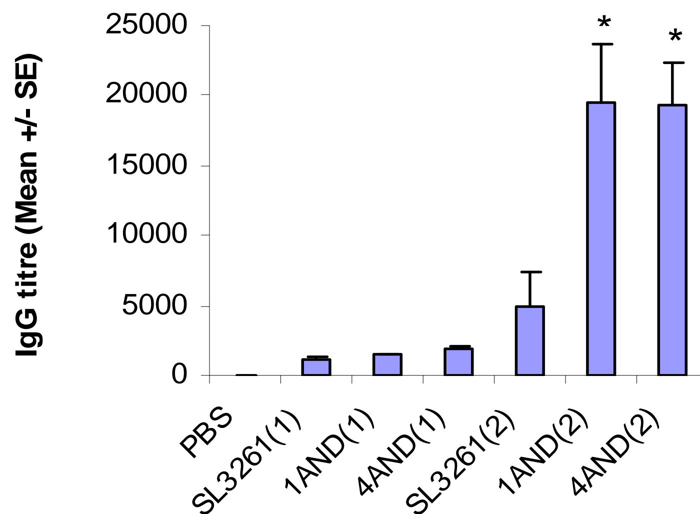


Figure 4-5 Comparison of the *anti-S. typhimurium* serum Ig G titres of mice immunized with inactivated *S. typhimurium* and fed APE for 14 or 28 days. Comparison of the *anti-S. typhimurium* serum Ig G titres of mice immunized with one dose (marked as 1 in parenthesis on the X-axis) and 2 doses (marked as 2 in parenthesis on the X-axis) of the inactivated *S. typhimurium* and fed AND for 14 or 28 days respectively. All values are means ± standard errors (SE) around the means. The asterisk * indicates significant difference of IgG titre ($P<0.05$) compared to groups of 1AND (1) or 4AND (1).

It can be seen that the *S. typhimurium*-specific serum IgG titre of mice treated with AND for 28 days and immunised with 2 doses of the inactivated Salmonella vaccine was significantly higher than that of mice treated with AND for 14 days and immunised with one dose of the inactivated *Salmonella* vaccine ($p<0.05$). As observed with the groups of mice treated with the APE (Figure 4-4), the anti-*S. typhimurium* serum IgG levels of mice immunised with one dose of SL3261 was lower than that of 2 doses of the inactivated *Salmonella* vaccine although the difference was not statistically significant ($p=0.108$).

4.4.5 Effects of 14 days APE or AND treatment on cytokine production in mice immunized with the inactivated *Salmonella* vaccine

The cultured splenocytes of immunized and APE or AND treated groups at all given doses secreted both IFN-γ and IL-4 type cytokines during 72 hour incubation in the presence of sonicated SL3261 (2μg/ml) (Figure 4-6). No IL-2 type cytokine was determined. The cultured splenocytes of immunization control

group (1vaccine) also secreted IFN-γ type cytokine, but IL-4 type cytokine was not detected (Figure 4-6).

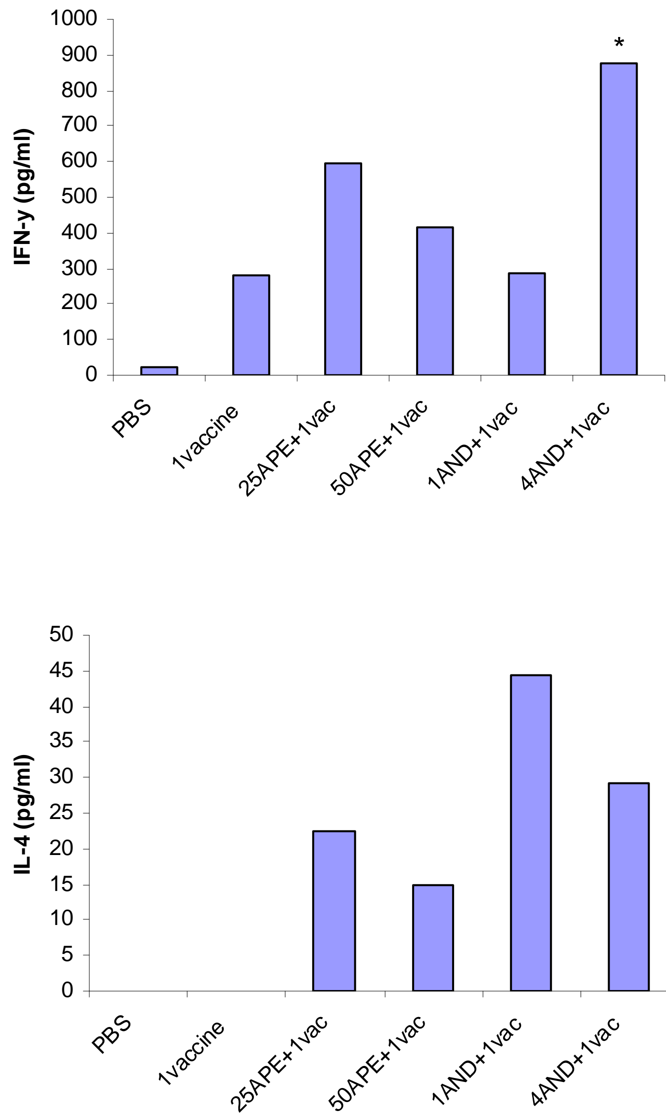


Figure 4-6 IFN-γ and IL-4 production by whole spleen cells after 72 hr stimulation with sonicated SL3261 antigen.
Spleens were removed 14 days after mice had been treated with APE or AND. Pooled spleens from groups of 5 mice were used. The plotted values represent mean values of duplicate wells. The asterisk * indicates significant difference of IFN-γ production ($P<0.05$) compared to 1vaccine group.

4.4.6 Effect of 28 days APE or AND treatment on cytokine production in mice immunized with the inactivated *Salmonella* vaccine

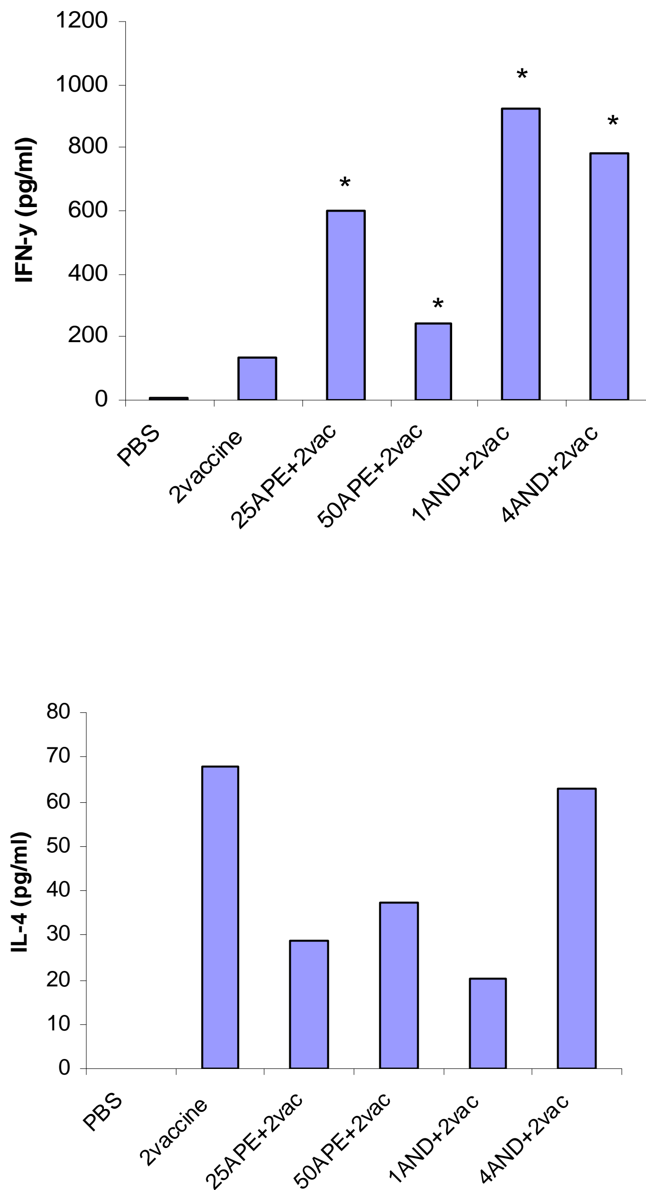


Figure 4-7 IFN-γ and IL-4 production by whole spleen cells after 72 hr stimulation with sonicated SL3261 antigen.

Spleens were removed 28 days after mice had been treated with APE or AND. Pooled spleens from groups of 5 mice were used. The plotted values represent mean values of duplicate wells. The asterisk * indicates significant difference of IFN-γ production ($P<0.05$) compared to 2vaccine group.

The cultured splenocytes with the exception of the PBS control group, secreted both IFN-γ and IL-4 type cytokines upon stimulation with sonicated SL3261 (2μg/ml) (Figure 4-7). All APE or AND treatment of vaccinated mice elicited significantly higher IFN-γ levels than the level elicited in 2vaccine group. No IL-2 was detectable.

4.5 Discussion

Results obtained in this investigation revealed that oral administration of either APE or AND to mice vaccinated with inactivated *S. typhimurium* for 28 days not only significantly enhanced anti- *S. typhimurium* serum IgG titres, but also induced high IFN-γ levels indicating induction of a CMI response. The serum anti-*S. typhimurium* IgG levels of mice vaccinated with 2 doses of the inactivated *Salmonella* vaccine and gavaged with either APE or AND for 28 days were significantly greater than the group of mice immunised with one dose of the inactivated *Salmonella* vaccine by the i/p route and gavaged with APE or AND for 14 days. The serum anti-*S typhimurium* IgG level of mice immunised with 2 doses vaccine was substantially higher than the group of mice receiving only one dose of the inactivated salmonella vaccine although the difference was not statistically significant (p=0.108). These results suggested that the APE or AND treatment period may potentially be an important factor influencing the magnitude of enhancement of antibody levels of immunised animals. However, no serum anti-*S. typhimurium* IgA was detected in any of the treated or untreated groups in this investigation.

Puri et al (1993) reported that feeding mice for 7 days with the ethanol extract of *A. paniculata* or injecting mice with andrographolide at 7 and 3 days prior to immunisation with sheep red blood cells resulted in a significant enhancement of haemagglutinating antibody and delayed type hypersensitivity as compared to untreated animals. These authors also reported that there was a significant enhancement [not induction] of non-specific or innate immune response as measured by an increase in macrophage migration indices, phagocytosis of ^{14}C labelled *E coli*, and increased incorporation of ^{3} H-thymidine in PHA (phytohaemagglutinin) - stimulated splenocyte supernatants. Furthermore it was also reported that administration of pure AND or neoandrographolide by the intraperitoneal route enhanced the haemagglutinating antibody levels and macrophage migration index significantly higher than when these compounds were administered by the oral route. In contrast, Iruretagoyena et al (2005) recently reported that AND administered by the intraperitoneal route down-modulated the antibody response to a thymus-dependent antigen and delayed-type hypersensitivity were drastically diminished in mice by AND treatment. These authors further reported that this molecule was able to interfere with T cell

proliferation and IFN-γ or IL-2 release *in vitro* in response to allogenic stimulation with MOG (mouse myelin oligodendrocyte glycoprotein). Whether the discrepancies observed in the effect of AND on the immune response to different antigens are due to the difference in the route of administration, viz., oral versus intraperitoneal, is not understood and clearly warrants further investigation.

However, results obtained in this investigation showed that both the APE or AND not only increased anti-*S. typhimurium* IgG titres, but also induced CMI to the inactivated salmonella vaccine. Except in the 14 day-1 mg/kg bw-AND treatment group, the IFN-γ levels of all other APE or AND treatment groups viz., 14 day or 28 day treatment groups, increased remarkably compared to the untreated vaccine controls ($P<0.05$) . Therefore, it was concluded that oral administration of APE or AND to vaccinated mice enhanced production of IFN-γ, indicating induction of CMI response potentially via Th1 cells.

The IL-4 levels in the APE or AND treatment groups ranged from 15 pg/ml to 62 pg/ml with the level in the 2 dose vaccine control group being higher than that in the one dose vaccine control group. Essentially no IL-2 was detectable in the splenocyte supernatants prepared from treated or untreated groups after 72 hr stimulation with *Salmonella* antigen.

This investigation differed from that of Puri et al. (1993) in the following respects:

i. Whereas the particulate antigen used in our investigation was an intracellular pathogen (*S. typhimurium*, used as a model for human typhoid for the last nearly 4 decades and that is also responsible for serious economic losses in food-producing animals), Puri et al. (1993) used a non-invasive particulate antigen viz., sheep RBCs in their study, that still required validation in an infectious disease model.

ii. The intracellular pathogen selected for this study did not induce CMI when used as an inactivated or killed vaccine, providing an opportunity to differentiate between induction and enhancement of an immune response to the intracellular pathogen. In the study by Puri et al. (1993), there was at least a basal, but not negative level, of DTH response to SRBCs, and hence their conclusion implying enhancement and not induction of DTH, not necessarily equating to potential protection (Tsukada et al., 1991).

iii. CMI response in our investigation was determined using the indicator cytokine, INF-γ, rather than DTH in order to avoid potential problem associated with the dissociated development of T-cell mediated DTH (IL-2+, IL-3+) versus protective T-cells (IL-2+, IL-3+, INF-γ) as reported for another intracellular pathogen, *Listeria monocytogenes* (Tsukada et al., 1991).

The investigation differed from that of Kumar et al. (2004) by the following facts:

i. This study investigated the antigen-specific induction of the CMI-indicator cytokine, IFN-γ, rather than an enhancement of IL-2 secretion by splenocytes stimulated with a T-cell mitogen viz., PHA.

ii. This study investigated enhancement or induction of antigen-specific antibodies or CMI respectively in vaccinated mice treated with andrographolide, rather than an enhancement of innate immune responses (unvaccinated mice).

The protective efficacy of live attenuated *Salmonella* vaccines in the mouse model has been well demonstrated to be due to both CMI and antibody responses (Viret et al., 1999; Harrison et al., 1997; Mastroeni et al., 1993; Mukkur et al., 1987; Mukkur et al., 1991a, 1991b). Killed vaccines were also observed to generally produce a less broad immune response than live attenuated vaccines in chicken (Babu et al., 2004; Babu et al., 2003; Thatte et al., 1993). Although Okamura et al. (2004; 2003) reported that killed *S. enteritidis* vaccine induced CMI response in chicken; the CMI response is mainly limited to an age-dependent manner. Our results showed that both APE and AND not only enhance the humoral (anti-*S. typhimurium* IgG), but also induced cellular (IFN-γ) immune responses, which are considered to be important for protection against salmonellosis. Whether the enhancement of antibody and cell-mediated immune responses induced by APE or AND in mice vaccinated with inactivated *Salmonella* vaccine is significant enough to protect mice against challenge infection with virulent *S. typhimurium*, the model intracellular pathogen used in this investigation, was further evaluated in work reported in the next chapter.

Rajgopal et al. (2003) reported that AND exerted direct anti-cancer activity on different types of cancer cell lines by cell cycle arrest at G_0/G_1 phase through the induction of inhibitory protein p27 and decreased expression of cyclin-dependent kinase 4 (CDK4), whereas other groups have suggested AND-induced cell death either through the apoptotic pathway, via activation of the caspase cascade (Kim et al., 2005) or regulation of expression of pre-apoptotic markers (Cheung et al., 2005 and Zhou et al., 2006). Rajgopal et al. (2003) and Kumar et al. (2004) also reported immunomodulatory activity of AND *in vitro* and suggested that the latter may indirectly contribute to its anti-cancer activity. On the other hand, other groups have reported immunosuppressant activity of AND *in vitro* (Burgos et al., 2005 and Quin et al., 2006). This study demonstrated that feeding mice with APE or AND and immunizing with the inactivated *Salmonella* vaccine resulted in stimulation of both humoral (anti-*S. typhimurium* IgG) and cellular (IFN-γ) immune responses of acquired immunity.

In conclusion, our results have provided evidence that both the *Andrographis* extract and AND significantly enhance serum anti-*S. typhimurium* Ig G levels and

induce CMI against *Salmonella* antigens as judged by the production of the indicator cytokine IFN-γ in antigen-stimulated splenocyte supernatants, the latter being a correlate of protection against salmonellosis (VanCott et al., 1998; Harrison et al., 1997; Nauciel et al., 1992).

Chapter 5 Protection against experimental salmonellosis of ethanol extract of *Andrographis paniculata* or andrographolide in mice vaccinated with inactive *S. typhimurium*

5.1 Introduction

Protection against infection with an intracellular pathogen involves both humoral and CMI. CMI plays a significant role in protection against salmonellosis (Gupta, 1998; Harrison et al., 1997; Mastroeni et al., 1993; Hahn and Kaufman, 1981; Matsui and Arai, 1992; Collins, 1973). Previous studies (see Chapter 4) carried out revealed that Salmonella-specific antibody response was enhanced and CMI against *S. typhimurium* was induced in mice vaccinated with a killed *Salmonella* vaccine if the vaccinated mice were orally dosed with an ethanol extract of AP or purified AND. The focus of this study was functionally validate the immune response-modifying potential of AP extract or AND against intracellular microbial infections using mouse salmonellosis as a model system. In this study, mice were orally administered with the optimal doses of APE or AND for 28 days and immunized with 2 doses of killed *S. typhimurium* by i/p route. This was followed by oral challenge with virulent microorganisms to evaluate if APE or AND, that were found to enhance humoral immunity and to induce CMI also provided protection against infection with the intracellular pathogen used in this investigation viz., *S. typhimurium*.

5.2 Materials and Methods

The experimental design for this study partly repeated the previous methods used. This time, mice were orally administered with 25mg/kg bw of APE or 4mg/kg bw of AND for 28 days and immunized with 2 doses of killed *S. typhimurium* by i/p route. The other main difference from the previous experiments was that most mice were followed by oral challenge with virulent microorganisms (*S. typhimurium*, ovine strain 12313) and after 28 days treatment with APE or AND. In order to ensure that the stimulation of both humoral (anti-*S. typhimurium* IgG) and cellular (IFN-γ) immune responses of acquired immunity reported in the previous chapter was due to treatment with APE or AND, and not simply to an

additional vaccine booster dose, age-matched female mice administrated with either APE or AND, were also used. Mice were sacrificed pre-challenge as well as post-challenge at 4 or 8 or 12 or 15 days to collect blood or spleen or liver samples for the relevant bio-assay.

5.2.1 Preparation of APE and isolation of andrographolide

The preparation of APE and isolation of AND were described in Chapter 4, Section 4.2.1 and Chapter 2, Section 2.2.4. The identification of AND was described in Chapter 2, Section 2.3.4.

5.2.2 Animals

The same mouse strain, age and maintenance conditions were used as mentioned in Chapter 4, Section 4.2.3, but this time mice were given two doses of the vaccine and treated with APE or AND for 28 days. All experiments with laboratory animals were carried out to comply with the Animal Care and Protection Act 2001 and approved by the Animal Ethics Committee of the University of Southern Queensland (approved number: 04REA252).

5.2.3 APE and AND treatment of mice

Treatments were essentially the same as outlined in Chapter 4, Section 4.2.4 except that only 25mg/kg bw of APE or 4mg/kg bw of AND, single dose/day were used for 28 days. Age-matched female mice, which were administrated with either APE or AND alone, were also used.

5.2.4 Bacteria

The avirulent *S. typhimurium aroA* strain SL3261 (obtained from stock in the Department of Biological and Physical Sciences, University of Southern Queensland) were used for all immunizations, preparation of ELISA antigen and preparation of lysate for splenocyte stimulation. The virulent *S. typhimurium* (strain 12313, oral 1.5×10^{6} cfu or 1.5×10^{5} cfu suspended in 250 µl PBS) was used for challenge.

5.2.5 Reagents and culture media

Reagents and culture media were the same as outlined in Chapter 4, Section 4.2.5.

5.2.6 Preparation of inactivated whole cell SL3261 vaccines for immunization

Avirulent *S. typhimurium aroA* strain SL3261 was used to prepare the inactivated whole-cell vaccine. The preparation was described in Chapter 4, Section 4.2.7.

5.2.7 Preparation of *S. typhimurium* (strain 12313) for challenge

Virulent *S. typhimurium* (strain 12313) was used**.** Ten µl of the stock culture of *S. typhimurium* (strain 12313) (stored at -70°C, Biofreezer, Forma Scientific) was streaked on LB agar and incubated o/n at 37°C. A single colony was picked up from this plate grown with virulent *S. typhimurium* (strain 12313) and inoculated in 10 ml LB broth, then incubated with agitation o/n at 37°C and 200rpm (Bioline Shaker Incubator, Edwards Instrument Co, Australia).1 ml o/n culture was transferred to 200ml LB broth and incubated with agitation at 37°C and 200rpm until an optical density at 600 nm reached 1.2 ($OD_{600}=1.2$).

A series of dilutions (10^{-4}-10^{-8}) prepared in LB broth, were plated out on LB agar plates and incubated o/n at 37°C to count the number of colony forming units per ml. The broth was centrifuged at 8500xg for 15 minutes at 4°C (Biofuge Primo-R, Heraeus, supplied by Radiometer Pacific) and the pellets were washed twice with sterile PBS. The final pellets were resuspended in 50ml sterile PBS and appropriately diluted in PBS. The preparation was made as fresh as possible.

5.2.8 Preparation of *Salmonella* antigen for ELISA

Avirulent *S. typhimurium aro*A strain SL3261 was used to prepare the antigen for ELISA of antibody levels as mentioned in Chapter 4, Sections 4.2.7 and 4.2.8.

5.2.9 Preparation of *S. typhimurium* lysate for splenocyte stimulation

Avirulent *S. typhimurium aro*A strain SL3261 was used to prepare lysate for splenocyte stimulation. The preparation was described in Chapter 4, Section 4.2.9.

5.2.10 Immunization and challenge

For immunization, two doses ($1x10^7$ cfu and $5x10^7$ cfu) of whole-cell killed *S. typhimurium* (on days 7 and 21) were administered in 250 µl PBS by the i/p route.

Groups of 9 mice were challenged by the oral route with a sublethal dose of virulent *S. typhimurium* (strain 12313, $1.5x10^5$ cfu suspended in 250 µl PBS) at 7 days post-booster vaccination according to Mukkur et al. (1987). At days 4, 8 and 12 post-challenge, 3 mice from each group were euthanased for collection of blood sample, small intestine, spleen (described in Chapter 4, Section 4.2.11, except for the spleen from day 12 post-challenge) and liver (described in Section 5.2.11 below). For the day 12 post-challenge, the spleen was removed under aseptic conditions, placed into 1 ml DMEM and mashed through a sieve with a 1 ml syringe plunger. After rinsing though a sieve 3 times using 1 ml DMEM media, 0.1 ml of suspensions were used for enumeration of bacteria in mouse spleen (see in Section 5.2.11). The remaining suspensions were pooled in group for T cell assay (see in Section 5.2.16).

Other groups of 4 mice were challenged by the oral route with $1.5x10^6$ cfu (10 fold of sublethal dose) virulent *S. typhimurium* (strain 12313) suspended in 250 µl PBS (Mukkur et al.,1987) at 7 days post-booster vaccination and survival was recorded for 15 days.

Groups of 5 mice were euthanased for collection of blood and small intestinal wash samples (described in Chapter 4, Sections 4.2.11.1, 4.2.11.3, 4.2.12 and 4.2.13) for measurement of antibody responses and spleens (described in Chapter 4, Sections 4.2.11.2, 4.2.14 and 4.2.15) for measurement of cytokine production before challenge (recorded as day 0 post-challenge or pre-challenge).

An outline of experiment design is tabled as the following:

Group	Treatment	Immunization	No. of mice challenged with strain 12313		
			0 (pre-challenge)	1.5×10^{5} cfu	1.5×10^{6} cfu
PBS	PBS		5	9	4
2vaccine		2 doses	5	9	4
AND	AND		5	9	4
AND+2vaccine	AND	2 doses	5	9	4
APE	APE		5	9	4
APE+2vaccine	APE	2 doses	5	9	4

5.2.11 Enumeration of bacteria in mouse organs

Around ¼ of liver and a whole spleen (except for the 12 day post-challenge; see in Section 5.2.10 above) were removed from each animal and weighed individually in aseptic condition. The liver and spleen (of 4 and 8 day post-challenge) were placed into a sterile 2.0 ml conical screw cap microtube (Quality Scientific Plastics) which contained 0.9 ml LB sterile broth and 1cm depth of 1.0 mm glass beads on ice. The tissues were homogenized using a mini bead beater (Biospec products) at 4600 rpm for 1 min. Each homogenate or 0.1 ml of suspension from Section 5.2.10 was serially diluted (ten-fold) up to 10^{-5} for each time point and plated on LB agar by spread-plate method, and then incubated at 37°C for 18 hours for counting. The colonies in the 80 to 200 range were selected to calculate the number of colonies for the specimen. The number of colonies counted on the selected plate multiplied by the dilution of the specimen contained on that plate equals the bacteria, or CFU (colony-forming units), count per gram of the specimen.

5.2.12 Measurement of the protection-inducing potential of APE or AND

Seven days after post-booster vaccination, groups of 4 mice were challenged orally with $1.5\text{x}10^{6}$cfu (10 fold of sublethal dose) virulent *S. typhimurium* (strain 12313) suspended in 250 µl PBS (Mukkur et al.,1987) and survival was recorded for 15 days. Survival of mice was recorded daily and percentage of live animals at each chosen interval was calculated. Any mice, which became moribund during the experiments, were considered as dead. Mice were euthanased immediately and their spleen and liver were weighed, then bacteria in these organs were enumerated as described previously (see in Section 5.2.11).

5.2.13 Serum collection

Serum was collected as described elsewhere in Chapter 4 (Section 4.2.11.1).

5.2.14 Preparation of small intestinal washes for estimation of anti-*S. typhimurium* IgG and IgA by ELISA

Small intestinal washes were collected and prepared as described in Chapter 4, Sections 4.2.11.3 and 4.2.12.

5.2.15 Measurement of antibody responses by ELISA

The measurement of antibody responses in serum and small intestinal washes by ELISA were described in Chapter 4, Section 4.2.13.

5.2.16 T-cell assay

The processes were essentially the same as described in Chapter 4, Section 4.2.14, except that the remaining suspensions of spleens from Section 5.2.10 were used.

5.2.17 Measurement of production of cytokines in the antigen-stimulated splenocyte supernatants

Levels of interleukin-2 (IL-2), IL-4 or interferon-gamma (IFN-γ) in *Salmonella* lysate-stimulated splenocyte supernatants were estimated using mouse cytokine ELISA kits (R & D Systems, USA) according to the manufacturer's instructions. Each sample was analyzed in duplicate by ELISA. The absorbances were read at 450 nm on the Bio-Rad 550 microplate reader. The final concentration of each sample was calculated by subtracting the concentration of splenocytes stimulated with protein buffer (PBS) from the concentration of splenocytes stimulated with the antigen.

5.2.18 Measurement of average gain (AG) after APE or AND treatment pre-challenge

The initial body weight of 5 mice was recorded per group from the first day of APE or AND treatment. Mouse weight was measured in the morning. AG was calculated as follows:

$$\text{AG} = \frac{Final\ group\ weight - inital\ group\ weight}{Total\ number\ of\ group}(g)$$

5.3 Statistical Analysis

All statistical analysis was conducted using SPSS 12.0 for Windows™. The data was analysed using one-side, independent samples t-tests. A value of $p< 0.05$ was considered significant.

5.4 Results

5.4.1 Antibody isotype titres

Seven week old female Balb/c mice were orally administered with the optimal doses of APE or AND for total 28 days and immunized with 2 doses of killed *S. typhimurium* (on days 7 and 21) by i/p route, followed by oral challenge with 1.5×10^{5} cfu *S. typhimurium* 12313. Antibody isotypes (IgG or IgA) were quantified for serum or small intestinal wash of the treatment groups at days 0, 4, 8 and 12 post-challenge with 1.5×10^{5} cfu *S. typhimurium* 12313. The results are presented in Figures 5-1, 5-2 and 5-3.

5.4.1.1 Analysis of serum antibody responses by ELISA

Levels of anti- *S. typhimurium* IgG for all vaccinated mice increased in 4 days post-challenge, and then reduced in 12 days post-challenge. Low or no serum IgG was detectable among unvaccinated mice in 12 day post-challenge (Figure 5-1). There was no significant change in IgG titres between day 0 and 12 post-challenge (Figure 5-1). It was discovered that neither APE nor AND induced anti-*S. typhimurium* IgG in the pre-challenge experiment. On the other hand, the serum IgA titres were only detectable in vaccinated mice at 4 and 8 days post-challenge (Figure 5-2). No serum IgA was detectable in unvaccinated mice at the tested time points.

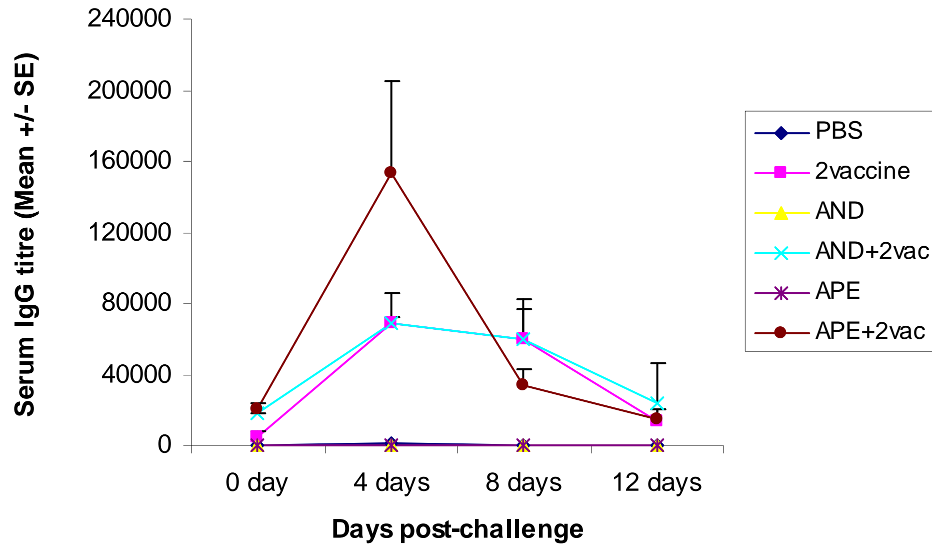


Figure 5-1 Serum IgG ELISA antibody titre in vaccinated mice at 0, 4, 8 and 12 days post-challenge with $1.5x10^5$ cfu *S. typhimurium* 12313.

Sera were taken from groups of 3 mice except for the pre-challenge groups (day 0), which were taken from groups of 5 mice. All values are the means ± standard error. PBS: mice treated with PBS. 2vaccine: mice vaccinated with 2 doses of inactivated *S. typhimurium*. APE+2vac: mice treated with 25 mg/kg bw of APE and immunized with 2 doses of killed *S. typhimurium*. APE: mice treated with 25 mg/kg bw of APE. AND+2vac: mice treated with 4 mg/kg bw of AND and immunized with 2 doses of killed *S. typhimurium*. AND: mice treated with 4 mg/kg bw of AND.

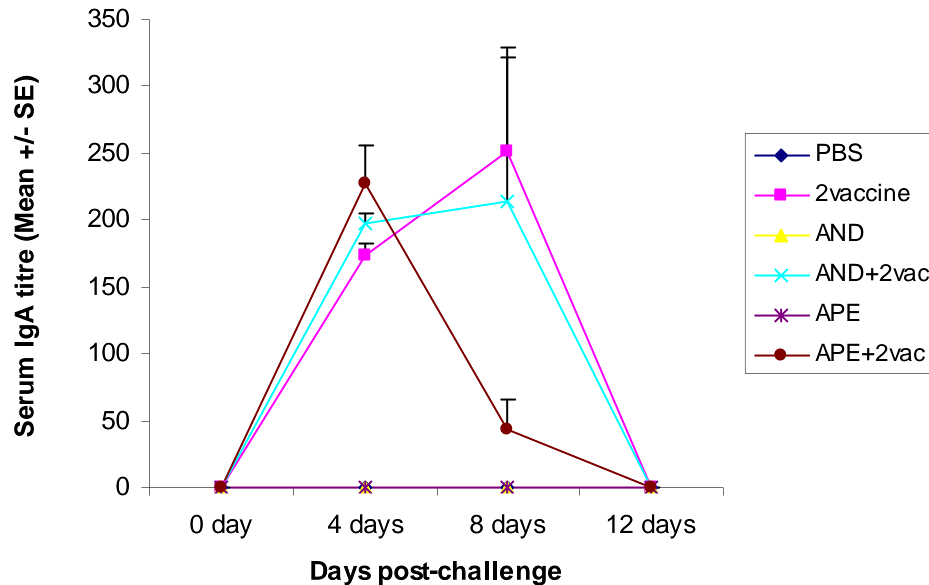


Figure 5-2 Serum IgA ELISA antibody titre in vaccinated mice at 0, 4, 8 and 12 days post-challenge with $1.5x10^5$ cfu *S. typhimurium* 12313.
Sera were taken from groups of 3 mice except for the pre-challenge groups (day 0), which were taken from groups of 5 mice. All values are the means ± standard error.

5.4.1.2 Determination of antibody responses in small intestinal washes by ELISA

No anti-*S. typhimurium* IgA was detected in all mice groups at 0 and 4 days post-challenge. Anti-*S. typhimurium* IgA was detectable in all mice groups at 8 and 12 days post-challenge (Figure 5-3). Anti-*S. typhimurium* IgA for all vaccinated mice increased in 4 to 8 days post-challenge, and then reduced in 12 days post-challenge. The vaccinated mice alone elicited higher level of anti- *S. typhimurium* IgA at 8 days post-challenge than the levels elicited in other groups of mice. On the other hand, levels of anti-*S. typhimurium* IgA for two control groups of mice (PBS and APE) were increased at 12 days post-challenge.

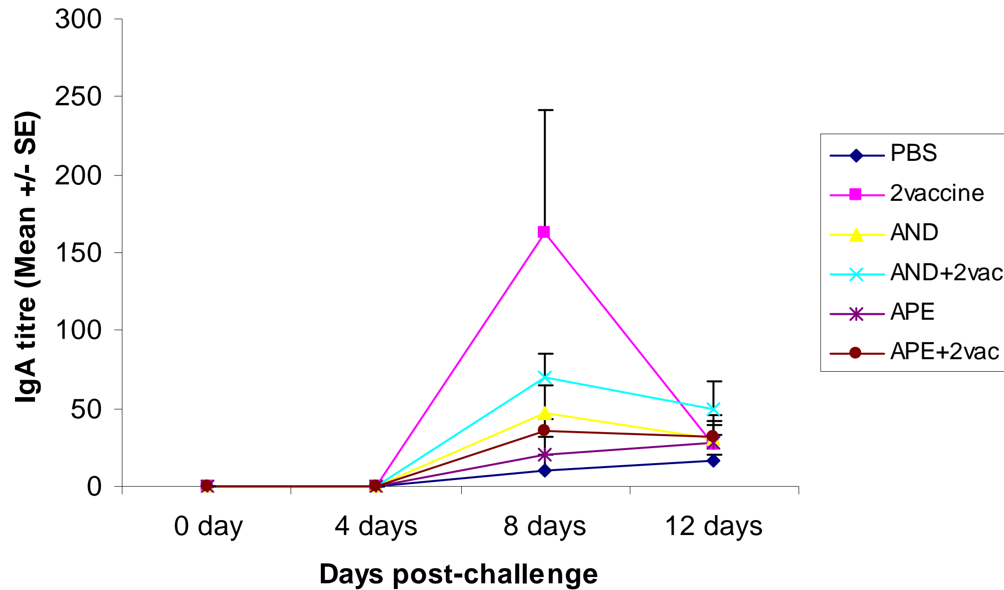


Figure 5-3 Small intestinal washes IgA ELISA antibody titre in vaccinated mice at 0, 4, 8 and 12 days post-challenge with $1.5x10^5$ cfu *S. typhimurium* 12313. Washes were taken from groups of 3 mice except for the pre-challenge groups, which were taken from groups of 5 mice. All values are the means ± standard error

5.4.2 Estimation of interferon-γ (IFN-γ), interleukin (IL-2) and IL-4 levels in the splenocyte culture supernatant

Mice of 12 day post-challenge were sacrificed on day 12 post-challenge with $1.5x10^5$ cfu *S. typhimurium* 12313 and their spleens were collected and processed as described in Section 5.2.16. IFN-γ, IL-2 and IL-4 were determined using pooled splenocyte culture supernatants in duplicate during 72 hour incubation in the presence of *Sallmonella* lysate (2µg/ml).

5.4.2.1 Measurement of IFN- γ levels by ELISA

The IFN-γ results are summarized below in Figure 5-4. The cultured splenocytes secreted IFN-γ type cytokine during 72 hour incubation in the presence of SL3261 lysate (2µg/ml).

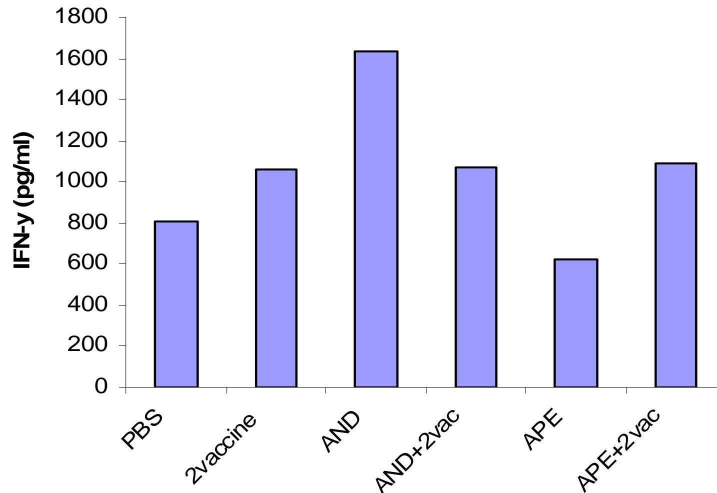


Figure 5-4 IFN-γ production by splenocytes after 72 hour stimulation with the *Salmonella* lysate antigen.

Spleens were removed 12 days post-challenge with $1.5x10^5$ cfu *S. typhimurium* 12313. Pooled spleens from groups of 3 mice were used. The plotted values represent mean values of duplicate wells.

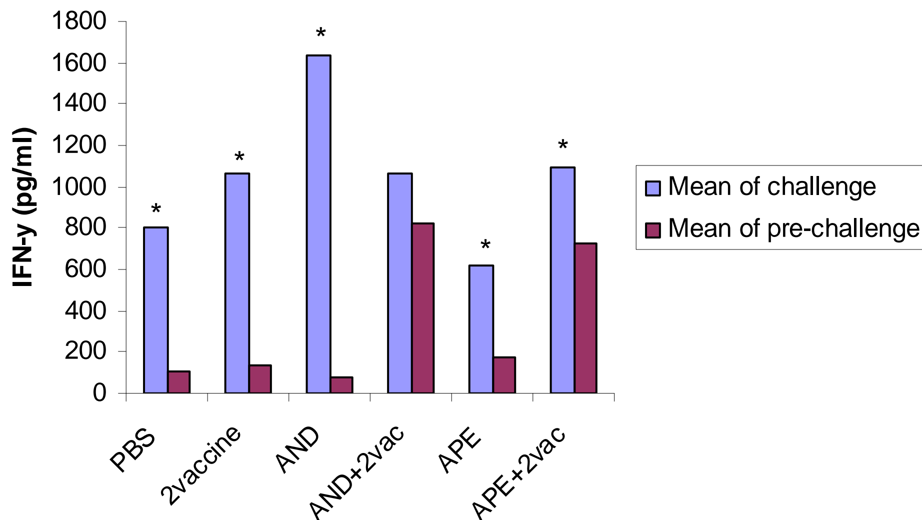


Figure 5-5 Comparison of IFN-γ levels of mice between 12 days post-challenge with $1.5x10^5$ cfu *S. typhimurium* 12313 and pre-challenge respectively.

IFN-γ was determined using pooled splenocyte culture supernatant in duplicate during 72 hour incubation in the presence of killed and sonicated SL3261. The plotted values represent mean values of duplicate wells. The asterisk * indicates significant difference of IFN-γ production ($p<0.05$) compared to groups of pre-challenge.

The splenocytes stimulated for 72 hours with the *Salmonella* lysate, secreted IFN-γ in all groups after 12 days post-challenge (Figure 5-4). After 12 days post-challenge, AND treatment of vaccinated mice (AND+2vaccine) elicited slightly higher IFN-γ levels after 72 hour stimulation with the *Salmonella* lysate than the levels elicited in pre-challenge mice ($p=0.145$), meanwhile, unvaccinated (PBS, APE and AND), vaccinated alone (2vaccine) and APE+2vaccine groups of mice after 12 days post-challenge elicited significantly higher IFN-γ levels than the levels elicited in pre-challenge ($p<0.05$) (Figure 5-5). In the pre-challenge experiment, groups of mice were not vaccinated but were gavaged with either APE or AND only. It was discovered that APE or AND induced only low levels of IFN-γ. Their IFN-γ levels were similar in magnitude to those in the PBS control group of mice. Whereas, the levels in mice immunized with 2 doses of the vaccine and fed either APE or AND were 75 to 90 percent higher than the levels detected in the PBS, APE or AND control groups of mice.

5.4.2.2 Measurement of IL-2 levels by ELISA

After 12 days post-challenge, IL-2 levels were detectable in all vaccinated mice splenocytes after the splenocytes were stimulated for 72 hours (Figure 5-6). On the other hand, no or low levels of IL-2 were detectable in PBS, AND and APE mice splenocytes. AND+2vaccine and APE+2vaccine groups of mice not only appear to elicit significantly higher IL-2 levels ($p<0.05$) than the AND or APE groups after 72 hour stimulation with the lysate, but also elicit significantly higher IL-2 levels ($p<0.05$) than the 2vaccine group (Figure 5-6). No IL-2 was detected in all groups of pre-challenge.

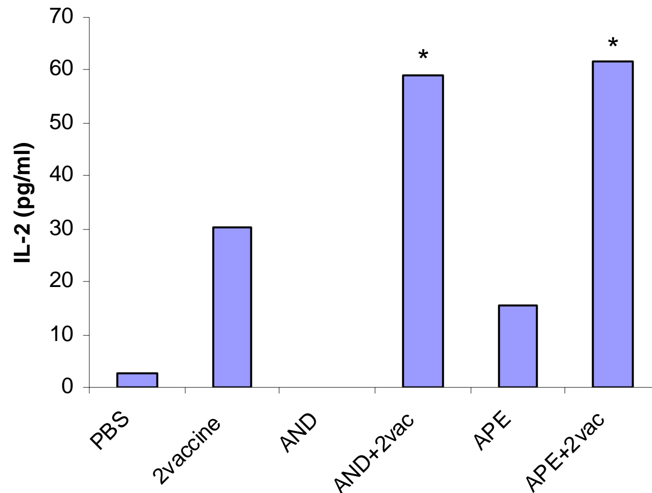


Figure 5-6 IL-2 production by splenocytes after 72 hour stimulation with the *Salmonella* lysate.

Spleens were removed 12 days post-challenge with 1.5×10^5 cfu *S. typhimurium* 12313. Pooled spleens from groups of 3 mice were used. The plotted values represent mean values of duplicate wells. The asterisk * indicates significant difference of IL-2 production ($p<0.05$) compared to group of 2vaccine.

5.4.2.3 Measurement of IL-4 levels by ELISA

After 12 days post-challenge, no IL-4 was detected in all groups after 72 hour stimulation with the *Salmonella* lysate.

5.4.3 Survival of mice after challenge with strain 12313, 1.5×10^6 cfu suspensions

Seven days after booster vaccination, groups of 4 mice were challenged orally with 1.5×10^6 cfu *S. typhimurium* 12313 and survival was recorded for 15 days. Survival of mice was recorded daily and is shown as percentage of animal's survival at each interval (Figure 5-7). No protection against strain 12313 was observed in both PBS and 2vaccine groups, as all eight mice died within 5-10 days of post-challenge. In contrast, a proportion of mice from groups of APE or AND treatment survived challenge infection and at least 50% mice were protected against challenge with strain 12313, no matter whether the mice were vaccinated with the killed *Salmonella* vaccine or not.

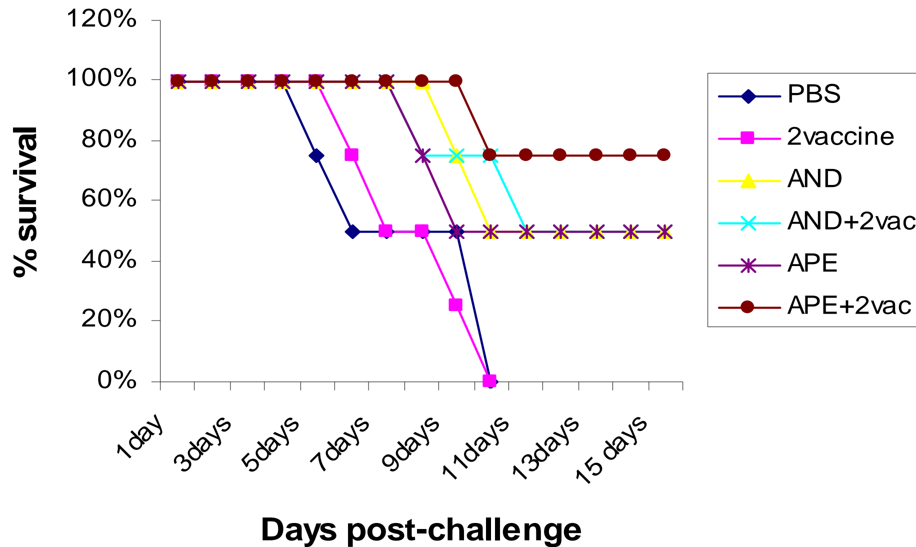


Figure 5-7 Percentage survival of mice in different groups, following challenge with $1.5x10^6$ cfu virulent *S. typhimurium.*
Survival was recorded daily and is shown as percentage of mouse survival at each interval. Groups consisted of 4 mice.

The enumeration of bacteria in individual mouse liver or spleen showed that the average numbers of viable bacteria in spleens or livers of dying mice were much higher (1.1×10^4 times) than those of surviving mice (data are shown in Appendices F and G). All mice with high enumeration of bacteria in spleens were accompanied by splenomegaly (spleen weights up to 2 to 4 times normal) (data are shown in Appendix H), whereas all spleen weights of surviving mice fell within the normal range.

5.4.4 Numbers of viable bacteria in the spleens and livers of mice post-challenge with $1.5x10^5$ cfu *Salmonella,* strain 12313

As shown in figures 5-8, numbers of viable bacteria from three control groups viz., 2vaccine, APE or AND increased significantly, particularly in liver. In contrast, the CFU in the APE+2vac and AND+2vac groups cleared the pathogen by 8 or 12 days post-challenge. A few viable bacteria from PBS group were counted only on days 4 post-challenge (data are shown in Appendices I, I.1, I.2 and I.3).

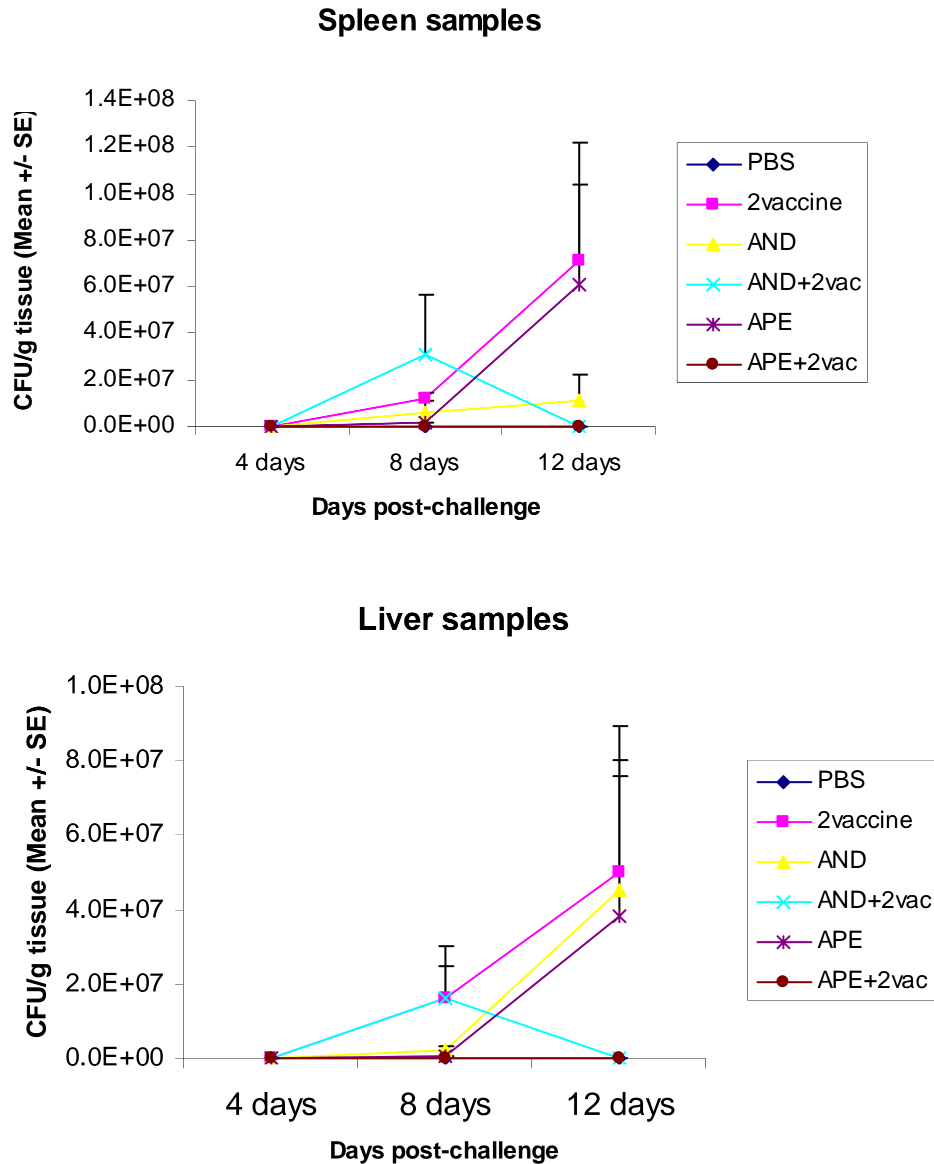


Figure 5-8 Determination of the CFU in mice challenged with *Salmonella,* 12313. Balb/c female mice were orally infected with 1.5×10^5 cfu *Salmonella,* 12313. Groups of 3 mice per time point were killed on days 4, 8 and 12 post-challenge. The spleen and liver were removed, homogenized and the CFU per gram of tissue determined. Values are shown the mean of CFU from three mice.

5.4.5 Average mouse weight and average weight gain

Five mice were weighed (g) in each group before challenge. The average weight gain (g) and average weight in the group (g) were calculated (Table 5-1 and

Figure 5-9) to evaluate if APE or AND at given doses affected mice adversely. None of the mice in any group lost weight indicating absence of apparent toxicity.

Table 5-1 The average gain (AG) (unit: g)

Groups	AG
PBS	1.6
APE+2vaccine	1.4
AND+2vaccine	1.5
APE	2.1
AND	1.6
2vaccine	1.2

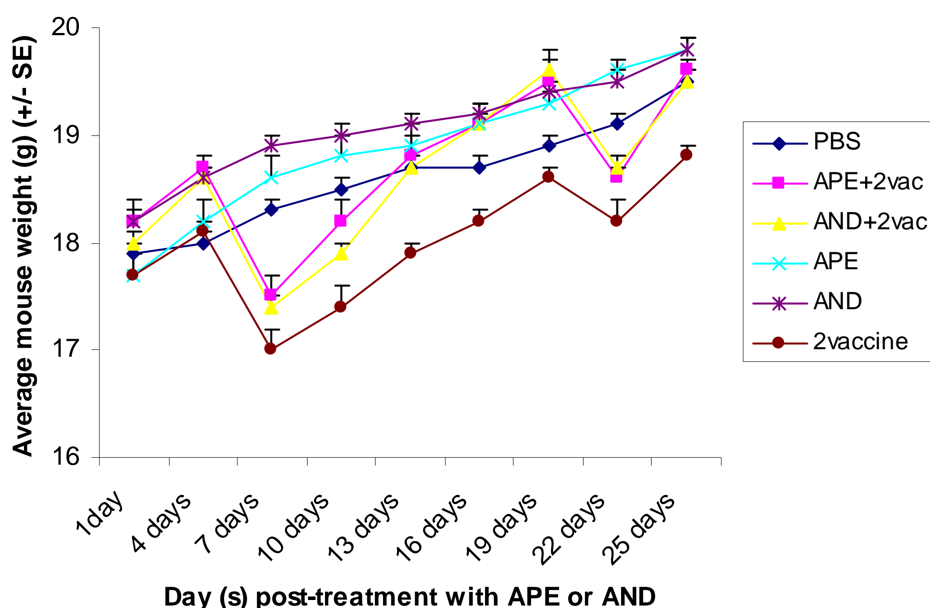


Figure 5-9 Average weight gains of mice in different group post-treatment with APE or AND. Each group consisted of 5 mice.

5.5 Discussion

The experimental mouse model was used to study the protective effect of APE or AND on mice vaccinated with killed *S. typhimurium* vaccine which had previously been reported to induce an antibody response (Mukkur et al., 1987; 1991a; 1991b). Results obtained in this investigation revealed that oral administration of either APE or AND to mice for 28 days substantially increased the protective efficacy on mice vaccinated with inactivated *S. typhimurium*. Fifty percent of mice from groups representing treatment with APE, AND or AND+2vac and 75% from APE+ 2vac group survived after being challenged by

the oral route with $1.5x10^6$ (10 fold of sublethal dose) cfu virulent *S. typhimurium* (strain 12313) for 15 days (Figure 5-7). Furthermore, clearance of *S. typhimurium* 12313 from mice challenged by the oral route with the sublethal dose of virulent *S. typhimurium* (strain 12313, $1.5x10^5$ cfu) showed that treatment of vaccinated mice with APE or AND promoted clearance of *S. typhimurium* on days 8 or 12 post-challenge respectively. In contrast, numbers of viable bacteria from three control groups of 2vaccine, APE or AND increased significantly (Figure 5-8).

In the pre-challenge investigation, mice were immunized with two doses of inactivated *Salmonella* vaccine and were treated with APE or AND for 28 days. Neither APE or AND alone induced an anti- *S. typhimurium* IgG antibody response. APE or AND control groups induced only low levels of IFN- γ, similar in magnitude to those in the PBS control group of mice. These discoveries suggested that the stimulation of both humoral (anti-*S. typhimurium* IgG) and cellular (IFN-γ) immune responses of acquired immunity, described in the previous chapter, were due to treatment with APE or AND, and not simply to an additional vaccine booster dose. APE and AND were confirmed to significantly increase IgG titres and IFN- γ production (Figure 5-10 and 5-11).

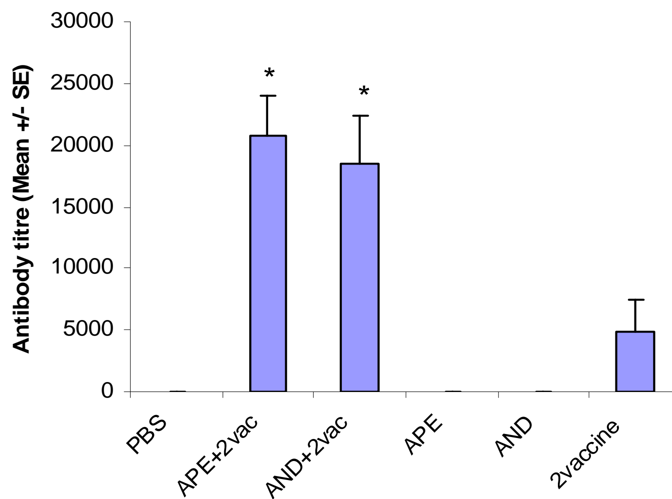


Figure 5-10 Anti-*S typhimurium* serum IgG titres of pre-challenge mice

Mice were treated with or without APE or AND for 28 days, and immunised with 2 doses of killed *S. typhimurium* vaccine. All values are means ± standard errors. The asterisk * indicates significant difference of IgG titre ($p<0.05$) compared to group of 2vaccine.

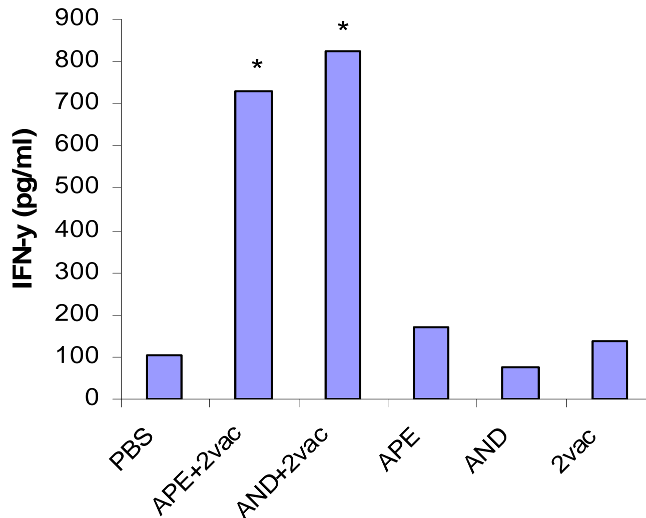


Figure 5-11 IFN-γ production of pre-challenge mice spleen cells

IFN-γ production by splenocytes after 72 hr stimulation with *Salmonella* antigen. Spleens were removed 28 days after mice had been treated with APE or AND. Pooled spleens from groups of 5 mice were used. The plotted values represent mean values of duplicate wells. The asterisk * indicates significant difference of IFN-γ production ($p<0.05$) compared to control groups of 2vaccine, PBS, AND and APE.

At 15 day post-challenge, fifty percent of unimmunized mice treated with APE and AND alone were found to be protected against oral challenge with virulent *S. typhimurium.* Given that no anti- *S. typhimurium* IgA or IgG antibody response was detected in mice pre-challenge with only basal level of IFN- γ (Figures 5-10 and 5-11), the protection observed could be contributed at least in part to innate immunity. This is an observation that clearly warrants further investigation.

Vaccinated mice responded with highest sera IgG titres against *S. typhimurium* on day 4 post-challenge, whereas, all control (unvaccinated) mice did not respond or responded with low sera IgG titres on days 8 and 12 post-challenge (Figure 5-1). These results confirmed that secondary response was rapid onset and greater production of IgG than the primary response.

IgA is thought to be important in the primary immunological defence against local gastrointestinal infection (Neutra, et al., 1996; Kraehenbuhl and Neutra, 1992). IgA of small intestinal washes was detectable on days 8 and 12 post-challenge for all tested groups, although IgA antibody isotype was low. The mechanism of

action of IgA might prevent the invading organism from attaching to and penetrating the epithelial surface (Coico et al., 2003), but this is yet to be proved.

Measurement of cytokine production from mouse splenocytes on day 12 post-challenge with a sublethal dose ($1.5x10^5$ cfu) of virulent *S. typhimurium* showed that IFN-γ was produced in all groups. This is because all groups were infected with live virulent *S. typhimurium*. As numbers of viable *S. typhimurium* in spleens of three control mice groups (2vaccine, APE and AND) were still high at 12 days post-challenge with virulent *S. typhimurium*, bacteria may still be present in splenocytes prepared from these mice, potentially increasing stimulant concentration resulting in higher cytokine production in these three groups. However, the results of cytokine production in AND+2vaccine and APE+2vaccine groups are not affected as no viable *S. typhimurium* was found in the spleens in these two groups of mice. High levels of IL-2 were only produced in vaccinated mice, but AND+2vaccine and APE+2vaccine groups of mice elicited substantially higher IL-2 levels ($p<0.05$) than the 2vaccine group (Figure 5-6). No IL-4 was detected in all groups after 72 hour stimulation with the *Salmonella* lysate.

Our results confirmed that inactivated *S. typhimurium* could not provide good protection against *S. typhimurium* (Mukkur et al., 1987; 1991a and 1991b), as all vaccinated control mice died within 5-10 days post-challenge with $1.5x10^6$ cfu virulent *S. typhimurium*. Although the serum IgG and IgA were produced within 12 days post-challenge with $1.5x10^5$ cfu virulent *S. typhimurium* and IFN-γ or IL-2 were detected at 12 days post-challenge, significantly large numbers of viable bacteria in spleens and livers were presented within 12 days post-challenge. The production of IL-2 may be one of the reasons leading to a failure to protect against *S. typhimurium* in this case as IL-2 levels in groups of APE+2vaccine and AND+2vaccine were significantly higher than group 2vaccine ($p<0.05$) (Figure 5-6). IL-2 is also involved in the induction of CMI (Gupta, 1998; Tsukada et al., 1991) suggesting that this may be the reason for such a response.

APE and AND did protect vaccinated mice against $1.5x10^6$ cfu virulent *S. typhimurium*, as 75% or 50% vaccinated mice survived from the challenge after 28 days APE or AND treatment. Furthermore, clearance of *S. typhimurium* 12313 in spleens or livers after mice were challenged by the oral route with a sublethal dose of virulent *S. typhimurium* (strain 12313, $1.5x10^5$ cfu) showed that APE or AND helped vaccinated mice eliminate *S. typhimurium* on days 8 or 12 post-challenge respectively, whereas, numbers of viable bacteria from three control groups of 2 vaccine, APE or AND increased significantly. This is despite the fact that the serum IgG and IgA antibody titres against *S. typhimurium* were increased within 8 days post-challenge with $1.5x10^5$ cfu virulent *S. typhimurium* and IFN-γ or IL-2 produced.

It was interesting to note that APE or AND alone also provided a degree of protection against $1.5 x 10^6$ cfu virulent *S. typhimurium*, as 50% mice survived from the challenge infection after 28 days APE or AND treatment. However, no serum IgG and IgA were detected and significantly large numbers of viable bacteria in the spleens and livers of mice were presented within 12 days post-challenge with $1.5 x 10^5$ cfu virulent *S. typhimurium*. Also either no or low IL-2 was detected at 12 days post-challenge, although IFN-γ was detected. No or low IL-2 determination may due to the splenocyte collection time. It may be too long for IL-2 determination after 72 hour stimulation with the *Salmonella* lysate, although it was good time for IFN-γ determination. Although the reason for the survival of mice could not be due to the direct anti-bacterial activity of APE or AND, because no direct anti-bacterial activity of APE or AND was detected in our previous study (see Chapter 3), it may be potentially due to innate immunity (Panossian et al., 2002; Rajagopal et al., 2003; Kumar et al., 2004). Another possible reason is some metabolites of APE or AND that are being generated *in vivo*. The metabolites could lead to their antibacterial activity. A further anti-bacterial activity study on metabolite of AND by LC MS/MS from mouse serum should be warranted.

Unvaccinated mice without APE or AND treatment (PBS group) were not protected following challenge with $1.5 x 10^6$ cfu virulent *S. typhimurium*, as all PBS mice died within 5-10 days post-challenge.

In conclusion, our results showed that both the *Andrographis* extract and andrographolide substantially enhance the protective efficacy against *Salmonella* on mice vaccinated with inactivated *S. typhimurium*. Consequently andrographolide might be a useful potential immunomodulator in the development of nonviable vaccines.

Chapter 6 Overall conclusions

Live attenuated vaccines elicit both humoral and cellular immunity similar to that elicited by the natural infection (Viret et al., 1999; Harrison et al., 1997; Mastroeni et al., 1993; Mukkur et al., 1991a, 1991b; Mukkur et al., 1987), but they may pose some risk such as reversion to pathogenicity within an immunized individual. On the other hand, inactivated vaccines may be safer than live vaccines, but their antigens elicit only antibody responses and essentially no CMI. The new type of DNA (or nucleic acid) vaccines are composed of a bacterial plasmid encoding foreign antigens cloned in eukaryotic expression vectors (Wolff et al., 1990; Dubensky et al., 1984; Will et al., 1982). This new type of vaccines is being developed because of the potential to induce both the humoral and the cellular arms of the immune response (Zinkernagel, 2003; Ulmer et al., 1993; Tang et al., 1992). If one could tailor the immune response towards CMI, the potential to develop safe inactivated vaccines capable of generating CMI that provide protection against intracellular infectious diseases would become possible.

Traditional herbal medicines are widely used for the treatment of many kinds of acute and chronic diseases in Asia. Some herbs, such as *Echinacea*, *Astragalus*, *Picrorrhiza*, *Phytolacca* and *Andrographis* has been claimed to be immune-enhancing herbs (Mills and Bone, 2000). However, herbal extracts are a mixture of bioactive and inactive chemical ingredients. Also, the chemical composition of extracts varies depending on geographical distribution, climate condition of cultivation and the method used for preparation of the extracts. Hence, it is desirable to validate these bioactive chemical ingredients responsible for the claimed therapeutic properties.

Extracts of whole plants of *Andrographis paniculata* have been reported to have anti-cancer, anti-inflammatory, anti-allergic, immunostimulatory, antithrombotic, hypoglycaemic and hepatoprotective activities (Kumar, 2004; ; Zhang & Tan, 1996 & 2000; Gupta et al., 1998; Habtemariam, 1998; Trivedi & Rawal, 1998; Matsuda et al., 1994; Puri et al., 1993; Kapil et al., 1993; Zhao & Fang, 1991; Handa & Sharma, 1990; Sawasdimongkol et al., 1990; Chantasutra & Limpapanichkul, 1989; Dutta & Sukul, 1982). Andrographolide has been reported to be anti-inflammatory, stimulate or suppress innate immunity as assessed by cytokine induction/deduction and anti-carcinogenic activity (Qin et al., 2006; Zhou et al., 2006; Burgos et al., 2005; Kumar et al., 2004; Rajagopal et al., 2003;

Panossian et al., 2002; Shen. et al., 2002; Madav et al., 1996) and either stimulated (Puri et al., 1993) or suppressed adaptive immunity (Iruretagoyena et al, 2006; 2005). However, the potential of this medicinal plant and AND in the induction or enhancement of immune response particularly, against infectious diseases had not been evaluated.

The first phase in this project involved purification and confirmation of andrographolide, the active principle from *Andrographis paniculata*. The second phase was to investigate the direct antimicrobial activity of AP extracts and AND *in vitro*. The third phase was to investigate if oral administration of an AP extract or AND was capable of inducing or promoting one or both effector arms of the immune response viz., the humoral and/or CMI on mice immunized with an inactivated vaccine. This inactivated vaccine was previously demonstrated to induce only an antibody response (Mukkur et al., 1991a; 1991b; 1987; Harrison et al., 1997) and unable to protect mice against challenge with virulent *S. typhimurium*. The final phase of this investigation was to extend the third phase and to validate the potential of the induced CMI in protecting mice against challenge with the intracellular pathogen, *S. typhimurium*.

A modification of the method reported by Rajani et al (2000) was used to isolate AND from AP in the first process. Before AND was purified from AP, preliminary and confirmatory tests were conducted to make sure the plant sample contained active diterpene lactones and AND was contained in AP. The purified AND was further confirmed by melting point, color test, UV λmax, TLC, ESI-MS and ^{1}H-NMR compared with commercial AND and the published data (http://www.plantpharm.net/new_page_25.htm; Cui et al., 2005; Medforth et al., 1990) to ensure the compound quality. All data from the experiments indicated that the plant sample and purified AND were qualified to be used. An aqueous and two ethanolic extracts of *Andrographis paniculata*, and AND, an active principle of *Andrographis paniculata*, were investigated for their antimicrobial activity against nine bacterial species including *Salmonella typhimurium*, *Escherichia coli, Shigella sonnei, Staphylococcus aureus, Pseudomonas aeruginosa*, *Streptococcus pneumoniae, Streptococcus pyogenes, Legionella pneumophila,* and *Bordetella pertussis*, using the disc diffusion method in the second investigation. Of all tested concentrations, direct antimicrobial activity of the two ethanolic *Andrographis paniculata* extracts was observed for only two human pathogens, *Legionella pneumophila* and *Bordetella pertussis*. However, no antibacterial activity of AND against any of the pathogens tested in this investigation was observed. Given that the TLC of *Andrographis paniculata* extracts showed that AND was present in all the three *Andrographis paniculata* extracts, it was concluded that the observed antimicrobial activity was due to other active principle(s) present in the extracts used in this investigation. This study was important because without this information, it would not be possible to

determine whether any protection observed was due to the direct antimicrobial activity or modulatory effect of APE or AND. In the third investigation, the immunomodulatory activities of an ethanolic extract of *Andrographis paniculata* and AND, administered by the oral route in mice vaccinated with inactivated *Salmonella typhimurium*, that by itself has been previously shown to induce only humoral and no CMI response (Harrison et al., 1997; Mukkur et al., 1987; Mukkur et al., 1991a, 1991b) were investigated. The results demonstrated that both the *Andrographis* extract and andrographolide enhanced not only anti-*S. typhimurium* IgG antibody levels in sera of vaccinated mice but also induced CMI as judged by production of the CMI indicator cytokine IFN-γ in antigen stimulated splenocyte supernatants. When these mice were challenged with the pathogen by the oral route, it was discovered that oral administration of either APE or AND to mice for 28 days substantially increased the protective efficacy in the mice, as fifty to 75 percent of mice from AND or APE treatment survived after challenge with the virulent organism (1.5×10^6 cfu *S. typhimurium*, strain 12313). Furthermore, clearance of *S. typhimurium* 12313 in spleens or livers from mice which were challenged by the oral route with a sublethal dose of virulent *S. typhimurium* suggested that treatment of vaccinated mice with APE or AND promoted clearance of *S. typhimurium* from challenged mice by days 8 or 12 post-challenge respectively, whereas, numbers of viable bacteria in the control groups (2vaccine, APE or AND) increased significantly. Meanwhile, the sera IgG, IgA and IFN-γ or IL-2 of the splenocytes were detected after the mice were challenged by the oral route with a sublethal dose of virulent *S. typhimurium* for 12 days. The investigations presented in this thesis warrant extension of these studies to other intracellular pathogens, particularly *Mycobacterium tuberculosis* and viral infectious agents such as acquired immunodeficiency syndrome virus because of (a) the clear demonstration that adaptive immune responses can be modified to induce CMI and enhance antibody responses as shown in this investigation, and (b) an increase in the $CD4^+$ T-cells in HIV-postive patients (Calabrese et al., 2000).

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Chapter 8 APPENDICES

Appendix A: White cell counting

White cell counting was performed using a haemocytometer with conversion factor of 10.000 for a counting chamber of depth 0.1 mm (Life technologies). Briefly, 10 µl cell suspension was added to 90µl CDMEM and then 10 µl was taken out and mixed with 10 µl of 0.14% trypan blue (Life technologies). The mixture was added to the haemocytometer to fill the chamber. The chamber consists of 4 large squares at the corners and each large square has 16 small squares. The unstained cells were counted in 4 small squares in each of the large square. The total number for each large square was calculated by multiplying the number of the cells from each 4 small squares by 4. The equation for viable cells per ml is: average cell count per large square × the dilution factor × haemocytometer conversion factor. And then, the remaining cell suspension was adjusted to 1×10^{7} cells/ml.

Appendix B: Determination of protein concentration

The protein concentration was determined using “Coomassie® Plus™ -the Better Bradford Assay kit” (Pierce, Rockford, USA). Briefly, a series of diluted bovine serum albumin (BSA) standards were prepared in the same diluent as the samples to 25, 125, 250, 500 and 750 µg/ml. 10 µl of each standard or unknown sample were pipetted into the appropriate 96 microplate wells. 300 µl of the Coomassie® Plus™ reagent was added to each well and mixed with plate shaker for 30 seconds. The plate was then incubated for 10 minutes at RT. The absorbance was measured at 595 nm (Bio-Rad 550, microplate reader, Hercules, CA), minus the reagent blank. A standard curve was plotted, from which the unknown protein concentration was determined. A standard curve is shown in the following figure.

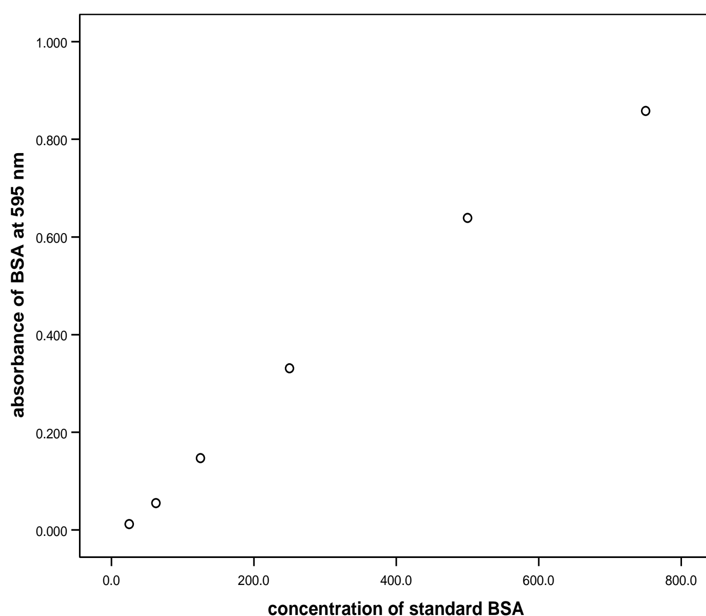

Figure A 1: a standard curve of BSA protein (unit: µg/ml)

Appendix C: Tissue culture media

(a) Without Fetal Bovine Serum (FBS) (used for washing cells)

To 500ml Dulbecco's Modified Eagle Media (Gibco, Invitrogen Corporation, Auckland, NZ) added 25mM HEPES, 200 units/ml penicillin-steptomycin, 50µM 2-Mecaptoethanol and 10µg/ml Polymyxin B (all final concentration).

(b) With 10% FBS (CDMEM media, used for culturing cells)

To the tissue culture media (a), add 10% fetal bovine serum (Gibco, Invitrogen Corporation, Auckland, NZ).

Appendix D: Borate saline coating Buffer

(a) Stock buffer
1.5 M NaCl (Sodium chloride)
0.5 M H_3BO_4 (Boric acid)

1.0 M NaOH (Sodium hydroxide)

(b) Working buffer (120mM NaCl, 50mM H_3BO_4 and 24 mM NaOH, pH 9.0)

To 80 ml of 1.5 M NaCl, 100 ml of 0.5 M H_3BO_4, 24 ml of 1.0 M NaOH,

add distilled water to about 1 litre and adjust pH to 9. And then adjust to 1 litre by distilled water. Make the buffer as fresh as possible.

Appendix E: Antigen-specific immune responses for *S. typhimurium*

E.1: Antigen specific antibody responses in mice treated with APE and AND for 14 days

E.1.1: Serum IgG titers against *Salmonella typhimurium aroA* SL3261 whole-cell lysate

Groups (5 mice/group)	Serum IgG titers
PBS-1	0
PBS-2	0
PBS-3	0
PBS-4	0
PBS-5	0
1vaccine-1	1100
1vaccine-2	1150
1vaccine-3	600
1vaccine-4	1550
1vaccine-5	1550
25APE+1vaccine-1	1050
25APE+1vaccine-2	2000
25APE+1vaccine-3	1050
25APE+1vaccine-4	900
25APE+1vaccine-5	1550
50APE+1vaccine-1	2000
50APE+1vaccine-2	2250
50APE+1vaccine-3	700
50APE+1vaccine-4	3000

50APE+1vaccine-5	1500
1AND+1vaccine-1	1500
1AND+1vaccine-2	1500
1AND+1vaccine-3	1500
1AND+1vaccine-4	1500
1AND+1vaccine-5	1550
4AND+1vaccine-1	2700,
4AND+1vaccine-2	1700
4AND+1vaccine-3	2000
4AND+1vaccine-4	1650
4AND+1vaccine-5	1100

E.1.2: Statistical analysis of serum antibody (IgG) titre for 1 dose *S. typhimurium*-vaccinated mice given oral doses of either APE or AND

Groups	Mean IgG titre ± 1SE	Standard Deviation	Independent-sample t-tests for equality means		
			t-statistic	P value	df
PBS	0				
1vaccine	1190±175	392	n/a	n/a	n/a
25APE+1vaccine	1310±204	457	-0.445	0.668	7
50APE+1vaccine	1890±383	857	-1.659	0.152	5
1AND+1vaccine	1510±10	22	-1.819	0.143	4
4AND+1vaccine	1830±261	584	-2.031	0.082	7

Note: SE=standard error, df=degrees of freedom, n/a=not applicable since comparison of means is made to this group, p value two tailed (equal variance not assumed)

E.2: Effects of 14 days APE and AND treatment on cytokine production in mice immunized with the inactivated *Salmonella* vaccine

E.2.1: *S. typhimurium*-specific IFN-γ production

(Duplicate culture wells were pooled from 5 mice)

Groups (5 mice/group)	IFN-γ production (pg/ml)
PBS	22.2
	23.8
1vaccine	245.2
	311.8
25APE+1vaccine	541.6
	647.6
50APE+1vaccine	395.8
	441.2
1AND+1vaccine	240.8
	332.8
4AND+1vaccine	870.4
	883.2

E.2.2: *S. typhimurium*-specific IL-4 production

(Duplicate culture wells were pooled from 5 mice)

Groups (5 mice/group)	IL-4 production (pg/ml)
PBS	0
	0
1vaccine	0
	0
25APE+1vaccine	22.0
	23.0

50APE+1vaccine	14.8
	14.8
1AND+1vaccine	40.2
	48.4
4AND+1vaccine	29.8
	28.4

E.3: Antigen specific antibody responses in mice treated with APE and AND for 28 days

E.3.1: Serum IgG titers against *Salmonella typhimurium aroA* SL3261 whole-cell lysate

Groups (5 mice/group)	Serum IgG titers
PBS-1	0
PBS-2	0
PBS-3	0
PBS-4	0
PBS-5	0
2vaccine-1	1800
2vaccine-2	2300
2vaccine-3	2350
2vaccine-4	15000
2vaccine-5	3100
25 APE+2vaccine-1	26000
25APE+2vaccine-2	1700
25APE+2vaccine-3	26000
25APE+2vaccine-4	26000
25APE+2vaccine-5	26000

50APE+2vaccine-1	26000
50APE+2vaccine-2	26000
50APE+2vaccine-3	26000
50APE+2vaccine-4	26000
50APE+2vaccine-5	1600
1AND+2vaccine-1	26000
1AND+2vaccine-2	26000
1AND+2vaccine-3	6400
1AND+2vaccine-4	12800
1AND+2vaccine-5	26000
4AND+2vaccine-1	25600
4AND+2vaccine-2	18000
4AND+2vaccine-3	13000
4AND+2vaccine-4	13000
4AND+2vaccine-5	27000

E.3.2: Statistical analysis of serum antibody (IgG) titre for 2 dose *S. typhimurium*-vaccinated mice given oral doses of either APE or AND

Groups	Mean IgG titre ± 1SE	Standard Deviation	Independent-sample t-tests for equality means		
			t-statistic	P value	df
PBS	0				
2vaccine	4910±2531	5659	n/a	n/a	n/a
25APE+2vaccine	21140±4859	10867	-2.962	0.025	6
50APE+2vaccine	21120±4879	10912	-2.949	0.026	6
1AND+2vaccine	19440±4142	9263	-2.993	0.022	6
4AND+2vaccine	19320±3000	6709	-3.671	0.007	7

Note: SE=standard error, df=degrees of freedom, n/a=not applicable since comparison of means is made to this group, p value two tailed (equal variance not assumed)

E.4: Effects of 28 days APE or AND treatment on cytokine production in mice immunized with the inactivated *Salmonella* vaccine

E.4.1: *S. typhimurium*-specific IFN-γ production

(Duplicate culture wells were pooled from 5 mice)

Groups (5 mice/group)	IFN-γ production (pg/ml)
PBS	6.8
	9.4
2vaccine	124.0
	152.4
25APE+2vaccine	573.6
	622.6
50APE+2vaccine	230.8
	258.8
1AND+2vaccine	882.0
	959.6
4AND+2vaccine	768.2
	799.2

E.4.2: *S. typhimurium*-specific IL-4 production

(Duplicate culture wells were pooled from 5 mice)

Groups (5 mice/group)	IL-4 production (pg/ml)
PBS	0
	0
2vaccine	70.6
	65.2
25APE+2vaccine	31.2

	26.2
50APE+2vaccine	40.2
	34.2
1AND+2vaccine	22.0
	18.4
4AND+2vaccine	60.4
	65.6

E.5: Antigen specific antibody responses in mice post-challenge with 1. $5x10^5$ cfu of *S. typhimurium* 12313

E.5.1: Serum IgG titers against *Salmonella typhimurium aroA* SL3261 whole-cell lysate on four time points

Groups	Serum IgG titers			
	0 day	4 days	8 days	12 days
PBS-1	0	1700	600	0
PBS-2	0	0	0	0
PBS-3	0	0	0	0
PBS-4	0			
PBS-5	0			
2vaccine-1	1800	102500	102400	3200
2vaccine-2	2300	51200	51200	12800
2vaccine-3	2350	51200	25600	25600
2vaccine-4	15000			
2vaccine-5	3100			
AND-1	0	0	0	0
AND-2	0	0	0	0
AND-3	0	0	0	0
AND-4	0			
AND-5	0			
AND+2vaccine-1	25600	102600	25600	51200
AND+2vaccine-2	18000	51300	102400	12800

AND+2vaccine-3	28000	51200	51200	6400
AND+2vaccine-4	14000			
AND+2vaccine-5	6700			
APE-1	0	0	0	0
APE-2	0	0	0	0
APE-3	0	0	0	0
APE-4	0			
APE-5	0			
APE+2vaccine-1	13800	204800	25400	25600
APE+2vaccine-2	26000	204900	25600	12800
APE+2vaccine-3	26000	51200	51200	6400
APE+2vaccine-4	26000			
APE+2vaccine-5	11800			

E.5.2: Serum IgA titers against *Salmonella typhimurium aroA* SL3261 whole-cell lysate on four time points

Groups	Serum IgA titers			
	0 day	4 days	8 days	12 days
PBS-1	0	0	0	0
PBS-2	0	0	0	0
PBS-3	0	0	0	0
PBS-4	0			
PBS-5	0			
2vaccine-1	0	160	380	0
2vaccine-2	0	170	260	0
2vaccine-3	0	190	115	0
2vaccine-4	0			
2vaccine-5	0			
AND-1	0	0	0	0
AND-2	0	0	0	0
AND-3	0	0	0	0
AND-4	0			
AND-5	0			
AND+2vaccine-1	0	180	170	0
AND+2vaccine-2	0	210	420	0

AND+2vaccine-3	0	200	50	0
AND+2vaccine-4	0			
AND+2vaccine-5	0			
APE-1	0	0	0	0
APE-2	0	0	0	0
APE-3	0	0	0	0
APE-4	0			
APE-5	0			
APE+2vac	0	250	10	0
APE+2vaccine-2	0	170	85	0
APE+2vaccine-3	0	260	35	0
APE+2vaccine-4	0			
APE+2vaccine-5	0			

E.5.3: Small intestinal washes (SIW) IgA titers against *Salmonella typhimurium aroA* SL3261 whole-cell lysate on four time points

Groups	SIW IgA titers			
	0 day	4 days	8 days	12 days
PBS-1	0	0	0	10
PBS-2	0	0	30	20
PBS-3	0	0	0	20
PBS-4	0			
PBS-5	0			
2vaccine-1	0	0	80	40
2vaccine-2	0	0	90	0
2vaccine-3	0	0	320	40
2vaccine-4	0			
2vaccine-5	0			
AND-1	0	0	40	60
AND-2	0	0	80	17
AND-3	0	0	20	15
AND-4	0			
AND-5	0			
AND+2vaccine-1	0	0	80	20

AND+2vaccine-2	0	0	90	50
AND+2vaccine-3	0	0	40	80
AND+2vaccine-4	0			
AND+2vaccine-5	0			
APE-1	0	0	40	20
APE-2	0	0	20	10
APE-3	0	0	0	55
APE-4	0			
APE-5	0			
APE+2vaccine-1	0	0	50	30
APE+2vaccine-2	0	0	30	30
APE+2vaccine-3	0	0	25	35
APE+2vaccine-4	0			
APE+2vaccine-5	0			

E.5.4: *S. typhimurium*-specific IL-2, IFN-γ and IL-4 production on day 12 post-challenge with 1. 5x10^5 cfu of *S. typhimurium* 12313

(Duplicate culture wells were pooled from 3 mice)

Groups (3 mice/group)	IL-2 level (pg/ml)	IFN-y level (pg/ml)	IL-4 level (pg/ml)
PBS	1.7	846.0	0
	3.7	765.4	0
2vaccine	28.4	1093.8	0
	32.4	1030.6	0
AND	0	1635.5	0
	0	1628.4	0
AND+2vaccine	58.5	1071.2	0
	59.9	1065.8	0
APE	9.5	500.6	0
	21.2	742.6	0
APE+2vaccine	63.4	1072.8	0
	59.9	1114.4	0

E.5.5: *S. typhimurium*-specific IFN-γ production of pre-challenge mice spleen cells

(Duplicate culture wells were pooled from 5 mice, 72 hours stimulated with antigen)

Groups (5 mice/group)	IFN-γ production (pg/ml)
PBS	94.1
	112.3
2vaccine	124.0
	155.2
APE	129.3
	210.1
APE+2vaccine	715.8
	738.6
AND	61.9
	91.0
AND+2vaccine	764.4
	879.6

Appendix F: The colony number (cfu)/gram liver sample, after challenge with 1.5× 10^{6} cfu *S. typhimurium* (strain 12313)

Groups	5 days	6 days	7 days	8 days	9 days	10 days	11 days	15 days
PBS-1	$1.8x10^{6}$							
PBS-2		$5. 2x10^{5}$						
PBS-3						$1. 0x10^{8}$		
PBS-4						$2.8x10^{5}$		
2vaccine-1		$1.3x10^{8}$						
2vaccine-2			$3. 1x10^{7}$					
2vaccine-3					$1. 0x10^{8}$			
2vaccine-4						$7. 5x10^{7}$		
AND-1					dead			
AND-2						$6. 1x10^{7}$		
AND-3								0
AND-4								$6. 2x10^{3}$
AND+2vaccine-1				dead				
AND+2vaccine-2							$5.1x10^{8}$	

AND+2vaccine-3								6.0×10^{3}
AND+2vaccine-4								0
APE-1				dead				
APE-2					1.4×10^{8}			
APE-3								7.2×10^{4}
APE-4								0
APE+2vaccine-1						dead		
APE+2vaccine-2								0
APE+2vaccine-3								0
APE+2vaccine-4								0

Appendix G: The colony number (cfu) / gram spleen sample, after challenge with 1.5x 10^{6} cfu *S. typhimurium* (strain 12313)

Groups	5 days	6 days	7 days	8 days	9 days	10 days	11 days	15 days
PBS-1	7.4x10^{6}							
PBS-2		3.5x10^{5}						
PBS-3						1.1x 10^{8}		
PBS-4						4.7x10^{5}		
2vaccine-1		1.3x10^{8}						
2vaccine-2			9.5x10^{7}					
2vaccine-3					1.7x10^{8}			
2vaccine-4						3. 0x10^{7}		
AND-1					dead			
AND-2						1.9x 10^{8}		
AND-3								0
AND-4								5.8x10^{3}
AND+2vacine-1				dead				
AND+2vaccine-2							6.0x10^{8}	
AND+2vaccine-3								1.3x10^{4}

AND+2vaccine-4								0
APE-1				dead				
APE-2					$1.6x10^{8}$			
APE-3								$1. 2x10^{5}$
APE-4								0
APE+2vaccine-1						dead		
APE+2vaccine-2								0
APE+2vaccine-3								0
APE+2vaccine-4								0

Appendix H: Mouse spleen weight (gram), after challenge with 1.5x 10^6 cfu *S. typhimurium* (strain 12313)

Spleen weight (g)

Groups	5 days	6 days	7 days	8 days	9 days	10 days	11 days	15 days
PBS-1	0.22							
PBS-2		0.40						
PBS-3						0.46		
PBS-4						0.27		
2vaccine-1		0.49						
2vaccine-2			0.25					
2vaccine-3					0.51			
2vaccine-4						0.59		
AND-1					0.36			
AND-2						0.47		
AND-3								0.11
AND-4								0.11
AND+2 vaccine-1				0.42				
AND+2vaccine-2							0.39	
AND+2vaccine-3								0.12
AND+2vaccine-4								0.12

APE-1				0.44				
APE-2					0.41			
APE-3								0.15
APE-4								0.17
APE+2vaccine-1						0.51		
APE+2vaccine-2								0.17
APE+2vaccine-3								0.17
APE+2vaccine-4								0.17

Appendix I: The colony number (cfu)/ gram sample, after challenge with 1.5x 10^5 *S. typhimurium* (strain 12313)

I.1: On day 4 post-challenge

Groups	whole spleen weight (g)	the colony number (cfu)/g spleen	liver sample weight (g)	the colony number (cfu)/ g liver
PBS-1	0.11	0	0.51	0
PBS-2	0.12	7.6×10^3	0.64	4.5×10^2
PBS-3	0.11	0	0.50	0
2vaccine-1	0.12	0	0.45	0
2vaccine-2	0.11	0	0.49	0
2vaccine-3	0.19	6.4×10^4	0.41	2.5×10^2
AND-1	0.14	3.0×10^3	0.42	3.1×10^3
AND-2	0.15	1.1×10^4	0.40	5.2×10^3
AND-3	0.09	0	0.38	0
AND+2vaccine-1	0.14	4.6×10^2	0.43	1.4×10^2
AND+2vaccine-2	0.11	8.3×10^3	0.32	7.5×10^4

AND+2vaccine-3	0.12	0	0.32	0
APE-1	0.09	8.0×10^{4}	0.35	4.4×10^{4}
APE-2	0.08	4.2×10^{3}	0.29	6.2×10^{3}
APE-3	0.14	0	0.39	1.0×10^{3}
APE+2vaccine-1	0.17	5.3×10^{5}	0.42	1.7×10^{5}
APE+2vaccine-2	0.13	0	0.41	0
APE+2vaccine-3	0.06	0	0.43	0

I.2: On day 8 post-challenge

Groups	whole spleen weight (g)	the colony number (cfu)/g spleen	liver sample weight (g)	the colony number (cfu)/ g liver
PBS-1	0.11	0	0.50	0
PBS-2	0.11	0	0.41	0
PBS-3	0.10	0	0.65	0
2vaccine-1	0.29	1.5×10^{7}	0.46	4.3×10^{7}
2vaccine-2	0.25	6.0×10^{6}	0.36	2.4×10^{6}
2vaccine-3	0.16	1.3×10^{7}	0.53	1.3×10^{6}
AND-1	0.34	3.7×10^{6}	0.52	1.7×10^{6}

AND-2	0.10	0	0.43	0
AND-3	0.33	$1.5x10^{7}$	0.58	$3.9x10^{6}$
AND+2vaccine-1	0.28	$8.2x10^{7}$	0.48	$3.1x10^{7}$
AND+2vaccine-2	0.43	$8.9x10^{6}$	0.44	$1.6x10^{7}$
AND+2vaccine-3	0.12	0	0.35	0
APE-1	0.49	$1.2x10^{6}$	0.46	$5.2x10^{5}$
APE-2	0.39	$1.6x10^{6}$	0.38	$2.6x10^{5}$
APE-3	0.39	$1.9x10^{6}$	0.38	$2.8x10^{5}$
APE+2vaccine-1	0.14	0	0.43	0
APE+2vaccine-2	0.13	0	0.45	0
APE+2vaccine-3	0.14	0	0.48	0

I.3: On day 12 post-challenge

Groups	whole spleen weight (g)	the colony number (cfu)/g spleen	liver sample weight (g)	the colony number (cfu)/ g liver
PBS-1	0.11	0	0.46	0
PBS-2	0.11	0	0.44	$1.0x10^{2}$
PBS-3	0.11	0	0.47	0

2vaccine-1	dead		$1.7x10^{8}$	dead		$1.0x10^{8}$
2vaccine-2	0.23	(close to die)	$4. 4x10^{7}$	0.52	(close to die)	$4.6x10^{7}$
2vaccine-3	0.16		0	0.35		0
AND-1	0.51	(close to die)	$3.1x10^{7}$	0.44	(close to die)	$1.3x10^{8}$
AND-2	0.25		$4.1x10^{4}$	0.48		$2.5x10^{4}$
AND-3	0.11		0	0.45		0
AND+2vaccine-1	0.12		0	0.39		$3.4x10^{2}$
AND+2vaccine-2	0.12		0	0.39		0
AND+2vaccine-3	0.13		0	0.31		0
APE-1	0.48	(close to die)	$1.8x10^{8}$	0.60	(close to die)	$1.1x10^{8}$
APE-2	0.13		0	0.30		0
APE-3	0.12		0	0.37		0
APE+2vaccine-1	0.11		0	0.37		0
APE+2vaccine-2	0.11		0	0.36		0
APE+2vaccine-3	0.12		0	0.33		0

Appendix J: Mouse weight post-treatment with APE or AND (gram)

Groups	1day	4 days	7 days	10 days	13 days	16 days	19 days	22 days	25 days
PBS-1	18.1	18.1	18.4	18.5	18.7	18.7	19	19	19.4
PBS-2	17.8	17.9	18	18.4	18.5	18.9	18.9	19.2	19.5
PBS-3	17.9	18.1	18.5	18.6	18.8	18.7	19.1	19.2	19.6
PBS-4	17.7	17.7	18.2	18.3	18.5	18.6	18.7	18.9	19.2
PBS-5	18	18.1	18.3	18.5	18.8	18.5	18.6	19	19.7
APE+2vaccine-1	18.2	18.8	17.4	18.3	19	19.4	19.8	18.7	19.7
APE+2vaccine-2	18	18.4	17.1	17.8	18.2	18.6	18.9	18.2	19.4
APE+2vaccine-3	18.7	18.9	18.1	18.7	19.1	19.3	19.9	18.6	19.6
APE+2vaccine-4	18.1	18.7	17.3	17.9	18.8	18.8	19.2	18.6	19.3
APE+2vaccine-5	17.9	18.5	17.5	18.2	18.9	19.3	19.7	19.1	19.8
AND+2vaccine-1	18.1	18.9	17.4	17.9	18.8	19.1	19.7	18.5	19.5
AND+2vaccine-2	18	18.7	17.1	17.7	18.6	18.6	19.2	18.4	19.1
AND+2vaccine-3	18.3	18.9	17.8	18.1	18.9	19.3	19.8	18.7	19.7
AND+2vaccine-4	17.9	18.3	17.2	17.6	18.5	18.9	19.2	18.5	19.4
AND+2vaccine-5	17.8	18.4	17.7	18.2	18.9	19.4	20	19.2	19.8
APE-1	17.8	18.4	18.8	18.8	18.9	19	19.4	19.5	19.9
APE-2	17.7	18.2	18.3	18.5	18.7	18.8	19.1	19.3	19.5
APE-3	18.5	18.9	19.2	19.3	19.4	19.3	19.5	20	20.1

APE-4	17.1	17.6	18.1	18.4	18.4	18.7	19	19.3	19.6
APE-5	17.6	18.1	18.6	19.1	19.3	19.5	19.7	19.8	20
AND-1	18.4	18.8	19	19.1	19.1	19.1	19.4	19.4	20.1
AND-2	18	18.3	18.7	19	18.9	19.2	19.2	19.3	19.6
AND-3	18.5	18.5	18.9	18.9	19	19	19.2	19.5	19.5
AND-4	18.6	18.9	19.2	19.4	19.4	19.7	19.9	20	20.2
AND-5	17.7	18.4	18.7	18.8	19	18.9	19.1	19.3	19.6
2vaccine-1	17.5	17.9	16.6	17.4	17.8	18.2	18.6	17.6	18.7
2vaccine-2	17.5	18	17.1	17.2	18.2	18.7	18.9	18	19.3
2vaccine-3	18.1	18.5	17.6	17.8	17.9	18.1	18.5	18.5	18.8
2vaccine-4	18.1	18.3	17.2	17.7	18	18.2	18.7	18.6	18.7
2vaccine-5	17.1	17.7	16.3	16.9	17.5	17.8	18.1	18.4	18.6

Appendix K: Chemicals

The companies and relevant chemicals used in this research project are listed in the followings:
Sigma-Aldrich: Standard andrographolide (98%); ethyl alcohol (food grade); charcoal activated; 3,5-dinitrobenzoic acid; potassium hydroxide; methanol; ethyl acetate; glycerol; ethylenediamine tetraacetic acid (EDTA); polyoxyethylenesorbitan monolaurate (Tween 20); phenylmethylsulfonyl fluoride (PMSF); polymyxin B; sodium chloride; formaldehyde (37 wt. %) solution in water
AnalaR: Dichloromethane
Ajax: Toluene; boric acid
Gibco: HEPES buffer solution (1M); phosphate buffered saline (PBS, pH 7.2)
BDH: 2-mercaptoethanol
ICN: Sodium hydroxide
Merck: A precoated plate of silica gel 60F254
Baxter: 0.9% sodium chloride (0.9% saline)
BBL®/ Becton Dickinson: Commercial antibiotic-impregnated disks as follows:
ST: streptomycin (100µg); P10: penicillin G (10IU); VA30: vancomycin (30µg); E15: erythromycin (15µg); FOX30: cefoxitin (30µg); SXT: sulfamethoxazole/trimethoprim (23.75:1.25µg); AM10: ampicillin (10µg); GM10: gentamicin (10µg)

Appendix L: Publications