IN VIVO EVALUATION OF IMMUNOMODULATORY PROPERTIES OF CRUDE EXTRACTS OF *ECHINACEA* SPECIES AND FRACTIONS ISOLATED FROM *ECHINACEA PURPUREA*

Katherine Marie Spence
Bachelor of Arts and Science
University of Southern Queensland
Toowoomba Queensland
1999

Submitted to the Faculty of Biological and Physical Sciences, University of Southern Queensland in partial fulfilment of the requirements for the Degree of Master of Science 2002

STRICTLY CONFIDENTIAL

Certification of Thesis

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

Signature of candidate Date

Endorsement

Signature of Principle Supervisor Date

ABSTRACT

IN VIVO EVALUATIONS OF IMMUNOMODULATORY PROPERTIES OF CRUDE EXTRACTS OF *ECHINACEA* SPECIES AND COMPOUNDS ISOLATED FROM *ECHINACEA PURPUREA*

Katherine Marie Spence
University of Southern Queensland
2002

Supervisors:
Prof T. K. Mukkur
Dr Ray Marshall

This thesis describes the *in vivo* evaluation of orally administered extracts *of Echinacea* species, and fractions obtained from *Echinacea purpurea* for specific immunostimulatory function induced in Balb/c mice by immunization with microbial vaccines. Two vaccines were used for this purpose in this study. The first vaccine used consisted of whole-cell formalin-killed *Salmonella typhimurium* vaccine, which is known to induce production of specific antibodies but no delayed-type hypersensitivity response considered as an indicator of the cell-mediated immune response. The second vaccine was a commercial acellular pertussis vaccine (DTaP) consisting of inactivated diphtheria and tetanus toxoids and native or inactivated virulence factors of *Bordetella pertussis* (the etiological agent of whooping cough) which is known to induce a humoral immune response but with controversial reports about performance in the induction of cell-mediated immunity. Both vaccines were administered intraperitoneally, whereas the herbal extracts and fractions were administered by oral gavage. The phytochemical profile of the ethanolic extracts of *Echinacea purpurea, Echinacea pallida, Echinacea angustifolia* and one commercial product "Echinacea Triplex" and three fractions prepared from *Echinacea purpurea,*

namely polysaccharides, phenolics and alkylamides, was determined by thin-layer chromatography, high-performance liquid chromatography and/or by chemical analytical techniques. Statistically significant increases in specific anti-*Salmonella typhimurium* serum antibody were obtained for the *Echinacea purpurea*, *Echinacea angustifolia* and Echinacea Triplex™ treatment groups, and also for groups receiving treatment with fractions obtained from *Echinacea purpurea,* namely, polysaccharide, alkylamide and phenolic fractions. Statistically significant increases in the anti-*B pertussis* serum antibodies were also obtained in the young Echinacea Triplex™ group vaccinated with the DTaP vaccine. Significant enhancement of serum interleukin-12 titres was observed in the Echinacea Triplex™ as well as the phenolic fraction treatment groups which were vaccinated with killed *Salmonella typhimurium* vaccine. Of the groups vaccinated with the DTaP vaccine, only Echinacea Triplex™ displayed statistically significant increases in mean interleukin-12 titre compared to the placebo in both young and old groups, however *Echinacea purpurea,* and *Echinacea angustifolia* both displayed significant increases in mean interleukin-12 titre in the young treatment mice. Increases in interferon-γ levels in mice orally dosed with phenolic, polysaccharide or alkylamide fractions of *Echinacea purpurea* and vaccinated with the killed *Salmonella typhimurium* vaccine were also observed, however these need to be repeated for confirmation, and statistical analysis.

Acknowledgments

I would like to thank my primary supervisor Professor T. K. Mukkur for his support, enthusiasm, motivation and guidance throughout this project. I also thank Dr Ray Marshall for his patience, good-humor and guidance. Thank you to the other members of T. K.'s research team, in particular Sonja Wapstra for her dedicated professionalism in animal handling, and technical assistance, and Scott Kershaw for his tireless technical assistance particularly near the culmination of the project. My thanks also go to Maryanne Genrich (Greenridge Botanicals), without her expertise a part of this project could not have been completed. I would like to thank Robert Watson (Greenridge Botanicals, a division of Thursday Plantation Laboratories, Pty Ltd) and Phillip Bradley (Thursday Plantation Laboratories Pty, Ltd) for their vision, guidance and assistance. For their assistance in animal maintenance, patience and congeniality, I extend my thanks to Julie Murphy and Sandra Sharp. I also thank Ruth Hilton for your patient assistance and guidance with administration conundrums.

This project would not have been possible without the financial assistance of Thursday Plantation Laboratories Pty Ltd.

Finally, but most importantly, I would like to thank my family for their support, especially Jarl. I would not be who I am today without you.

There are many people who remain unnamed whose friendship and encouragement helped me through a very difficult time.

Abbreviations

*aro*A	mutant unable to make aromatic amino acids
BP	British principles
C	Centigrade
CFU	Colony-forming units
CMI	Cell-mediated immunity
DBA	Diphenylboric acid, beta-ethylamino ester
DTaP	Diptheria, tetanus, acellular pertussis vaccine
EDTA	Ethylenediamine Tetraacetic Acid
ELISA	Enzyme-linked immunosorbent assay
FHA	Filamentous haemagglutin
FBS	Fetal bovine serum
FP	Fresh plant
HRP	Horseradish Peroxidase
HPLC	High performance liquid chromatography
IFN-γ	Interferon gamma
IgA	Immunoglobulin Class “A”
IgE	Immunoglobulin Class “E”
IgG	Immunoglobulin Class “G”
IgM	Immunoglobulin Class “M”
IL	Interleukin
KLH	Keyhole limpet hemocyanin
LB	Luria Bertani
Lf	Floculating units

LPS	Lipopolysaccharide
M	Molar
m	milli (10^{-3})
μ	micro (10^{-6})
MIC	minimal inhibitory concentration
min	Minutes
MHC	Major histocompatibility complex
n	nano (10^{-9})
NaOAc	Sodium acetate
NK	Natural killer
OMP	Outer membrane protein
PEG	Polyethylene glycol-4000
PBS	Phosphate buffered saline
PT	Pertussis toxoid
R_f	Retardation factor
rpm	Revolutions per minute
SE	Standard error
SRBC	Sheep red blood cells
Tc	Cytotoxic T cells
T_H1	T helper 1 type lymphocyte
T_H2	T helper 2 type lymphocyte
TNF	Tumor necrosis factor
URTI	Upper respiratory tract infections
WP	Whole plant

CONTENTS:

List of figures

List of tables

Abbreviations

*aro*A	aromatic-dependent mutant
BP	British Pharmacopoeia
C	Centigrade
CFU	Colony-forming units
CMI	Cell-mediated immunity
DBA	Diphenylboric acid, beta-ethylamino ester
DTaP	Diptheria, tetanus, acellular pertussis vaccine
EDTA	Ethylenediamine tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FHA	Filamentous haemagglutin
FBS	Fetal bovine serum
FP	Fresh plant
HRP	Horseradish peroxidase
HPLC	High performance liquid chromatography
IFN-γ	Interferon gamma
IgA	Immunoglobulin Class A
IgE	Immunoglobulin Class E
IgG	Immunoglobulin Class G
IgM	Immunoglobulin Class M
IL	Interleukin
KLH	Keyhole limpet hemocyanin
LB	Luria Bertani
Lf	Flocculating units

LPS	Lipopolysaccharide
M	Molar
m	milli (10^{-3})
μ	micro (10^{-6})
MIC	minimal inhibitory concentration
min	Minutes
MHC	Major histocompatibility complex
n	nano (10^{-9})
NaOAc	Sodium acetate
NK	Natural killer
OMP	Outer membrane protein
PEG	Polyethylene glycol-4000
PBS	Phosphate buffered saline
PT	Pertussis toxoid
R_f	Retardation factor
rpm	Revolutions per minute
SE	Standard error
SRBC	Sheep red blood cells
Tc	Cytotoxic T cells
T_H1	T helper 1 type lymphocyte
T_H2	T helper 2 type lymphocyte
TNF	Tumor necrosis factor
URTI	Upper respiratory tract infections
WP	Whole plant

Chapter 1: Introduction

This study represents a novel approach to the *in vivo* evaluation of the immunomodulatory properties of *Echinacea* species, and purified components of *Echinacea purpurea*. Unlike previous studies, which have used sheep red blood cells (SRBC), human serum, keyhole limpet hemocyanin (KLH) or killed tumour cells as antigens, it was decided to determine the immunomodulatory effect of *Echinacea* species and major components on specific immunity induced in mice by immunisation with microbial vaccines. Two vaccines were used for this purpose in this study. Whole-cell formalin-killed *Salmonella typhimurium* vaccine is known to induce production of specific antibodies, but not delayed-type hypersensitivity (DTH) used as an indicator of cell-mediated immunity (CMI). If the CMI was promoted by the administration of *Echinacea* species by the oral route, mice vaccinated with the killed *Salmonella typhimurium* vaccine should induce mediators such as interleukin-12 or interferon-γ, which are considered to be indicators of CMI. The second vaccine was an acellular pertussis vaccine (DTaP), the CMI-inducing potential of which is still under scrutiny. If the oral administration of *Echinacea* species in DTaP-vaccinated mice promoted both the CMI and humoral immune responses, an induction or increase in the cytokine responses and humoral antibody response would be expected.

This study is also distinguished from others by the method of application of the *Echinacea* extracts. In many studies, the extracts have been administered by

intramuscular, intravenous, or intraperitoneal routes, which is in direct conflict with the recommended way of delivering traditional medicines. In other studies, the extracts have been given orally via drinking water, soaked pellets or by oral gavage in many cases for a short period of a few days. These methods of application make calculation of the quantity of herb or extract consumed difficult, which is often complicated by the fact that the phytochemical profile of the extract has not been presented. Mice in this study received a daily oral gavage with known quantities of the herb or fraction for a period of 35 days (7 days prior to vaccination, and 28 days post-vaccination) in a regime intended to mimic the recommended dose regime of commercial extracts for human use.

Another important aspect of this study was the focus on characterising the phytochemistry of the extracts. However, in the initial stages of this study the extracts were not chemically analyzed. This decision was taken for experimental logistical reasons because if the extract did not have any immunomodulatory activity, knowledge of the phytochemical profile would have been futile. This first study did, however, narrow the search for the bioactive principles or compound in a step-wise process i.e., from the extract, to crude fractions, to purified fractions and compounds, which ensured a logical and progressive elimination of compounds and isolation of all biologically active compounds as will become evident from the final outcomes of this project.

Chapter 2: Literature Review

2.1 *Echinacea* Species

2.1.1 Botanical aspects and traditional use

The genus *Echinacea* Moench (Compositae) belongs to the *Heliantheae* that is the largest tribe within the Compositae (Asteracae) family and is endemic to North America, where it occurs in the Great Plains between the Appalachian Mountains in the east and the Rocky Mountains in the west. Of the 11 species of *Echinacea,* three are commonly used for medicinal purposes. These are *Echinacea purpurea, Echinacea angustifolia* and *Echinacea pallida.* However, historically, other species may have been used (Bauer, 2000). The use of *Echinacea* can be traced to the native Americans, who used the expressed juice to treat wounds, snakebites, headache and the common cold (Moreman, 1998).

In the mid-1800's *Echinacea* was introduced to European settlers in America, distributed by HGF Meyer, a German quack doctor. The extract was an *Echinacea angustifolia* tincture, named "Meyers Blood Purifier," which was purported to be effective in the treatment of rheumatism, neuralgia, headache, erysipelas, dyspepsia, tumours, boils, open wounds, vertigo, scrofula, "bad eyes," "poisoning by herbs" and rattlesnake bites (Bauer, 2000; Lloyd, 1904). *E pallida* was introduced to the European settlers during the early 1900's, when the Lloyd Brothers started to produce large-scale tinctures. The roots of both species were officially listed in the monograph of the National Formulary of the US in 1916, resulting in the two species

often being used interchangeably even in recent times (Bauer and Wagner, 1991; Schindler, 1940). *Echinacea purpurea* has been used by Europeans in America to treat saddle-sores on horses and syphilis since the mid-1800's, but its usage was less common than the other two species (Bauer, 2000). Identification and authentication of the three medicinal species of *Echinacea* has been problematic in the past, not only with the confusion between species, but also with adulteration of extracts with *Parthenium integrifolium* roots. In fact, sesquiterpene esters which characterise *Parthenium integrifolium* were originally described as constituents of *Echinacea purpurea* (Bauer et al., 1985). Subsequently, chemical identification techniques to ensure the integrity and authenticity of extracts have been devised.

2.1.2 Chemistry and standardisation methods

The achenes (fruits) of *Echinacea* species can be differentiated by their chemical constituents. All three species produce dodeca-2E,4E,8Z,10E(10Z)-tetraenoic acid isobutylamide as well as a number of other alkylamides during germination. However, the 2-monoene alkylamides and polyacetylenes that characterise the mature plants are not found in the achenes at germination (Schulthess et al., 1991).

The mature *Echinacea purpurea* plant contains at least nine alkylamides in the roots (Bauer and Remiger, 1989; Bauer et al., 1988a; He et al., 1998). Fifteen alkylamides have been isolated, and characterised from the roots of *Echinacea angustifolia*, while *Echinacea pallida* roots are almost void of amides (Bauer et al., 1989). However, the aerial parts of these three species are very similar in terms of alkylamide content (Bauer and Remiger, 1989). The three species can be characterised by thin-layer chromatography and high-performance liquid chromatography, by comparing not

only the alkylamides, but also comparing polyacetylenes, polyenes, and the caffeic acid derivatives (phenolic acids) for which each species has its own "fingerprint" (Bauer and Remiger, 1989; Pomponio et al., 2002). All three medicinal species of *Echinacea* also contain polysaccharides, of particular interest being arabinogalactans (Proksch and Wagner, 1987). However there is currently no method available for routine quantification of these polysaccharides. Although several methods have been suggested (Proksch and Wagner, 1987; Wagner, 1997), none possess both the efficiency and specificity necessary for a routine analysis technique. It has been suggested that a method utilising fluorescence or radioactivity labeled polysaccharides or antibodies for a specific assay would be more appropriate for the quantification of specific polysaccharides (Bauer, 2000).

Knowledge of the chemical profiles of the three medicinal species has allowed the assessment of the potential of other *Echinacea* species for medicinal use. For example *E paradoxa* contains several ketoalkenyes, and echinacoside (found in *E angustifolia*) (Facino et al., 1991) and has almost identical constituents as *E pallida. E stimulata* contains alkylamides and echinacoside found in *E angustifolia* and also ketoalkenynes as in *E pallida* (Bauer and Foster, 1991).

2.1.3 Biological activity of *Echinacea* extracts

Much of the work into the biological activity of *Echinacea* extracts is compromised by the fact that the phytochemical profile of the extracts is unknown. For many of the extracts, the extraction procedure is not specified or the extract is a non-standardised complex mixture of unknown compounds, and in some cases a mixture of more than

one herb. Also, many experiments testing for immunomodulatory action discussed below have not tested the oral efficacy of the extract, as per the traditional use.

2.1.3.1 Toxicology

After 4 weeks of oral administration of doses equivalent to many times the human therapeutic dose, the expressed juice of *Echinacea purpurea* proved virtually non-toxic to rats and mice. *In vitro* tests for mutagenicity and carcinogenicity also gave negative results (Mengs et al., 1991).

2.1.3.2 Non-specific and cellular immune systems

A purified compound obtained from an aqueous extract of *E angustifolia*, "Echinacosid," was reported to have no detectable effect on non-specific and cellular immunity of the mouse after intraperitoneal, intravenous or peroral application, as gauged by the carbon-clearance test (Schumacher and Friedberg, 1991). This was in contrast to the results obtained by Wagner and Juric (1991) who compared a mixture of ethanolic-aqueous extracts of *Echinacea angustifolia, Eupatorium perfoliatum, Baptisia tinctoria* and *Arnica montana* to a pure *Echinacea angustifolia* extract for increased stimulation of phagocytosis, tested using the *in vitro* granulocyte and the *in vivo* carbon-clearance tests in the mouse model. The extract combination showed, in both test models, a higher efficiency than the pure *Echinacea angustifolia* extract alone (Wagner and Juric, 1991). A major weakness of this study was their failure to characterise the phytochemistry of the extracts.

A more recent study investigated the macrophage-activation properties of standardised *Echinacea angustifolia* extracts using a simulated digestion protocol to

emulate oral dosing. The extracts were standardised to phenolic acid or echinacoside content and were shown to be inactive as macrophage-activation agents, as measured by secretions of tumor-necrosis factor alpha, interleukin (IL) -1alpha, IL-1beta, IL-6, IL-10 and nitric oxide (Rininger et al., 2000). However, in the same study non-standardised *Echinacea angustifolia* herb and root powders were shown to stimulate murine macrophage cytokine secretion as well as significantly enhance the viability and proliferation of human peripheral blood mononuclear cells *in vitro,* measured using the same immunological parameters (Rininger et al., 2000). However, a similar study conducted using tumor patients, measuring almost identical immune parameters, concluded that the extract tested (a mixture of aqueous-ethanolic extracts from *Echinacea angustifolia, Eupatorium perfoliatum,* and *Thuja occidentalis,* "Echinacea complex") had no detectable immunological effect (Elsasser-Beile et al., 1996). Again the complex mixture of herbal extracts had not been chemically characterised.

A study conducted by Bauer et al. (1988b) showed that aqueous-ethanolic extracts of *Echinacea purpurea, E pallida,* and *E angustifolia* roots all enhanced phagocytosis significantly, and that the lipophilic fractions of the extracts showed greater activity than polar fractions, when assessed by the carbon clearance test. Significantly, all extracts in this experiment were analysed by high performance liquid chromatography, allowing direct association between a phytochemical profile and biological effect. Another study supporting the efficacy of *Echinacea* as a stimulator of non-specific or cellular immune systems, showed that oral doses of powdered *Echinacea purpurea* leaves increased the mean survival age of experimental AKR/J

mice, and also increased their production of endogenous interferon-γ, while levels of interleukin-12 and tumor-necrosis factor-α were unaffected (Hayashi et al., 2001).

2.1.3.3 Specific antibody responses

Echinacea extracts have been shown to have an effect on specific immunity in several studies, although the experimental design in some or analysis in others compromised interpretation of the data obtained. Rehman et al. (1999) showed that the oral treatment of rats with a commercially available aqueous-ethanolic extract of *Echinacea angustifolia* root (Eclectic Institute, Inc, Sandy) significantly increased primary and secondary IgG response to a novel antigen, keyhole limpet hemocyanin (Rehman et al., 1999). While the extract was administered orally, it was presented in the drinking water, making calculation of the dosage of the extract consumed difficult. Also, the phytochemical profile of the extract was not determined, and the extraction procedure not specified. Similarly, Bodinet and Freudenstein (1999) showed that a mixture of aqueous-ethanolic extracts of *Thuja occidentalis* herba, *Baptisie tinctoriae* radix, *Echinacea purpurea* radix and *Echinacea pallidae* radix augmented the specific antibody response to sheep blood cells in mice, as well as inducing and increasing the numbers of splenic plaque forming cells. Again, determination of the quantity of the extract consumed was difficult as the extracts were administered via the drinking water; and the unknown phytochemical profile complicated by the presence of multiple herbs. A recent study by South and Exon (2001) however, found no evidence of enhanced specific antibody response in rats fed a commercial *Echinacea* preparation, consisting of Nabisco crackers soaked in glycerol or ethanolic extracts. Conversely, a study of chickens immunised with human serum found a significant increase in three classes of antibody in the group fed *Echinacea angustifolia* extract

(Schranner et al., 1989). However, the phytochemical profile of the extract was not presented, and the chickens only received two oral doses of the extract, rather than a continuous regime as recommended for many commercial preparations.

2.1.3.4 Delayed-type hypersensitivity and natural killer cell activity

South and Exon (2001) also assessed a commercial *Echinacea* product for promotion of natural-killer (NK) cell activity and T cell-mediated delayed-type hypersensitivity, and found no evidence for the enhancement of either of these activities in rats. However, Currier and Miller (2002) found that natural-killer cells were significantly elevated in leukemic mice immunised with killed erythroleukemia cells, receiving *E purpurea* in their diet compared to those receiving untreated chow. The mice receiving daily treatment with *E purpurea* also had longer life spans, than those not receiving *E purpurea*. Unfortunately no comparison between these investigations could be made owing to the lack of information on the phytochemical profiles of the *Echinacea* preparations.

See et al. (1997) reported enhancement of natural killer cell function and antibody-dependent cellular cytotoxicity *in vitro* by human peripheral blood mononuclear cells against human herpesvirus 6 infected H9 cells that had been stimulated with extracts of *Echinacea purpurea*. Sun et al. (1999) found that dietary addition of *Echinacea* root increased cell numbers of natural killer cells and monocytes in the bone marrow and spleen of mice after only week of treatment. However, the phytochemical profile of the herb was not presented. Coeuginet and Elek (1987) also described the *in vitro* stimulation of cell-mediated immunity by extracts of *Viscum album* and *Echinacea*

purpurea, as judged by lymphokine production and transformation test, again the phytochemical profile of the extract was not presented.

2.1.4 Biological activity of isolated compounds

2.1.4.1 Biological activity of polysaccharides

Bonadeo et al. (1971) (extracted from Tragni et al., 1985) who observed a skin-repairing action of polysaccharides found in *Echinacea* species, proposed that the skin-repairing action of the roots may be due to the formation of a complex between hyaluronic acid and a polysaccharidic principle. The polysaccharides obtained from *Echinacea* have also been shown to possess anti-inflammatory activity, with 0.5mg kg^{-1} intravenous almost completely inhibiting the carrageenan-induced oedema over 8 h and also inhibiting mouse ear oedema induced by croton oil, when topically applied (Tubaro et al., 1987).

Polysaccharides isolated from large-scale plant cell cultures of *Echinacea purpurea*, have been shown to activate a broad variety of cytotoxic and secretory functions of murine and human macrophages (Leuttig et al., 1989; Roesler et al., 1991a; Roesler et al., 1991b; Stimpel et al., 1984) *in vitro*. Roesler et al. (1991a, b) found that human macrophages were activated to secrete TNF-α, IL-6, and IL-1, while class II expression was unaffected on stimulation with polysaccharides purified from *E purpurea*. In the same study, the purified polysaccharides were also shown to inhibit growth of *Candida albicans, in vitro* (Roesler et al., 1991a). Furthermore, *in vivo* the polysaccharides, when administered intravenously, induced increased proliferation of phagocytes in spleen and bone marrow and migration of granulocytes to the peripheral blood, which resulted in excellent protection of mice against lethal

infections with *Listeria monocytogenes* and *C. albicans* (Roesler et al., 1991b). However, the antibody response to sheep red blood cells and to Listeria (DTH) were not affected by the polysaccharides (Roesler et al., 1991b).

Leuttig et al. (1989) confirmed the results reported by Roesler et al. (1991b) that the arabinogalactans, administered intraperitoneally to mice induced macrophages to produce tumor necrosis factor (TNF-α), interleukin-1, and interferon-β2. However, no activation of B cells, or induction of T cells to produce IL-2, or interferon- β2 was observed (Leuttig et al., 1989). Proksch and Wagner (1987) showed similar results, which showed that a homogenous 4-*O*-methyl-glucuronoarabinoxylan purified from *Echinacea purpurea* enhanced phagocytosis, as assessed by the *in vitro* granulocyte test.

While a crude hemicellulosic polysaccharide fraction was observed to enhance phagocytosis of carbon particles *in vivo* (Wagner et al., 1985), no enhancement was observed *in vivo* using the homogenous 4-*O*-methyl-glucuronoarabinoxylan isolated by Proksch and Wagner (1987). On the other hand, arabinogalactans purified from *Echinacea purpurea* according to the method developed by Wagner et al. (1984b), have been shown to stimulate macrophages, increasing their cytotoxicity to tumor targets (Stimpel et al., 1984; Wagner et al., 1988). Arabinogalactans belong to the group of water-soluble, acidic branched-chain heteroglycans, and have molecular weights in the range 25 000 to 500 000 (Wagner et al., 1985). They have been shown to increase the production and secretion of oxygen radicals and interleukin 1 by macrophages, with no effect on T lymphocytes (Stimpel et al., 1984; Wagner et al., 1985).

In mice with a defective T-cell system caused by CsA [cyclosporin A affects specific responses of the immune system as it blocks the stimulating function of CD4-positive lymphocytes during an antigen-specific immune response (Steinmuller et al., 1993)] or CP [cyclophosphoamide blocks the proliferation of precursors or PMN leukocytes (Steinmuller et al., 1993)], polysaccharides isolated from *Echinacea purpurea* were shown to enhance host defense against microbial infections. Also, after injection of CP, the mice treated with polysaccharide displayed an earlier reconstitution of the number of peripheral granulocytes than the controls (Steinmuller et al., 1993). The polysaccharides were also shown *in vitro* to activate macrophages to function as cytotoxic effector cells against WEHI 164 fibrosarcoma tumour targets and also against *Leishmania enrietti* (Steinmuller et al., 1993).

Antibodies raised in rabbits against arabinogalactan-proteins isolated from extracts of *Baptisia tinctoria, Echinacea purpurea* and *Thuja occidentalis* were shown to possess structural differences in their antigenic regions. The specific antibodies generated were uninhibited by the other glycoprotein antigens or by lipopolysaccharide isolated from *Salmonella typhimurium* and from *Escherichia coli* in a competitive enzyme-linked immunosorbent assay (ELISA) (Egert and Beuscher, 1992).

2.1.4.2 Biological activity of alkylamides

Muller-Jakic et al. (1994) isolated and tested eight alkylamides from *Echinacea angustifolia* for inhibitory activity in *in vitro* 5-lipoygenase and cyclooxygenase assays. [The latter two enzymes are the key enzymes of the two major pathways of arachidonic acid metabolism, responsible for the formation of leukotrienes and

prostaglandins respectively (Church and Robinson, 1988)]. It was discovered that the inhibitory activity was dependent on the structure of the alkylamides, with the polyunsaturated alkylamides yielding the greatest inhibition.

A rat-model was used by Goel et al. (2002) to examine the immunomodulatory effects of three components of *Echinacea purpurea,* namely cichoric acid, polysaccharides, and alkylamides. Following administration of the purified extracts by oral gavage, (two times/day for 4 days), it was reported that alkylamides derived from *E. purpurea* significantly increased phagocytic activity as well as phagocytic index of the alveolar macrophages, and an increase in TNF-α and nitric oxide production by the alveolar macrophages after *in vitro* stimulation with lipopolysaccharide (LPS). None of the components at any concentration had a measurable effect on the release of IFN-gamma or IL-2 by the splenocytes.

2.1.4.3 Biological activity of phenolics

The main polyphenols present in considerable amounts in the polar fractions of *Echinacea* species are the caffeoyl derivatives (echinacoside, chlorogenic acid, chicoric acid, cynarine, and caffeic acid). Caffeoyl derivatives, in particular echinacoside, have been shown to have a strong anti-oxidative effect judged by the protection of Type III collagen from free-radical-induced degradation through a scavenging effect on reactive oxygen species and/or C-, N-, S- centered secondary radicals (Facino et al., 1995).

"Echinacina B", a purified fraction obtained from an aqueous extract of *E angustifolia* roots, was found to be more potent than the positive control, benzidamine, for anti-

inflammatory action when tested using the croton-oil ear oedema test in mice (Tragni et al., 1985). While no attempt to isolate a specific active principle was made in this study, a subsequent study using *Echinacea* extracts, chemically standardised to phenolic acid or echinacoside content and fresh pressed juice preparations, were also found to display anti-inflammatory and antioxidant properties (Rininger et al., 2000).

2.1.5 Clinical trials

2.1.5.1 Allergy

With fifty percent of Australians using complementary and alternative medicines (other than vitamins) in any 12-month period (Eisenberg et al., 1998; Eisenberg et al., 1993; Maclennan et al., 1996), allergy and cross-reactivity with other drugs has become a concern (Pinn, 2001). Echinacea is perhaps the most commonly used plant therapy in Europe and USA, with sales in the USA of $300 million in 1998 (Brevort, 1998). Obviously knowledge of allergenic properties, and cross-reactivity of *Echinacea*-derived compounds and extracts with drugs is extremely important. In 2002, Mullins and Heddle reviewed 26 cases suggestive of possible immunoglobulin E-mediated hypersensitivity to *Echinacea* products (4 anaphalaxis, 12 acute asthma, 10 urticaria/angioderma) of which 78% were female and greater than 50% of these patients were known to be atopic. Some of these atopic subjects yielded positive skin-prick test results in the absence of known exposure to *Echinacea* products. Mullins and Heddle (2002) concluded that, given *Echinacea's* widespread (and largely unsupervised) community use, even rare adverse events become inevitable. It has been shown that there is cross-reactivity between antigenic components of *Echinacea* and some related, and even non-related plants that may cause allergic reaction (Pham and Baldo, 1995). Perhaps of greater concern is evidence that

Echinacea products are influencing the development of antibiotic resistance in bacteria. Products tested by Ward et al. (2002) were reported to be causing large increases in the minimal inhibitory concentration (MIC) to ampicillin over the baseline values.

2.1.5.2 Treatment of upper respiratory tract infections (URTI)

A recent overview of 16 trials of upper respiratory infection involving some 3400 patients given *Echinacea* extracts suggested some benefit, but was considered inconclusive (Melchart et al., 1999). Many of the trials were compromised because the extracts tested were not standardised, and as such may have had different chemical constituents that may have affected the trial outcomes. Also different species of *Echinacea* are used in the trials, and so comparison between trials is difficult (Pinn, 2001).

Giles et al. (2000) conducted a critical review of clinical trials published from 1961-1997. Twelve studies published in that period concluded that *Echinacea* was efficacious for treating the common cold, but the results were unclear due to inherent flaws in study design such as small population size, poorly designed diagnostic criteria, or non-standardised extracts. Of the five trials published since 1997, two showed that *Echinacea* did not prevent, or reduced the severity/duration of URTI symptoms, and three concluded that *Echinacea* was effective in reducing the frequency, duration and severity of common-cold symptoms. However, it was not possible to draw definitive conclusions because of either small populations in the trials, not clearly defined diagnostic criteria, and/or the use of non-standardised

extracts (Giles et al., 2000). A similar opinion had been put forward by Schumacher and Friedberg, (1991) who suggested that most reports which declared the stimulating biological activity of *Echinacea* could not resist any critical assessment of their respective experimental designs, and/or analyses.

Even the more recent clinical trials are plagued with similar problems. Lindenmuth and Lindenmuth (2000) conducted a random assignment double-blind placebo-controlled study to determine the efficacy of a commercial *Echinacea* tea preparation in reducing the severity and duration of cold or flu symptoms. The study concluded that the preparation was effective in relieving cold and flu symptoms in a shorter period of time than a placebo. However the dosages were not standardised, the phytochemical profile of the extract not determined, and only a self-scoring questionnaire was used to arrive conclusions about at the efficacy of the treatments

Another randomised, double-blind, placebo-controlled clinical trial was conducted by Schulten et al. (2001). The median time of illness in the experimental group was 6.0 days compared to 9.0 days in the placebo-control group (one-sided $p=0.0112$). It was concluded that the *Echinacea* extract was clinically effective in alleviating symptoms more rapidly than placebo in patients with a common cold. However the population size was small, and the phytochemical profile of the extract not determined. These results are in contrast to those obtained by Turner et al. (2000) who found that a standardised *Echinacea* extract containing 0.16% cichoric acid, and almost no echinacosides or alkylamides had no significant effect on either the occurrence of the common cold due to rhinoviruses or the severity/duration of the illness.

However, a trial conducted by Kim et al., (2002) which also tested *E purpurea* and *E angustifolia* extracts standardised according to the phenol content, showed an increased production of complement properdin after four weeks of treatment compared to the control group. The extract did not have any measurable effect on blood pressure, radial pulse, respiration rate, temperature, total white blood cell count, NK cell count, TNF-α, Epstein-Barr Virus viral capsid antigen, IgG antibody or on a subjective report on health-related quality of life.

2.2 Vaccines used in this investigation

Previous investigations on the immunomodulatory properties of *Echinacea* species have either involved determination of their effect on phagocytosis *in vitro* or clearance of carbon particles (non-specific immunity) *in vivo* or the use of keyhole limpet hemocyanin (KLH), heterologous sheep red blood cells (SRBC) or human serum as antigens. In this project, it was decided to determine the immunomodulatory effect of *Echinacea* species and major components, delivered by the oral route on specific immunity induced in mice by immunisation with microbial vaccines. The two types of vaccines used in this project were (a) whole-cell formalin-killed *Salmonella typhimurium* vaccine and (b) acellular pertussis vaccine, which is currently used for the prevention of whooping cough in children.

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

Despite eliciting high antibody titres, the whole-cell killed *Salmonella typhimurium* vaccine does not provide protection from challenge by virulent organisms, nor does it elicit delayed-type hypersensitivity even where multiple doses are used for

vaccination, whereas live attenuated (*aro*A) *S typhimurium* vaccines protected mice following immunisation with 1-3 doses, depending upon the route of vaccination (Harrison et al., 1997; Mukkur et al., 1987; Mukkur et al., 1995; Mukkur et al., 1991b). In another study, mice immunised with live, attenuated aromatic-dependent S. serotype Bovismorbificans were found to be protected and elicited significant delayed-type hypersensitivity responses to the microorganism (Mukkur et al., 1991a). Similar results were obtained in sheep vaccinated with the live attenuated mutant microorganisms (Begg et al., 1990; Mukkur and Walker, 1992).

Live vaccines have been shown to elicit higher interleukin-2, and interferon-gamma responses in spleens cells than killed vaccines, and also higher IgG2a, indicating a Th1 response (Harrison et al., 1997). Interleukin-12 has been shown to be stimulated by vaccination with the attenuated *S typhimurium* SL3261 *aro*A strain. In fact administration of anti-interleukin-12 antibodies was found to exacerbate mild infections in mice (Mastroeni et al., 1998). Interferon-γ levels were also reduced in sera and tissue homogenates in anti-IL-12 treated mice compared to those in control animals. Inducible nitric oxide synthase and IFN-γ mRNA production were also found to decrease in anti-IL-12 treated mice, with an increased production of IL-10 mRNA. However, administration of recombinant IFN-gamma to anti-IL-12 treated mice restored host resistance (Mastroeni et al., 1998).

It is thus clear that if cell mediated immunity was promoted by the administration of *Echinacea* species by the oral route, mice vaccinated with the killed *Salmonella typhimurium* vaccine should emulate the highly effective CMI response generated by

live attenuated *Salmonella* vaccines and hence the rationale for use of the killed vaccine in this project.

2.2.2 Acellular pertussis vaccine

Two types of pertussis vaccines, which are currently being used, are the killed whole cell vaccine consisting of killed *B pertussis*, chemically-inactivated diphtheria toxoid and genetically- or chemically-inactivated tetanus toxoid (also referred to as DTwP). However the severe side-reactions including encephalopathy, albeit in a small number of cases (1/70,000) caused a reduction in the uptake of immunisation with this vaccine in the community resulting in re-emergence of the whooping cough (pertussis) outbreaks. This led various research groups worldwide to develop an acellular vaccine (referred to as DTaP) although the DTwP is still used quite extensively in the developing or undeveloped countries.

The DTaP vaccine contains three to five (depending upon the manufacturer) components. The three major virulence factors included in the vaccine are filamentous haemagglutinin (FHA), inactivated pertussis toxin (PTxoid) and pertactin (a membrane protein, PRN) (Mills et al., 1998) and was recently introduced into the marketplace. Some vaccines also include 2 other minor adhesins viz., Fim 1 and 2. These factors are combined and emulsified in an aluminium hydroxide adjuvant and sold as the acellular vaccine (referred to a DTaP). Although this vaccine was claimed to cause milder side-reactions and to generate antibody responses in vaccinated mice or children, information on its capacity to induce cell-mediated immune responses, considered to be a requirement for generation of long-term protection, are not

convincing. More recently it has been revealed that an unacceptable number of children given booster vaccinations with the acellular vaccines sold by different manufacturers led to extensive swelling after booster doses of acellular pertussis-tetanus-diphtheria vaccines. This swelling was recently shown to be due to the generation of PT-specific IgE (Rennels et al., 2000) and one possible suggestion made to reduce this side effect was to reduce the number of booster vaccinations but accepting the risk of generating a reduced level of protection (Mills et al., 1998).

The reason for using the DTaP as a vaccine in this project was to determine if the products containing Echinacea extracts, Echinacea extracts alone or the major highly purified components were capable of inducing one or both the effector arms viz., the humoral and/or cell-mediated arms of the immune response as measured by the induction of specific antibody production and production of cell-mediated immunity (CMI)-indicator cytokines, interleukin (IL)-12 and interferon (IFN)-γ respectively.

Chapter 3: Materials and Methods

3.1 Bacterial strains and growth media

Salmonella typhimurium (CS332, *aro*A) and *Bordetella pertussis* Tohama I (1950's Japan/vaccine), kept as stocks in the Department of Biological and Physical Sciences, University of Southern Queensland, were used in this project. Both bacterial strains were maintained in a Microbank® (Prolab, Austin, Texas) storage system at -70ºC in a Biofreezer (Forma Scientific). *Salmonella typhimurium* was subcultured onto Miller's Luria-Bertani (LB) agar (Difco, Detroit, MI), and grown in Miller's Luria-Betani (LB) broth (Difco, Detroit, MI) where necessary. *Bordetella pertussis* was subcultured and grown on Bordet-Gengou agar (Difco, Detroit, MI).

3.2 Herbs

Echinacea purpurea and *Echinacea pallida* were obtained from Greenridge Botanicals (Toowoomba, Qld), whereas *Echinacea angustifolia* was obtained from Woods and Woods (Sydney, NSW). Echinacea Triplex™, which consists of a mixture of *E purpurea, E angustifolia* and *E pallida* extracts (see Section 3.5.5), is a product of Greenridge Botanicals (Toowoomba, Qld); a sample of batch number 50851C (expiry 05/03) was donated by Greenridge Botanicals (Toowoomba, Qld), a Division of Thursday Plantation Laboratories Pty Ltd (Ballina, NSW).

3.3 Animals

Female, specific pathogen free Balb/c mice purchased from the Animal Resource Centre, Perth, WA were used for all experiments in this project. Particulars of the ages, and numbers of mice used are shown in Section 3.7, "*In vivo* evaluations of

immunomodulatory properties of crude herbal extracts or purified fractions." All mice were housed in mouse cages (5 mice per cage) in the general housing area of the Animal House at the University of Southern Queensland. Mice received 12 hours light and 12 hours dark, unlimited mouse/rat chow and water and the temperature was kept constant at 22ºC.

3.4 Vaccine preparation

3.4.1 Whole-cell killed *Salmonella typhimurium* (CS332) vaccine

Ten microlitres (μL) of the stock culture of *aro*A *S. typhimurium* (stored at -70ºC) was streaked for colonies on Miller's LB agar (Difco, Detroit, MI) and incubated overnight at 37ºC. Two hundred millilitres (mL) of Miller's LB broth (Difco, Detroit, MI) was inoculated with a single colony from this plate and incubated with agitation at 37ºC, 150 rpm (Bioline Shaker Incubator, Edwards Instrument Co, Australia) for eighteen hours. A series of dilutions (10^{-4} – 10^{-8}), prepared in LB broth, were plated out onto LB agar and incubated overnight at 37ºC to determine the number of colony forming units (CFU) per mL. Formalin (ICN Biomedicals, Aurora, Ohio) to a final concentration of 1% was added to the broth, which was re-incubated with agitation for 4 hours at 37ºC at 150 rpm. One hundred microlitres of the broth culture was then plated out on LB agar to check for sterility. The remaining broth was centrifuged at 7000 rpm for 20 minutes at room temperature (Beckman Avanti J251 centrifuge, Beckman Instruments, Palo Alto, CA), and washed twice with sterile phosphate buffered saline (PBS, see Appendix A for formulation). The final pellet was resuspended in 50 mL sterile PBS, and stored at 4ºC.

3.4.2 Acellular pertussis vaccine (Infanrix™)

Infanrix™ (SmithKline Beecham, Dandenong, Vic) is a commercial vaccine intended for use on infants for the prevention of whooping cough. Based on the product information, each 5 mL dose contains not less than:

25 flocculating units (Lf) of diphtheria toxoid, (inactivated diphtheria toxin produced by *Corynebacterium diptheriae*)

10 Lf of tetanus toxoid, (inactivated tetanus toxin produced by *Clostridium tetani*)

25 μg of filamentous haemagglutinin (FHA),

25 μg pertussis toxoid (PT), and

8 μg 69 kDa outer membrane protein (OMP), (*Bordetella pertussis*).

The above antigenic components are adsorbed onto 0.5 mg aluminium hydroxide, and are suspended in an isotonic sodium chloride solution.

3.5 Preparation and purification of herbal extracts

3.5.1 Confirmation of authenticity of herbs

The authenticity of the herbs was confirmed by thin-layer chromatography (Appendix B, Thin Layer Chromatography, Method 1).

3.5.2 *Echinacea purpurea* dried whole plant extract ("*Echinacea purpurea* WP 1:1")

The *Echinacea purpurea* whole plant (that is, flowers, stems, leaves, roots) extract was prepared according to the protocol used by Greenridge Botanicals, Pty Ltd for the preparation of their commercial product (Watson, personal communication). Five

hundred grams of milled, dried, whole plant herb was steeped in 2.25 L of 65% ethanol (Recochem, Lytton) for seven days in a sealed container. The herb material was then pressed extensively using nylon gauze to express the maximum volume of extract. The volume recovered was 1060 mL, which, allowing for a 20% press loss, is equivalent to a recovery of 59%. For a 1:1 extract (where 1 g of start herb is equivalent to 1 mL of final extract) the final volume of the extract was calculated to be 295 mL. The extract was then rotary evaporated to 171 mL in a Labrota 4001 (Heidolph) rotary evaporator. 124 mL of 95% food grade ethanol (Recochem, Lytton) was added for a final ethanol concentration of 40%.

3.5.3 *Echinacea pallida* dried roots extract ("*Echinacea pallida* roots 1:1")

The *Echinacea pallida* extract was also prepared according to the protocol used by Greenridge Botanicals, Pty Ltd for use in their commercial products (Watson, personal communication). Five hundred grams of milled, dried roots was steeped in 1.5 L of 45% ethanol in a sealed container for seven days. The herb material was pressed extensively, as previously described, and 905 mL of extract was recovered. Allowing for a 20% press loss, 905 mL is equivalent to a 75% recovery. The calculated final volume was therefore 375 mL. The extract was concentrated to 178 mL in a rotary-evaporator (Labrota 4001, Heidolph), and 197 mL of 95% ethanol (Recochem, Lytton) added for a final ethanol concentration of 50%.

3.5.4 Echinacea angustifolia dried roots extract ("Echinacea angustifolia roots 1:1")

The *Echinacea angustifolia* extract was prepared according to a protocol devised by Robert Watson, Quality Assurance Manager for Greenridge Botanicals Pty Ltd (Division of Thursday Plantation Laboratories Pty Ltd). Three hundred and fifteen grams of milled, dried roots were steeped in 1.26 L of 60% ethanol (95% BP, food grade, Recochem, Lytton) in a sealed container for seven days. The herb was pressed extensively as previously described, and 645 mL extract was recovered. Allowing for a 20% press loss, the recovery was 64%. For a 1:1 extract the final volume was calculated to be 202 mL. The extract was rotary-evaporated (Labrota 4001, Heidolph) to 117 mL, and 85 mL of 95% ethanol (95% BP, food grade, Recochem, Lytton) was added for a final ethanol concentration of 40%.

3.5.5 Echinacea Triplex™

Echinacea Triplex™ is a commercial herbal preparation produced by Greenridge Botanicals, Pty Ltd, Toowoomba, Qld. All experiments with this product were carried out using Batch number 50851C. Echinacea Triplex™ is a mixture of other Greenridge Botanicals extracts, namely: "*Echinacea pallida* 1:1" (Section 3.5.3 for extraction procedure), "*Echinacea angustifolia* 1:1", "*Echinacea purpurea* bolster 3:1", and "*Echinacea purpurea* FP (Fresh plant) 1:2" in the ratio 3:3:4:40. "*Echinacea purpurea* bolster 3:1" is prepared by steeping milled, dried aerial parts of *Echinacea purpurea* in 95% ethanol for seven days. The extract recovered from the herb is then concentrated to produce an extract equivalent to 3 g dry herb per mL. The extract has a final ethanol concentration of 70%. "*Echinacea purpurea* FP 1:2" is

a mixture of three separate extracts. Firstly, the fresh (not dried) aerial parts of *Echinacea purpurea* are extracted in 25% ethanol for 24 hours. The supernatant is then removed from the herb. Secondly, additional ethanol is then added to the herb to a final concentration of 60% ethanol. The herb is allowed to steep for 72 hours, after which it is pressed and added to the initial extract. The third extract is made from the fresh, milled roots of *Echinacea purpurea*, and is carried out in 50% ethanol. The aerial and root extracts are combined in a ratio of 9:1 dry weight equivalent of the herb. The finished liquid contains 40% ethanol and 5% glycerin.

3.5.6 "Triplex innovation"

"Triplex innovation" was a mixture of equal volumes of *Echinacea purpurea* WP 1:1, *Echinacea angustifolia* roots 1:1, and *Echinacea pallida* roots 1:1. It was used to observe any synergistic effect that the compounds from different species may have, and to compare to Echinacea Triplex™ (Greenridge Botanicals, Toowoomba, Qld), which is made from the same herbs but extracted by an entirely different method (see Section 3.5.5).

3.5.7 Purification of polysaccharides

Polysaccharides were purified from an alkaline extract according to Proksch and Wagner (1987). Five hundred and eighty-one grams of ground, dried, whole-plant *Echinacea purpurea* (Greenridge Botanicals, Toowoomba, Qld) was exhaustively extracted in methanol (Sigma, St Louis, MO) under reflux in a Soxhlet apparatus for 4 days and the extract discarded. The remaining plant residue was dried for 48 hours at 40ºC. The dried plant residue (539 g) was twice extracted overnight with 0.5 M

aqueous NaOH (5 mL/g plant material) without agitation, at 4ºC. These extracts were combined, then centrifuged at 10 000 rpm for 10 minutes (Beckman Avanti J251 centrifuge, Beckman Instruments, Palo Alto, CA) to remove insoluble material, leaving a volume of 2.55 L. 4 volumes 95% non-denatured, food-grade ethanol (95% BP, food grade, Recochem, Lytton) was then added to the extract, under constant stirring. The mixture was allowed to stand for 24 hours at 4ºC, then centrifuged at 10 000 rpm for 10 minutes. The supernatant was discarded, and the pellet resuspended in 1.7 L water. Under constant stirring over ice, aqueous 15% trichloro-acetic acid was added (Sigma, St Louis, MO), then the mixture allowed to stand for 1 hour at room temperature. After centrifugation at 10 000 rpm for 10 minutes, 3 volumes of 95% food grade ethanol was added to the supernatant under constant stirring. The mixture was allowed to stand for 24 hours at 4ºC, then centrifuged again at 10 000 rpm for 10 minutes. The supernatant was discarded, and the pellet resuspended in 500 mL 2% aqueous NaOAc. The solution was centrifuged to remove any insoluble material at 10 000 rpm for 10 minutes. 1 volume 95% food-grade ethanol (Recochem, Lytton) was then added under constant stirring, the solution allowed to stand for 62 hours at 4ºC. The solution was centrifuged at 10 000 rpm for 10 minutes, and the supernatant discarded. The precipitate was dissolved in 295 mL water, and dialysed against water for 3 days. The concentration of total carbohydrates in this crude polysaccharide fraction was estimated by the phenol-sulphuric total carbohydrate method (see Appendix B).

The crude polysaccharide fraction was analysed by further fractionating on an anion exchange chromatography on a DEAE Sepharose Fast Flow column (Amersham Pharmacia Biotech, Uppsala, Sweden). The crude polysaccharide fraction was first

dialysed against water for three days, then against start buffer of 0.02 M phosphate buffer solution (see Appendix A for reagent compositions) for 24 hours. The solution was applied to the column (2.1 x 15 cm) pre-equilibrated with start buffer. The linear flow rate was 5.3 cm/hour, and fractions were collected in 10 mL aliquots. Elution started with 2 column-volumes start buffer, followed by a gradient of 0.0-2.0M NaCl. Fractionation was monitored by total carbohydrates, determined by phenol-sulphuric method (see Appendix B). Fractions containing the highest concentrations of total carbohydrates were examined using thin-layer chromatography (see Appendix B, Thin layer chromatography, Method 2).

The crude polysaccharide fraction was also examined by thin-layer chromatography for the presence of contaminants such as phenolics and alkylamides (see Appendix B, Thin layer chromatography, Method 1).

3.5.8 Purification of alkylamides and phenolic fractions

Purified alkylamide and phenolic fractions were prepared from a mixture of two *Echinacea purpurea* extracts, "*Echinacea purpurea* fresh plant 1:1" and "*Echinacea purpurea* 3:1 bolster," which are also components of Echinacea Triplex™ (see Section 3.5.5, Echinacea Triplex™). The two *Echinacea* extracts were combined in a 9:1 ratio, as per the ratio in Echinacea Triplex™, and the final ethanol concentration of this mixture calculated to be approximately 43%.

While the fractions purified from this mixture do not share cross-contaminants, they do not represent pure compounds, but rather a complex mixture. The fraction names

merely indicate the target compounds used for separation; while the fractions contain these target compounds, other compounds are present.

The *Echinacea* extract mixture was partitioned against an equal volume of hexane in a separating funnel (Sigma, St Louis, MO), to form two fractions; the lower aqueous fraction is subsequently referred to as the phenolic fraction, and the upper hexane fraction the alkylamide fraction. The lower phenolic (aqueous) fraction was allowed to flow through the separating funnel, and collected. The fluid at the junction of the two layers was discarded (approximately 1 mL). The hexane layer remaining in the funnel was collected in an evaporating dish. The phenolic (aqueous) fraction was washed a total of five times in this way, each time the hexane fraction was reserved, and added to the previous. Both the phenolic and alkylamide fractions were then evaporated over a water-bath, and then allowed to dry overnight at 50ºC. The phenolic fraction was resuspended in 15 mL 43% ethanol, and syringe-filtered with a 0.45 μm filter (Schleicher and Schuell). The hexane fraction was resuspended in 15 mL 95% food-grade ethanol (Recochem, Lytton), and syringe-filtered with a 0.45 μm filter.

Removal of polysaccharides from the phenolic (aqueous) fraction was achieved by adding ethanol to a final concentration of 70%, as per the Prokch and Wagner (1987) method for purifying polysaccharides (see Section 3.5.7 Purification of polysaccharides). The sample was then centrifuged in a Biofuge Primo R centrifuge (Heraeus, supplied by Radiometer Pacific) at 7000 rpm for 10 min at 4ºC. The supernatant was removed and concentrated in a rotary-evaporator (Labrota 4001,

Heidolph), ethanol was again added to 43% (as per original solution) so that the extract represented its approximate original volume.

High-performance Liquid Chromatography, performed by Ms Maryanne Genrich of Thursday Plantation Laboratories Pty Ltd, verified the purity and the concentration of the fractions (see Appendix B, High performance liquid chromatography).

3.6 Calculation of dosages

Dosage rates for the commercial products are proportional by weight to the maximum recommended doses suggested by Greenridge Botanicals. If a 40 kg child is optimally to take 3 mL/day of Echinacea Triplex™ (equivalent to 7.5 x 10^{-2} μL/g of body weight) then a 20 grams mouse should have approximately 2 all/day. "*Echinacea purpurea* WP 1:1," "*Echinacea pallida* roots 1:1," and "*Echinacea angustifolia* roots 1:1" also recommend a dose of 3 mL/day as suitable for a 40 kg child, therefore they also received 2 μL/mouse/day. The dosage for the phenolic and alkylamide fractions was calculated by comparing their HPLC titres to that of Echinacea Triplex™. Dosage for the polysaccharide fraction was calculated by precipitating out the polysaccharides from Echinacea Triplex™ at the same ethanol concentration used for their purification (approximately 70%). This amount of polysaccharide was then quantified by the same phenol-sulphuric method as used to quantify the purified polysaccharide (see Appendix B, Chemical analytical techniques). This figure was confirmed by calculating the percentage yield obtained from the polysaccharide extraction, and assuming a similar yield in the Echinacea Triplex™ to calculate expected polysaccharide concentration.

3.7 *In vivo* evaluations of immunomodulatory properties of crude herbal extracts or purified compounds

3.7.1 STUDY 1: *In vivo* evaluation of *Echinacea* species for specific immunostimulatory function following vaccination with the killed *S. typhimurium* vaccine

60 six-week-old female, specific pathogen-free Balb/c (Animal Resource Centre, Perth WA) were allowed to acclimatise and mature until a weight of approximately 20 g, followed by random assignment in groups of five mice per cage. In this study there were 6 treatment and 2 placebo control groups each containing 5 mice/group. Two further controls groups used were hyperimmunised and non-immunized global controls (10 mice per group).

The control groups used in this project were treated as follows:

1. Placebo group: Mice in this group received identical treatment to the experimental group, except that it did not receive any herbal extract. Since the treatment of the groups was staggered, two placebo controls groups (receiving identical treatment) were used: one group began treatment before all other groups, and the second began treatment last of all groups. The purpose of having two placebo control groups was to observe any effect on the immune response that was due to age, since the last group to be treated was a month older than the start group.
2. Hyperimmune group: This group of mice was given two vaccinations, at 0 days, and 14 days and bled at 28 days and constituted a standard for normalisation of the serological data.

3. Global control/Normal group: This group did not receive any experimental treatment and it represented the immunological response to uncontrollable variables such as environmental factors.

Experimental mice received 2 μL (see Section 3.6 "Dosage calculation") of herbal extract dissolved in 250 microlitres of phosphate buffered saline daily, administered daily by the oral route using a flexible plastic gavage (plastic sheath of 24 G ¾", Insyte™ Catheter, Becton-Dickinson, Eight Mile Plains, Qld) for a total period of 35 days. Placebo mice received 250 μL phosphate buffered saline with no herbal extract added, also administered daily by the oral route, for a period of 35 days. On the seventh day of this period, both placebo and experimental groups received a single intraperitoneal vaccination with the whole-cell killed *Salmonella typhimurium* (CS332) vaccine (2.5×10^7 colony forming units). On the last (35^{th}) day of this period, serum was collected, and the mice euthanased as follows. Firstly, the adult mice were anaesthetised with 80 μL of a mixture of ketamine and xylazine (40 μL ketamine (100mg/ml, Troy Laboratories P/L, Smithfield, NSW) + 40 μL 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.

3.7.2 STUDY 2: *In vivo* evaluation of fractions obtained from *Echinacea purpurea* extract for specific immunostimulatory function following vaccination with the killed whole-cell *Salmonella typhimurium* vaccine

Experimental design for this was the same as used in Study 1. Four experimental groups were used in this study, the non-fractionated extract (Echinacea Triplex™) as a positive control and three groups, which represented three crude fractions obtained from the fresh-plant *Echinacea purpurea* extract, namely polysaccharides, alkylamides and phenolics. Again there were three control groups, a placebo control, hyperimmunised control and non-immunized control. Mice were nine weeks old at the start of this experiment.

3.7.3 STUDY 3: *In vivo* evaluation of Echinacea species for specific immunostimulatory function following vaccination with Infanrix™, an acellular pertussis vaccine (DTaP)

The basic experimental design for this study was the same as the previous two. Mice received the same herbal doses, and controls were set up in the same manner (see section 3.6 for dosage calculation). The main difference was that the vaccine used was commercial DTaP vaccine, Infantrix™ (SmithKline Beecham, Dandenong, Vic). Also, for reasons discussed later, several treatment groups were omitted from this study, namely *Echinacea angustifolia* roots 1:1 extract, and "Triplex innovation". The treatment of the mice was not staggered as significantly (all groups commenced

treatment within one week of each other) as the first study, and so the second placebo control was omitted. However, the additional variable of age was introduced in this study. Although there were ten mice per group, they were two different ages (referred to subsequently as 'young' and 'old' treatment groups); consequently, each group had two sub-groups of five 'young' mice (8 weeks old at start of treatment), and five 'old' mice (24 weeks old at start of treatment). These groups, subsequently referred to as 'young' and 'old' groups, were housed separately and treated as separate groups for subsequent analysis.

3.8 Collection of serum, tracheal wash, and splenocyte culture supernatant

3.8.1 Serum collection and storage

Mice were anaesthetised, and a cardiac puncture performed as described in Section 3.7.1 Study 1. The blood was collected in microfuge tubes (Axygen Scientific, Union City, CA), and allowed to clot for 15 minutes at 4ºC. The blood was centrifuged at 3000 xg for five minutes (Mikro 12-24, Hettich Zentrifugen, Tuttlingen), and the serum (supernatant) removed into 2 microfuge tubes in equal volume and stored at -20ºC.

3.8.2 Trachea collection and storage

Trachea were dissected out in a class II Biological Safety Cabinet (BH2000 Series Biohazard, Clyde-Apac, Darra, Qld), and placed straight into 200 μL PBS containing 1 mM phenylmethylsulfonyl fluoride (PMSF, Gibco Life Technologies, Grand Island,

NY), and 2 mM magnesium chloride (Sigma, St Louis, MO). Trachea were ground using sterile plastic pestles for exactly 1 minute, and then stored at -20ºC.

3.8.3 Preparation of splenocyte culture

5 spleens per group were dissected out immediately following cardiac puncture and placed into approximately 2 mL tissue culture media (a) (see Appendix A for composition of media) per spleen. Spleens and tissue culture media (a) were poured into a sterile cell strainer (Becton-Dickinson, Eight Mile Plains, Qld) that was on top of a sterile 50 mL tube (TPP, Switzerland). A sterile 1 mL syringe plunger (Becton-Dickinson, Eight Mile Plains, Qld) was used to mash the cells through the strainer. The cells were twice washed through the strainer with about 5 mL tissue culture media (a). Cells were pelleted out by centrifugation at 1000 rpm for 10 minutes at room temperature (Biofuge Primo-R, Heraeus, supplied by Radiometer Pacific). The supernatant was discarded, and the cells washed with about 10 mL tissue culture media (a). Again the cells were pelleted at 1000 rpm for 10 minutes, and the supernatant discarded. The cells were then resuspended in exactly 10 mL tissue culture media (b) (see Appendix A for composition of media). 10 µL of the cell suspension was added to 90 µL tissue culture media (a). 10 µL of this solution was added to 10 µL tryptan blue, and mixed well. 10 µL of this mixture was pipetted into the haemocytometer, and the white blood cells counted. The concentration of cells was adjusted to 5 x 10^6 cells/ml and 2 mL (10^7 cells) dispensed into 6 wells of a 24 well plate (TPP, Switzerland). One hundred microlitres of *Salmonella typhimurium* (CS332) lysate (see Section 3.8.4, Preparation of *S typhimurium* lysate for splenocyte stimulation), equivalent to approximately 2 µg protein/ml, was added to each of the six wells. The plate was covered with a loose-fitting lid and incubated at 37ºC, and

supernatant removed at 96 hours, the triplicates pooled and stored at -20ºC in microfuge tubes for subsequent analysis of cytokine levels (Axygen Scientific, Union City, CA). All tissue culture procedures were conducted in a Class II Biological Safety Cabinet (Email Air Handling, BTR Environmental).

3.8.4 Preparation of *S typhimurium* lysate for splenocyte stimulation

S typhimurium lysate was prepared according to Villarreal (1992) with several modifications. *Salmonella typhimurium* (CS332) was first grown on motility-test agar (Appendix A) overnight at 37ºC and bacteria with the highest motility used to inoculate 400 mL tryptic soy broth (Difco, Detroit, MI), which was incubated overnight at 37ºC without agitation. The broth was then centrifuged at 13 000 xg for 10 minutes, and the supernatant discarded. The pellet was washed with phosphate buffered saline (PBS) containing 5 mM EDTA (ethylenediamine tetraacetic acid, Sigma, St Louis, MO), centrifuged as previously, and washed again. The cell pellet was resuspended in 1/150 of the original volume, and sonicated for three 2-minute bursts at 60 duty-cycle, output 7 (Branson Sonifier 450, Branson Ultrasonics Corporation, Danbury, CT). Cell disruption was confirmed by gram stain. The cell lysate was further diluted to 1/3 original volume in sterile PBS, and centrifuged at 13 000 xg for 10 minutes to remove insoluble material. The supernatant was syringe-filtered (0.2 μm, Schleicher and Schuell) and stored in a sterile tube at 4ºC. Protein concentration was determined using the Coomassie® Plus Protein Assay Reagent Kit (Pierce, Rockford, IL, See Appendix B, Determination of protein concentration).

3.9 Analysis of serum, tracheal wash, and splenocyte supernatants for total antibody and antibody isotypes, Interleukin-12 and Interferon-γ levels

3.9.1 Preparation of antigens used for determination of antibody titres by enzyme-linked immunosorbent assay (ELISA)

3.9.1.1 Whole-cell *Salmonella typhimurium* (CS332) antigen

200 mL LB broth was inoculated with a colony of *Salmonella typhimurium CS332* previously streaked on LB agar and grown for 18 hours at 37ºC with agitation (150 rpm, Bioline, Edwards Instrument Co, Aust). A series dilution was prepared in Miller's LB broth (Difco, Detroit, MI), and dilutions 10^{-4} – 10^{-8} plated out on Miller's LB agar (Difco, Detroit, MI) for overnight incubation at 37ºC (Memmert, Schwaback, Germany). The culture was then formalised to 1% final concentration, and incubated under the same conditions for 4 hours. 100 µL culture was plated onto LB agar and grown overnight to confirm sterility. The remaining broth was centrifuged at 7000 rpm for 20 minutes at room temperature (Beckman Avanti J251), and washed twice with sterile phosphate buffered saline (see Appendix A for composition). The final pellet was resuspended in 0.2 µm syringe-filter sterilised coating buffer (see Appendix A for composition) to an optical density of 0.4 at 610 nm, and stored at 4ºC until used.

3.9.1.2 Whole cell *Bordetella pertussis* antigen

10 Bordet-Gengou agar (Difco, Detroit, MI) plates were streaked with 10 µL *Bordetella pertussis* Tohama I stock culture. Plates were incubated in a moist environment at 37ºC for 48 hours. Cultures were then scraped into sterile coating

buffer (Appendix A), to an optical density of 0.4 at 610 nm. Whole-cell antigen was stored at 4ºC until used.

3.9.2 Enzyme-linked immunosorbent assay (ELISA)

One hundred microlitres of antigen (OD 0.4 at 610 nm) suspended in coating buffer (see Appendix A for composition) was dispensed into columns 2-10 inclusive of a 96 well plate (Study 1: ELISA tray, Disposable Products, Technology Park, SA; Study 2/3: BD Falcon™ Microtest™, Becton Dickinson, Eight Mile Plains, Qld). The plate was covered with polyethylene wrap, and incubated over-night at 4ºC. Without emptying the wells, an additional 100 µL of 0.2% gelatin (Sigma, St Louis, MO) in coating buffer (Appendix A) was added to all test wells (columns 2-10) to block the plate. 200 µL of 0.2% gelatin in coating buffer was added to column 11 as antigen control. The plate was again covered with polyethylene wrap and incubated over-night at 4ºC. The plate was then emptied, tapped dry on absorbent paper, and flooded with wash buffer (see Appendix A for composition). The plate was allowed to stand for 3 minutes, then the wash procedure was repeated two more times.

During the incubation of the 0.2% gelatin in coating buffer a series dilution for each serum sample was prepared in phosphate buffered saline (Appendix A). For polyvalent antibody (total IgG, IgA, and IgM), and for IgG titres dilutions of 1/100 – 1/12800 were prepared. For the lower titres of IgA, dilutions of 1/10 – 1/1280 were prepared. Within each study, each plate was run with an internal standard, to normalize for plate-to-plate variation. Pooled hyperimmune serum, or pooled hyperimmune tracheal washes at the same concentration were used as the internal controls.

After washing the plate, 100 μL of the serum/tracheal wash series dilutions are plated out, with 1/100 in column 2, 1/200 in column 3, and so on. Column 11 (antigen control) received 100 μL of the 1/100 dilution of the test sera. 1/100 dilution of non-vaccinated mouse serum was aliquoted into column 10 as an antibody control. The plate was covered with polyethylene and incubated at 37ºC for 1 hour.

The plate was then washed three times, as described previously, allowing the wash buffer to stand for 3 minutes for each wash. One hundred microlitres of the secondary antibody was then added to columns 2-11 inclusive. Goat-anti-mouse polyvalent immunoglobulins (IgG, IgA, IgM) (Peroxidase conjugate, Sigma, St Louis, MO) was prepared as 1/3000 dilution in PBS (Appendix A). IgG Goat-anti-mouse, Fc specific, peroxidase conjugate (Sigma, St Louis, MO) was diluted 1/2000 in PBS, and IgA goat-anti-mouse, α-chain specific, peroxidase conjugate (Sigma, St Louis, MO) was diluted 1/1000 in PBS (see Appendix A for composition). The plate was covered and incubated at 37ºC for 1 hour.

The plate was washed three times, as previously described, and 100 μL Sigma-fast™ (Appendix A, Sigma, St Louis, MO), prepared according to the instructions, was added to all wells including the chromagen blanks. Plate was covered with aluminium foil, and incubated at 37ºC for exactly 30 minutes. The plate was then read in a micro-plate reader (Model 550, Biorad, Hercules, CA) at 450 nm. 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 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 standardised according to the internal standard (hyperimmune) titre. (Mean of all hyperimmune titres divided by the plate hyperimmune titre, multiplied by test sample titre).

3.9.3 Interleukin-12 titre

Interleukin-12 (IL-12) concentrations were titrated using a Cytoscreen Mouse IL-12 + p40 kit (Biosource International, Camarillo, CA). The procedure was conducted as per instructions. Briefly, standards were prepared using a series dilution of mouse IL-12. All samples, standards and controls were aliquoted into the pre-coated plate, along with the biotin conjugate, as per instructions. The plate was then allowed to incubate for 2 hours at room temperature, followed by four washes using the wash buffer provided. The plate was then incubated with Streptavidin-HRP for 30 minutes at room temperature, followed by four washes. After a final incubation with the colour substrate for 30 minutes, the stop solution was added and the plate read at 450 nm. A standard curve was plotted from the standard data, and IL-12 concentrations for samples and standards extrapolated.

3.9.4 Interferon-γ titre

Interferon-γ (IFN-γ) titres were determined using a Cytoscreen Mouse IFN-γ kit (Biosource International, Camarillo, CA).). The procedure was conducted as per instructions. Briefly, standards were prepared using a series dilution of mouse IFN-γ.

All samples, standards and controls were aliquoted into the pre-coated plate, along with the biotin conjugate, as per instructions. The plate was then allowed to incubate for 2 hours at room temperature, followed by four washes using the wash buffer provided. The plate was then incubated with Streptavidin-HRP for 30 minutes at room temperature, followed by four washes. After a final incubation with the colour substrate for 30 minutes, the stop solution was added and the plate read at 450 nm. A standard curve was plotted from the standard data, and IFN-γ concentrations for samples and standards extrapolated.

3.10 Statistical analysis of results

All statistical analysis was conduced using SPSS 11.0, for Windows™. Normalised (mean standard titre divided by plate standard titre multiplied by test titre gives normalised titre) antibody titres were analysed using one-sided, independent samples t-tests, comparing each group to the corresponding placebo (PBS) group. Interleukin-12 titres were also analysed using a one-sided, independent samples t-tests, comparing each group to the placebo (PBS) group. Equal variance was only assumed where the standard deviations of all groups were within three times the lowest standard deviation.

Chapter 4: Results

4.1 Confirmation of authenticity of herbs

Authenticated herb samples and the herbs used for extract preparation were analysed by thin-layer chromatography for confirmation of authenticity (Figure 1).

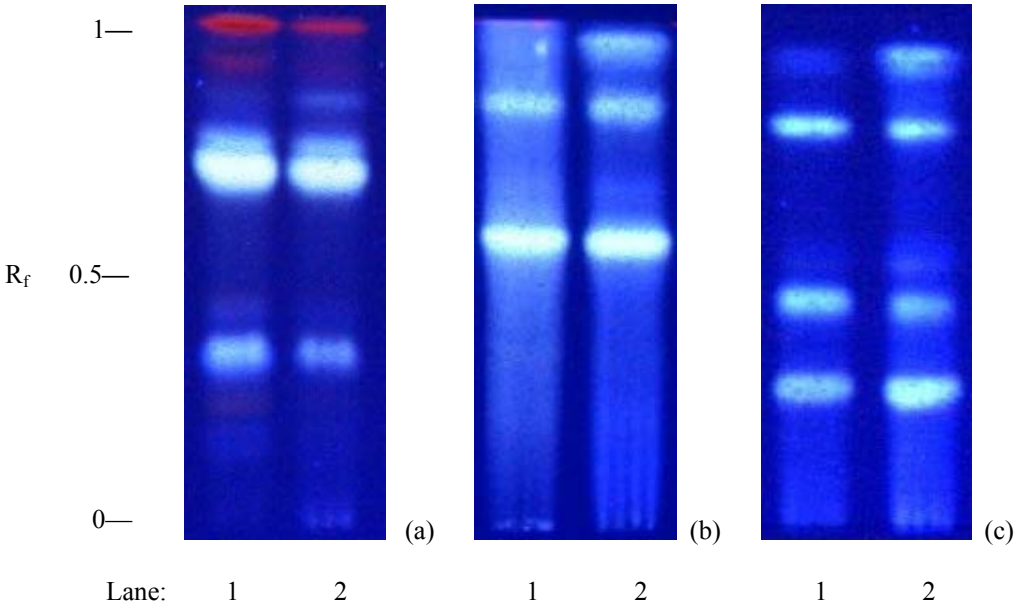

Figure 1: Thin-layer chromatograms of authenticated (lanes 1) and sample (lanes 2) *Echinacea* species (a) *Echinacea purpurea,* (b) *Echinacea pallida,* (c) *Echinacea angustifolia* (Glass-backed silica C_{60} F_{254} plates; Solvent: 11:11:27:100, CH_3COOH: Formic Acid: H_2O: Ethyl Acetate; Spray: 1% w/v methanolic DBA (diphenylboric acid), followed by 5% w/v ethanolic PEG (polyethylene glycol); viewed under 365 nm.)

Chromatogram (a), representing the *Echinacea purpurea* extract, shows bands consistent between sample and reference herbs at R_f 0.35, 0.66, 0.74 0.81 and 0.98. Chromatogram (b), representing the *Echinacea pallida* extract, shows bands

consistent between sample and reference herbs at R_f 0.54, 0.68 and 0.82. Chromatogram (c), representing the *Echinacea angustifolia* extract, shows bands consistent between sample and reference herbs at R_f 0.24, 0.44, 0.77 and 0.92. According to Wagner, et al. (1984a) the blue bands in all three chromatograms are likely to represent caffeic acid derivatives.

4.2 Purification of polysaccharides from *Echinacea purpurea* fresh plant extract

4.2.1 Estimation of the polysaccharide content of Echinacea Triplex™ and purified polysaccharide fraction

The polysaccharide content in Echinacea Triplex™ (Greenridge Botanicals, Pty, Ltd, Toowoomba) or fractionated components of Echinacea Triplex™ was determined by phenol-sulphuric assay using glucose as a standard (see Appendix C, Figure 2). The total carbohydrate concentration in Echinacea Triplex™ was estimated at approximately 22 mg/mL. Although Echinacea Triplex™ contains glycerin, glycerin did not give a positive reaction in this assay. Of the total carbohydrates in Echinacea Triplex™, only a small amount represented the high molecular weight polysaccharides in the purified fraction (prepared according to Proksch and Wagner 1987). To determine the concentration of these high molecular weight polysaccharides in Echinacea Triplex™, and subsequently calculate the dosage rate for the purified polysaccharide fraction, the polysaccharides in Echinacea Triplex™ were precipitated out according to the Proksch and Wagner (1987) method. Of the total carbohydrates in Echinacea Triplex™ (22 mg/mL), only 1 mg/mL (± 2 mg) represented high molecular weight polysaccharides.

The total carbohydrate in the purified polysaccharide fraction, which was prepared from dried *Echinacea purpurea* herb according to Proksch and Wagner (1987), was calculated to be 17 mg/ml.

4.2.2 Assessment of the purity of the polysaccharide fraction

The purity of the crude polysaccharide fraction was assessed by thin layer chromatography (Figure 2).

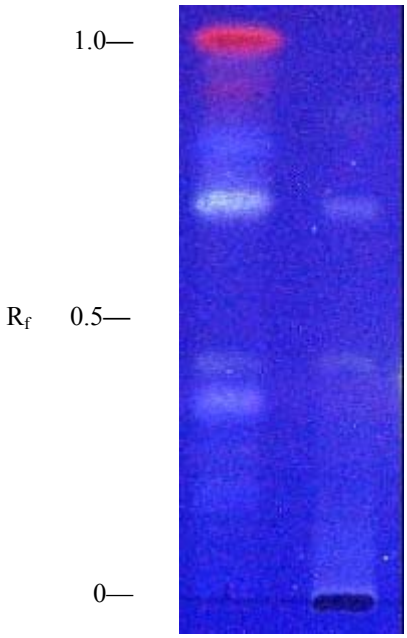

Figure 2: Comparison of methanolic *Echinacea purpurea* extract, lane 1, with purified polysaccharide fraction, lane 2, (C_{60} F_{254}; Solvent: 11:11:27:100, CH_3COOH: Formic Acid: H_2O: Ethyl Acetate; Spray: 1% w/v methanolic DBA, followed by 5% w/v ethanolic PEG; viewed under 365 nm).

Low levels of a phenolic acid derivative are seen in the polysaccharide chromatogram at R_f 0.67, this contaminant may be cichoric acid (Wagner et al., 1984).

4.2.3 Purification of the polysaccharide fraction from *E purpurea*

The crude polysaccharide fraction, extracted from whole plant *Echinacea purpurea* according to Proksch and Wagner (1987), was further fractionated on a DEAE Sepharose Fast Flow (anion-exchange) column using a gradient elution of 0.0-2.0 M NaCl in 20 mM phosphate buffer, with a linear flow rate of 5.3 cm/hour (Appendix A). The concentration of polysaccharides in the collected fractions was monitored by the phenol-sulphuric total carbohydrate assay (Figure 3). Eight major peaks were resolved.

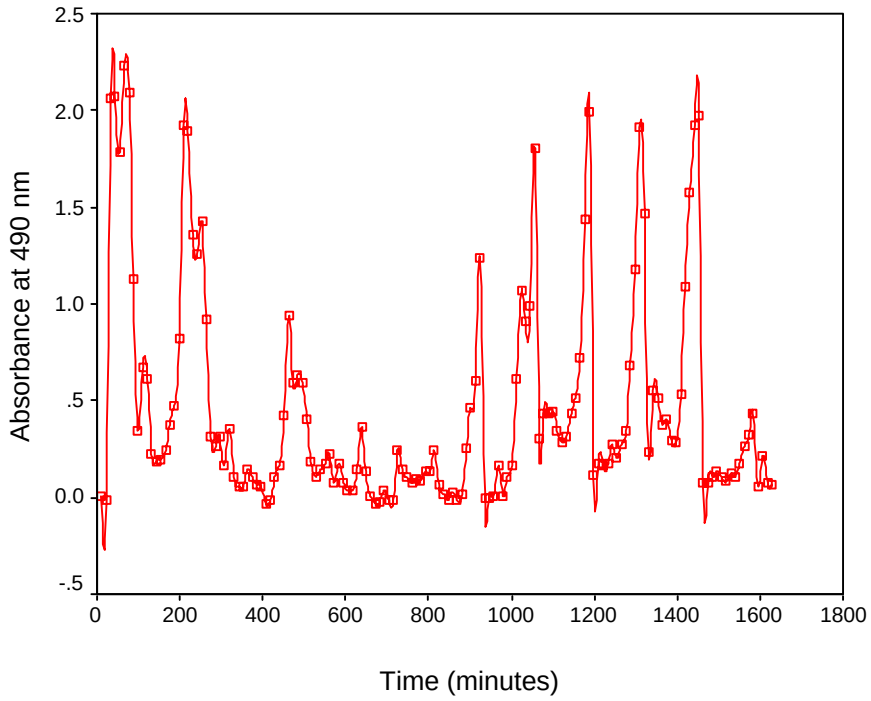

Figure 3: Elution profile of crude *Echinacea purpurea* polysaccharide on DEAE-Sepharose column (gradient elution of 0.0-2.0 M NaCl in 20 mM phosphate buffer, linear flow rate of 5.3 cm/hour)

The major peaks shown by the phenol-sulphuric total carbohydrate assay were analysed by thin layer chromatography. All peaks showed diffuse chromatographic profiles, with bands overlapping making it difficult to draw any definitive conclusions about the purity of the fractions (figure not shown).

4.3 Purification of alkylamide and phenolic fractions

The phenolic (retention time, 3.5 minutes) and alkylamide fractions (retention time, 31 minutes) were prepared by partitioning the fresh-plant *Echinacea purpurea* components of Echinacea Triplex™ against hexane. The ethanolic-aqueous fraction was shown by HPLC analysis to contain 11.2 mg/mL total phenolics (Figure 4), based on chicoric acid standard (not shown). Peaks were identified by their spectra (Appendix C, Figure 1). All chromatograms were scaled to absorbance 3, and total phenolics calculated using the peak data (see Appendix C, Figure 1).

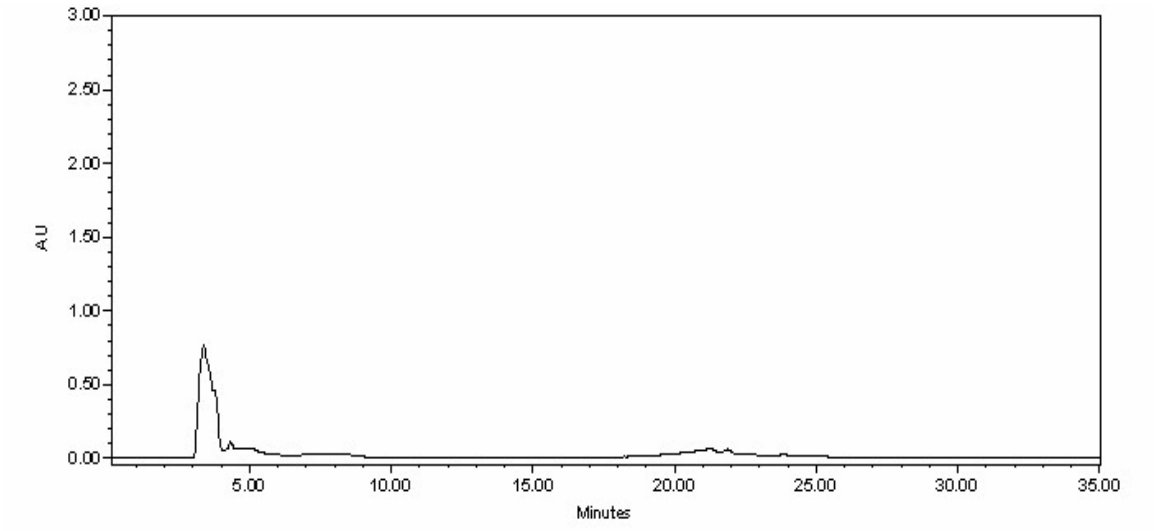

Figure 4: HPLC chromatogram of phenolics in purified phenolic fraction (for peak data and spectra, see Appendix C, Figure 1) AU denotes absorbance at 300 nm.

Echinacea Triplex™ was also analysed by HPLC, and was shown to contain 54.7 mg/ml total phenolics (Figure 5, see also Appendix C, Figure 2 for peak data).

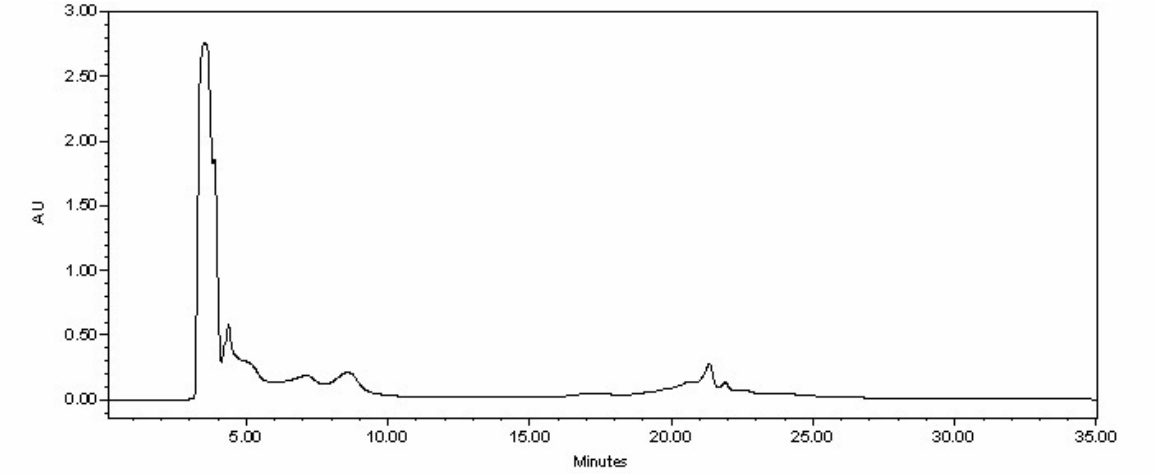

Figure 5: HPLC chromatogram of phenolics in purified phenolic fraction (for peak data, and spectra see Appendix C, Figure 2) AU denotes absorbance at 300 nm.

The alkylamide fraction was estimated to have 0.64 mg/ml total alkylamide (Figure 6; see also Appendix C, Figure 3 for peak data and spectra), calculated using peak data and identified using spectral profile, compared with 0.50 mg/ml in the Echinacea Triplex™ (Figure 7; see also Appendix C, Figure 4).

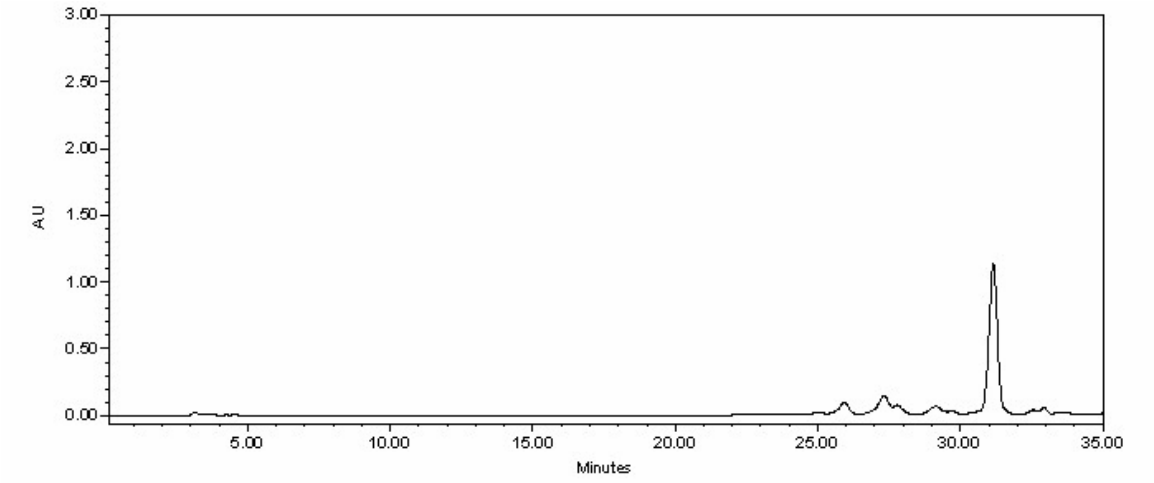

Figure 6: HPLC chromatogram of total alkylamide in purified alkylamide fraction (for spectra and peak data see Appendix C, Figure 3) AU denotes absorbance at 254nm.

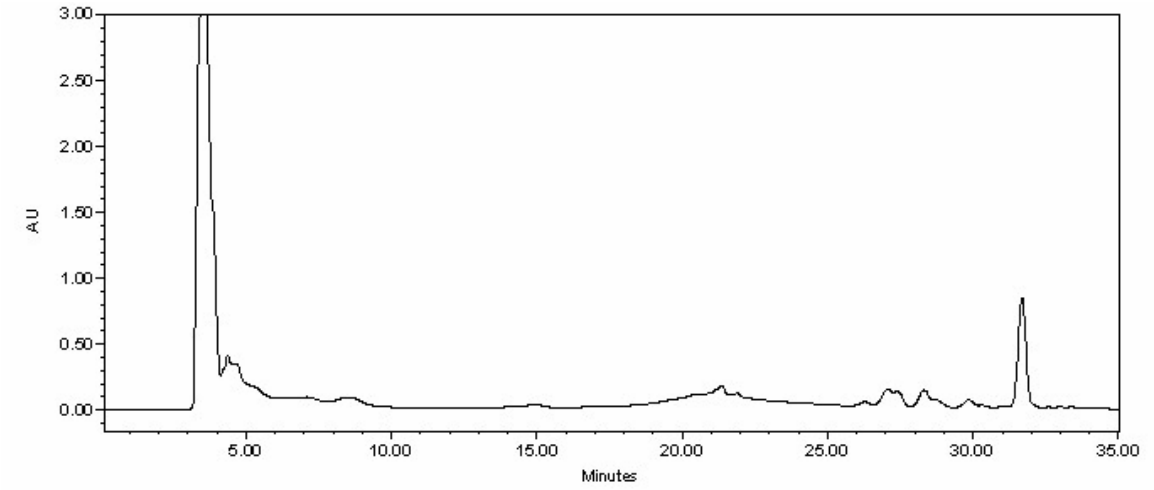

Figure 7: HPLC chromatogram of total alkylamide in Echinacea Triplex™ (for spectra and peak data see Appendix C, Figure 4) AU denotes absorbance at 254nm.

4.4 Dosage Calculations

Since Echinacea Triplex™ was shown to contain approximately 1 mg/ml high molecular weight polysaccharides (as determined by the phenol-sulphuric method), and the purified fraction contained 17 mg/ml, the dose of purified polysaccharide was calculated as 0.35 µL purified polysaccharide stock solution/mouse/day, which is equivalent to the daily polysaccharide dose received in Echinacea Triplex™. Similarly, Echinacea Triplex™ was shown to contain approximately 54.7 mg/ml total phenolics, as determined by HPLC, while the purified phenolic fraction contained 11.2 mg/mL total phenolics. Therefore the dose of purified phenolic fraction was calculated as 9.8 µL stock solution/mouse/day, which is equivalent to the daily polysaccharide dose received in Echinacea Triplex™. The total alkylamide, determined by HPLC analysis, in Echinacea Triplex™ was shown to be approximately 0.50 mg/ml. While the purified alkylamide fraction contained 0.64 mg/ml. Therefore the dosage for purified alkylamide fraction was calculated as 1.6 µL, which is equivalent to the daily dose of alkylamide received in Echinacea Triplex™.

4.5 STUDY 1: *In vivo* evaluation of *Echinacea* species for specific immunostimulatory function following vaccination with the killed *Salmonella typhimurium* vaccine

In this study mice were treated with either Echinacea Triplex™, *E purpurea* whole plant (WP) 1:1, *E angustifolia* 1:1, or *E pallida* 1:1, and vaccinated with killed *Salmonella typhimurium* vaccine. Only the serum of these mice was collected for serological analysis. Mean antibody titres (± 1 standard error, SE) of the treatment groups are shown in Table 1 below (also see Figure 8 for graphic representation of the data; Appendix D, Table 1 for individual titres, and Appendix E, Figures 1-9 for titre determination curves).

Table 1: Serum antibody titres for *S typhimurium*-vaccinated mice given oral doses of either *Echinacea pallida* 1:1, *Echinacea angustifolia* 1:1, Echinacea Triplex™, "Triplex innovation," *Echinacea purpurea* 1:1, or placebo (PBS)

			Independent-samples t-tests for equality of means		
Group	**Mean antibody titre ± 1SE**	**Standard Deviation**	**t statistic**	**p value**	**df**
Echinacea pallida 1:1	1409.6 ± 386.7	864.6	-1.632	0.079	8
Echinacea angustifolia 1:1	1473.0 ± 117.7	263.2	-3.670	0.004	8
Echinacea Triplex™	3689.0 ± 773.1	728.7	-3.751	0.009	8
"Triplex innovation"	1001.0 ± 286.7	641.0	-0.840	0.216	8
Echinacea purpurea 1:1	1911.0 ± 250.6	560.3	-3.946	0.003	8
Placebo (PBS)* Control 1	722.0 ± 167.4	374.4	n/a	n/a	n/a
Placebo (PBS)* Control 2	510.0 ± 270.0	605.2	0.666	0.262	8

SE = standard error
df = degrees of freedom
n/a = not applicable since comparison of means is made to this group
p value is one tailed (equal variance not assumed)
* Placebo (PBS) = mice vaccinated but not given the herbal extract

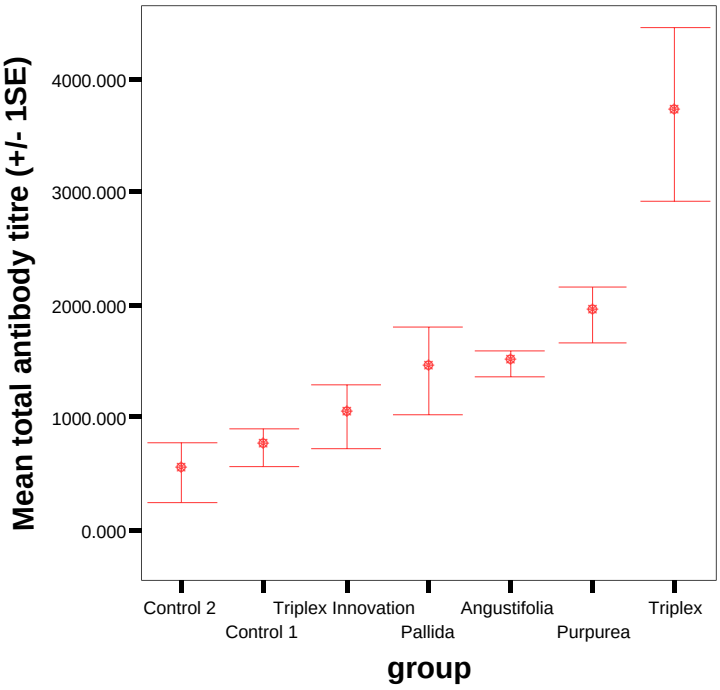

Figure 8: Comparison of anti-*S typhimurium* serum antibody titres for *S typhimurium* vaccinated mice treated with oral doses of either *Echinacea pallida* 1:1, *Echinacea angustifolia* 1:1, Echinacea Triplex™, "Triplex innovation," *Echinacea purpurea* 1:1, or placebo (PBS)

The t-tests were carried out in comparison to placebo (PBS) control group 1, which had the higher mean antibody titre of the two placebo control groups. The error bars (Figure 8) represent the mean plus or minus one SE. In other words the probability that the *population* mean will be in the bar region given by the *sample* mean was 68% since this represented one standard deviation from the mean. It can be seen from Figure 8 that, although the mean antibody titres of *Echinacea pallida* 1:1 and "Triplex innovation" treated groups were higher than both the placebo control groups, the differences observed may have been due to chance (*E pallida*/control t=-1.632, p=0.079, one tailed, equal variance not assumed, df=8; "Triplex innovation"/control

t=-0.840, p=0.216 one tailed, equal variance not assumed, df=8). However *E angustifolia,* Echinacea Triplex™ and *E purpurea* groups all displayed differences in means, when compared to placebo control group 1, that were unlikely to have been due to chance alone (*E angustifolia*/control t=-3.670, p=0.004, one tailed, equal variance not assumed, df=8; Echinacea Triplex™/control t=-3.751, p=0.009, one tailed, equal variance not assumed, df=8; *E purpurea*/control t=-3.946, p=0.003, one tailed equal variance not assumed, df=8).

4.6 STUDY 2: *In vivo* evaluation of fractions obtained from *Echinacea purpurea* extract for specific immunostimulatory function following vaccination with the killed whole-cell *Salmonella typhimurium* vaccine

In addition to serum levels of total antibody (IgG + IgA + IgM) which were determined in the initial study (see Section 4.5 Study 1), this second study also quantified several other immunological parameters of the mice vaccinated with killed *S typhimurium* and treated with either Echinacea Triplex™, phenolic fraction, polysaccharide fraction, alkylamide fraction or placebo (PBS). Levels of interleukin-12 were determined in the pooled serum samples whereas interferon-γ levels were estimated in the stimulated splenocyte supernatants.

4.6.1 Total antibody titres

The results of the total (IgG + IgA + IgM) antibody titres are summarised in Table 2 (see Figure 9 for graphic representation of the data; Appendix D, Table 2a for individual titres; Appendix F, Figures 1-7 for curves used to calculate titres).

Table 2: Serum anti-*S typhimurium* antibody titres for *S typhimurium* vaccinated mice given oral doses of either Echinacea Triplex™, phenolic fraction, alkylamide fraction, polysaccharide fraction, or placebo (PBS)

			Independent-samples t-tests for equality of means		
Group	**Mean antibody titre ± 1SE**	**Standard Deviation**	**t statistic**	**p value**	**df**
Echinacea Triplex™	1867.9 ± 344.7	1034.0	3.453	0.002	18
Phenolic fraction	1179.9 ± 242.7	797.3	1.875	0.039	18
Polysaccharide fraction	2210.0 ± 395.5	1311.7	3.571	0.001	18
Alkylamide fraction	1790.4 ± 155.2	490.7	6.075	0.000	18
Placebo (PBS)* control	693.1 ± 92.5	292.5	n/a	n/a	n/a

SE = standard error
df = degrees of freedom
n/a = not applicable since comparison of means is made to this group
p value is one tailed (equal variance not assumed)
* Placebo (PBS) = mice vaccinated but not given the herbal extract

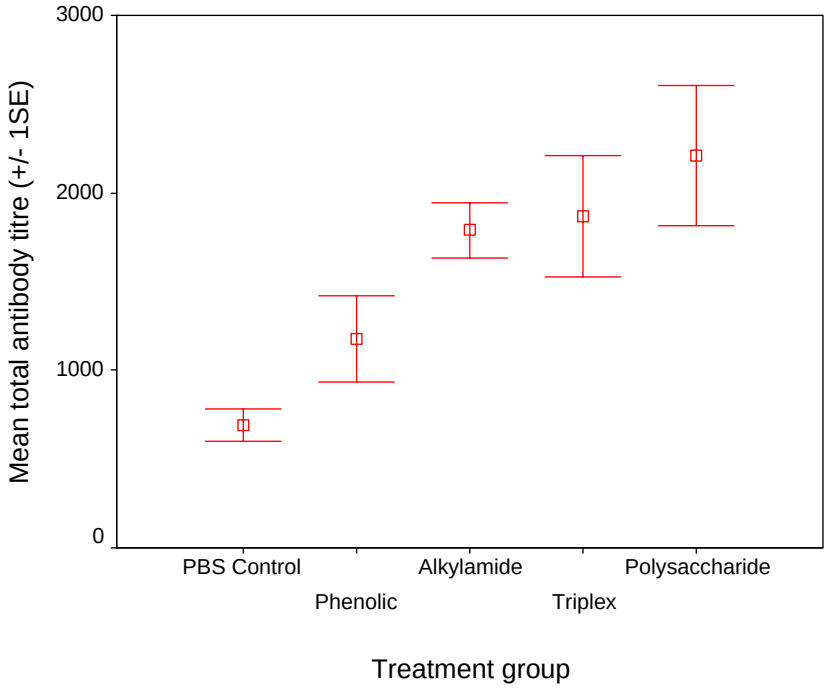

Figure 9: Comparison of serum anti-*S typhimurium* antibody titres of vaccinated mice treated with oral doses of either Echinacea Triplex™, phenolic fraction, alkylamide fraction, polysaccharide fraction, or placebo (PBS)

As can be seen from Table 2 and Figure 9, the total antibody titres against *S typhimurium* were significantly greater than those in the placebo control. While the alkylamide group did not have the highest mean antibody titre, it had the lowest p-value. The probability that the observed difference between the placebo (PBS) control and the alkylamide group was due to chance alone is <0.1% (t=6.075, df=18, p=0.000). On the other hand, the levels of anti-*S typhimurium* antibodies in mice given Echinacea Triplex™ were also statistically significant (Table 2, Figure 9). The probability that the observed differences between the placebo and Echinacea Triplex™ groups were due to chance alone is 0.2% (t=3.453, df=18, p=0.002, one tailed, equal variance assumed). The polysaccharide group had the highest mean antibody titre, and the results were also significant statistically despite of the high

variance of this group compared to the control group. However the probability that the differences observed between these two groups was due to chance alone is 0.1% (t=3.571, df=18, p=0.001, one tailed, equal variance assumed). The probability that the differences observed between the phenolic group and the control were due to chance alone is 3.9% (t=1.875, df=18, p=0.039, one tailed, equal variance assumed).

4.6.2 Antibody isotype titres

Antibody isotypes (IgG and IgA) were also quantified for the pooled sera of these treatment groups. These results are summarized in Figure 10 (see Appendix F, Figures 8-9 for curves used to determine titres).

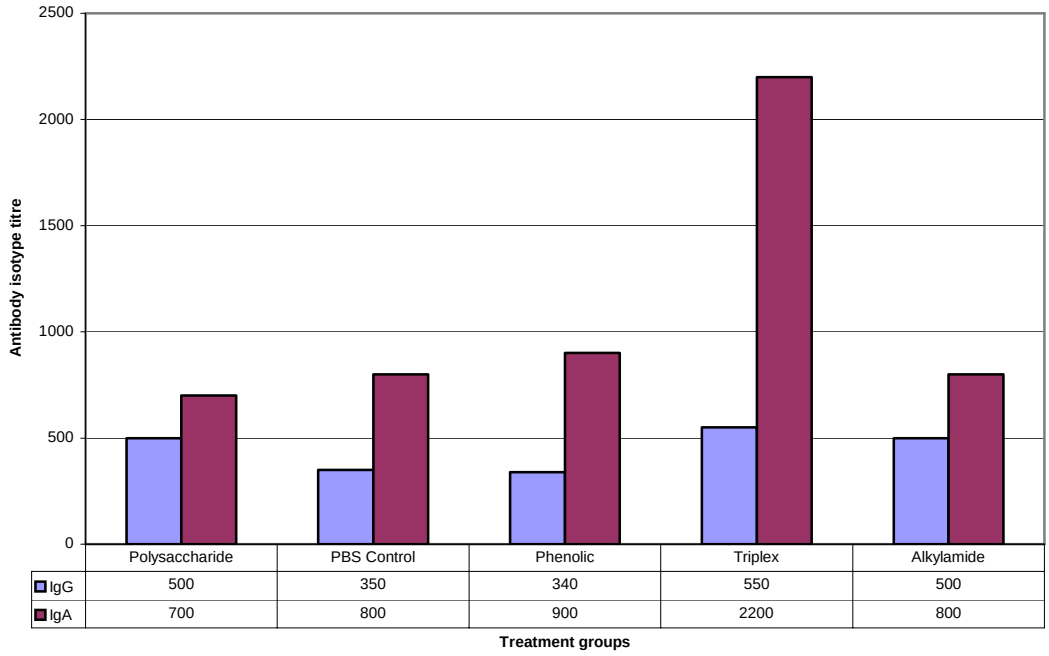

Figure 10: Comparison of anti-*S typhimurium* antibody isotypes IgG and IgA in pooled sera obtained from vaccinated mice treated with oral doses of either Echinacea Triplex™, phenolic fraction, alkylamide fraction, polysaccharide fraction, or placebo (PBS)

There was no apparent substantial difference in the IgG isotype titres between the groups given Echinacea Triplex™, polysaccharide, phenolic, alkylamide or PBS (placebo). However, the IgA antibody titre in mice given Echinacea Triplex™ were substantially greater than that observed in other groups.

4.6.3 Serum interleukin-12 (IL-12) titres

It was discovered that the mean IL-12 titres were significantly greater in mice given Echinacea Triplex™ or the phenolic fraction than those observed in the placebo (PBS) control group (see Table 3 below; Figure 11 for graphic representation of the data; Appendix D, Tables 2 for individual titres; Appendix F, Figure 9 for standard curve used to calculate the titres).

Table 3: Serum IL-12 titres for *S typhimurium* vaccine immunised mice given oral doses of either Echinacea Triplex™, phenolic fraction, alkylamide fraction, polysaccharide fraction, or placebo (PBS)

			Independent-samples t-tests for equality of means		
Group	**Mean IL-12 titre ± 1SE**	**Standard Deviation**	**t statistic**	**p value**	**df**
Echinacea Triplex™	2462.0 ± 125.9	389.0	1.773	0.047	18
Phenolic fraction	2458.0 ± 127.1	401.9	1.747	0.049	18
Polysaccharide fraction	2225.0 ± 65.8	208.0	0.732	0.237	18
Alkylamide fraction	2243.0 ± 29.9	94.4	0.884	0.194	18
Placebo (PBS)* control	2096.0 ± 163.6	517.4	n/a	n/a	n/a

SE = standard error
df = degrees of freedom
n/a = not applicable since comparison of means is made to this group
p value is one tailed (equal variance not assumed)
* Placebo (PBS) = mice vaccinated but not given the herbal extract

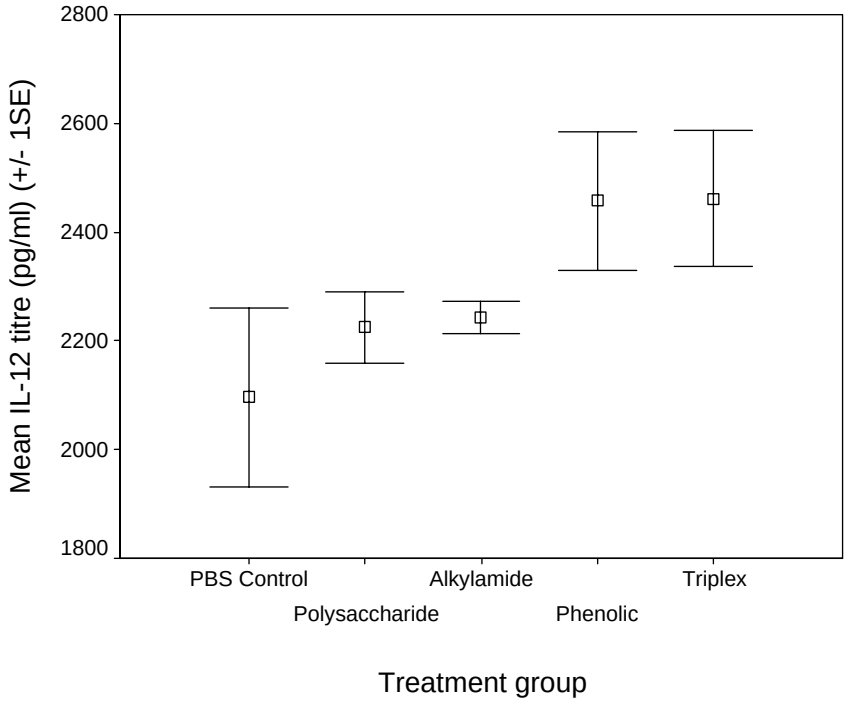

Figure 11: Comparison of serum IL-12 titres for *S typhimurium* vaccinated mice treated with oral doses of either Echinacea Triplex™, phenolic fraction, alkylamide fraction, polysaccharide fraction, or placebo (PBS)

The statistically significant results obtained in both phenolic and Echinacea Triplex™ groups displayed differences from the placebo control that were probably not due to chance alone. The probability that the differences observed between Echinacea Triplex™ and placebo (PBS) control groups was due to chance alone is 4.65% (t=1.773, df=18, p=0.047 one tailed, equal variance assumed). The probability that the differences observed between the phenolic and placebo control groups was due to chance alone is 4.9% (t=1.747, df=18, p=0.049, one tailed, equal variance assumed). Neither the alkylamide nor the polysaccharide groups yielded statistically significant differences in mean IL-12 levels (Alkylamide/Placebo: t=-0.884, df=18, p=0.194, one tailed, equal variance assumed; Polysaccharide/Placebo: t=-0.732, df=18, p=0.237, one tailed, equal variance assumed).

4.6.4 Estimation of interferon-γ (IFN-γ) levels in the splenocyte culture supernatant

Interferon-γ (IFN-γ) titres were determined using pooled splenocyte culture supernatant in duplicate. These results are summarized below in Table 4 (see Figure 12 for graphic representation of the data; Appendix D, Table 2c for replicate titres; Appendix F, Figure 10 for standard curve used to calculate titres).

Table 4: IFN-γ titres of the stimulated splenocyte culture supernatants from mice immunised with *S typhimurium* and given oral doses of either Echinacea Triplex™, phenolic fraction, alkylamide fraction, polysaccharide fraction, or the placebo (PBS)

Group	Mean IFN-γ titre (pg/mL) ± 1SE	Standard Deviation
Echinacea Triplex™	1.5 ± 0.5	0.7
Phenolic fraction	9.5 ± 0.5	0.7
Polysaccharide fraction	10.0 ± 1.0	1.4
Alkylamide fraction	7.0 ± 3.0	4.2
Placebo (PBS)* control	5.0 ± 0.5	0.7

SE = standard error

* Placebo (PBS) = mice vaccinated but not given the herbal extract

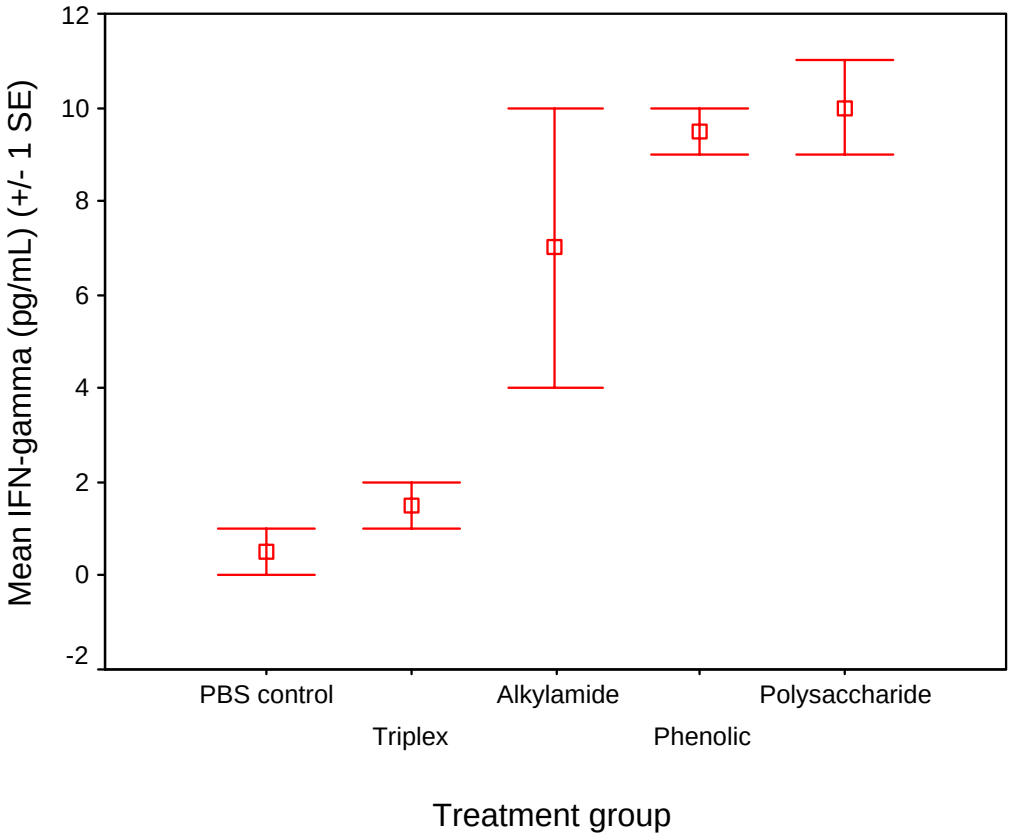

Figure 12: Comparison of splenocyte culture supernatant IFN-γ titres for *S typhimurium* vaccinated mice treated with oral doses of either Echinacea Triplex™, phenolic fraction, alkylamide fraction, polysaccharide fraction, or the placebo (PBS)

T-test analysis on IFN-γ levels of splenocyte culture supernatant from mice vaccinated with killed *S typhimurium* vaccine and given either Echinacea Triplex™, phenolic fraction, polysaccharide fraction, alkylamide fraction or placebo (PBS) was not possible since means were obtained from two replicates of the same pooled sample, rather than from individual splenocyte culture samples. However, alkylamide, polysaccharide and phenolic groups did have higher levels of IFN-γ than that of the placebo (PBS) control group. Whereas there was no apparent difference observed between Echinacea Triplex™ treatment group and the placebo (PBS) control group (Figure 12).

4.7 STUDY 3: *In vivo* evaluation of *Echinacea* species for specific immunostimulatory function following vaccination with Infanrix™, an acellular pertussis vaccine (DTaP)

4.7.1 Young DTaP-vaccinated mice

4.7.1.1 Total serum antibody titres

Total antibody titre (IgG + IgA + IgM) in serum was quantified for the acellular pertussis vaccinated mice that had been fed daily doses of *Echinacea purpurea* 1:1, Echinacea Triplex™, *Echinacea angustifolia* 1:1 or placebo (Ab titres calculated using Appendix G Figures 1-4). It was decided for this study to use mice of two different ages to observe the effect of age on immune response to DTaP of mice treated with *Echinacea* species. Consequently, this additional variable was considered during statistical analysis. The results for the young mice are summarized in Table 5 (see Figure 13 for graphical representation of the data; Appendix D, Table 3a for individual titres).

Table 5: Serum anti-*B pertussis* antibody titres of young DTaP-vaccinated mice given oral doses of either *Echinacea angustifolia* 1:1, Echinacea Triplex™, *Echinacea purpurea* 1:1, or the placebo (PBS)

			Independent-samples t-tests for equality of means		
Group	**Mean antibody titre ± 1SE**	**Standard Deviation**	**t statistic**	**p value**	**df**
Echinacea Triplex™	1040.0 ± 283.9	634.8	2.871	0.02	8
Echinacea purpurea 1:1	590.0 ± 378.0	845.1	1.014	0.182	8
Echinacea angustifolia 1:1	385.0 ± 174.6	390.4	0.982	0.185	8
Placebo (PBS)* control	200.0 ± 158.1	158.1	n/a	n/a	n/a

SE = standard error
df = degrees of freedom
n/a = not applicable since comparison of means is made to this group
p value is one tailed (equal variance not assumed)
* Placebo (PBS) = mice vaccinated but not given the herbal extract

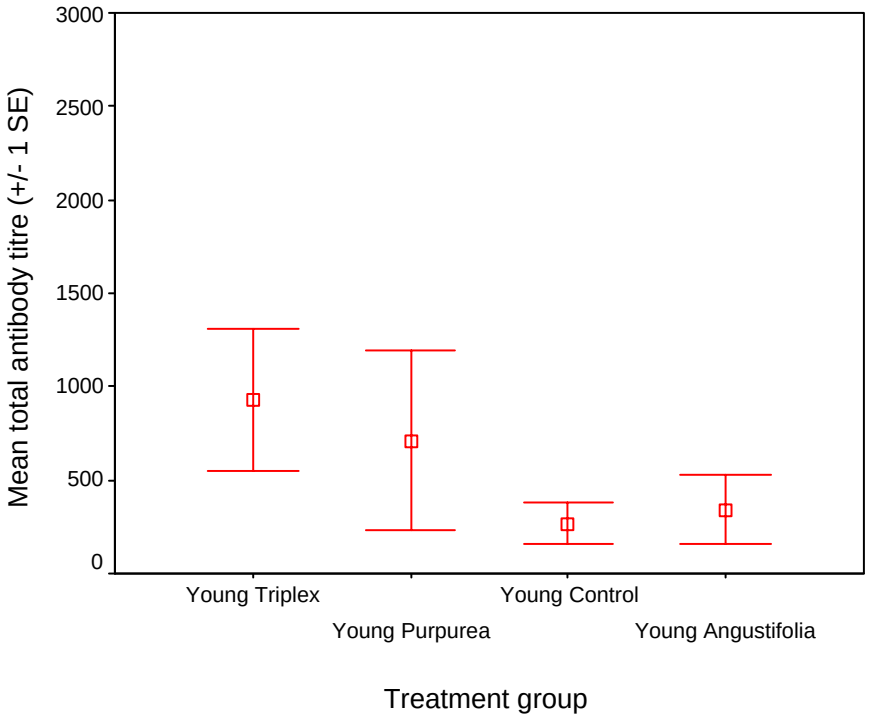

Figure 13: Comparison of anti-*B pertussis* serum antibody titres for young DTaP-vaccinated mice given oral doses of either Echinacea Triplex™, *Echinacea purpurea* 1:1, *Echinacea angustifolia* 1:1 or placebo (PBS)

The independent-samples t-test comparison of the total antibody (IgG + IgA + IgM) levels in DTaP-vaccinated young mice revealed that antibody titres of mice that received Echinacea Triplex™ were significantly greater than those of the placebo (PBS) control. The probability that the observed difference in mean antibody level observed between the young placebo (PBS) control and the young Echinacea Triplex™ treated group was due to chance alone was 2% (t=2.871, df=8, p=0.02, one-tailed). Surprisingly and contrary to results obtained for mice vaccinated with killed *Salmonella typhimurium* vaccine, the differences in mean antibody titre displayed by *Echinacea purpurea* 1:1, and *Echinacea angustifolia* 1:1 in young mice were not statistically significant (*E purpurea* t=1.014, p=0.182 one tailed, equal variance not assumed, df=8; *E angustifolia* t=0.982, p=0.185, one tailed, equal variance not assumed, df=8).

4.7.1.2 Antibody isotype titres

Following quantification of total antibody (IgG + IgA + IgM) in the serum samples of the young DTaP-vaccinated mice, antibody isotypes (IgG and IgA) were quantified by ELISA using pooled sera samples from the treatment or placebo (PBS) control groups and also for the hyperimmunised group. These results are summarised in Figure 14 (see Appendix G, Figures 5 and 6 for curves used to determine titres).

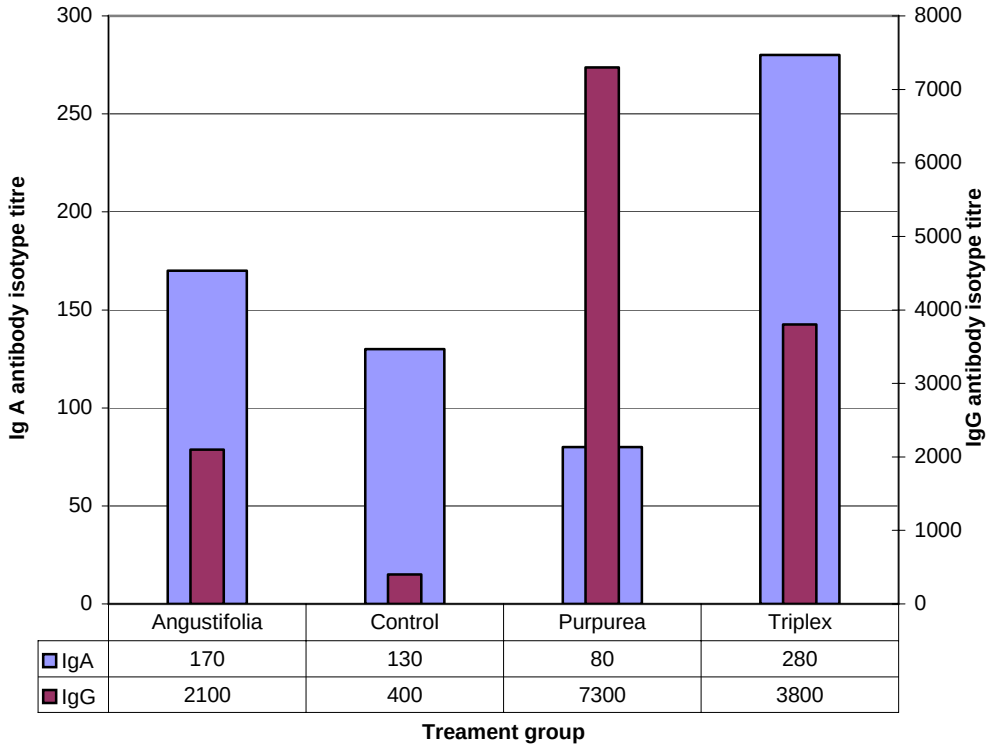

Figure 14: Comparison of antibody isotypes IgG and IgA in pooled sera between young DTaP-vaccinated mice treated with oral doses of either Echinacea Triplex™, *Echinacea purpurea* 1:1, *Echinacea angustifolia* 1:1 or placebo (PBS) control

As can be seen from Figure 14, the antibody levels of IgG were greater than those of IgA in magnitude. IgA levels in some treatment groups were greater than the placebo (PBS) control group, however the differences were not remarkable in terms of probable biological significance. On the other hand, antibody titres of the IgG isotype were substantially greater in magnitude in all the treatment groups in comparison to the placebo control group, the greatest increase being observed in the *E purpurea* group, although surprisingly, the increase seen in IgG antibody titre in the *E purpurea* group was not reflected in the total antibody titre (Table 5).

4.7.1.3 Interleukin-12 (IL-12) titres

The IL-12 levels of the serum samples obtained from young mice vaccinated with DTaP were determined using ELISA, which revealed that the IL-12 levels were significantly greater in all the treatment groups (Echinacea Triplex™, *E purpurea* 1:1, and *E angustifolia* 1:1) than in the placebo (PBS) control group. (see Table 6 for a summary of these results; Figure 15 for graphic representation of the data; Appendix D, Table 3b for individual titres; Appendix G, Figure 15 for standard curve used for calculation of titres).

Table 6: Serum IL-12 titres of young DTaP-vaccinated mice given oral doses of either *Echinacea angustifolia* 1:1, Echinacea Triplex™, *Echinacea purpurea* 1:1, or the placebo (PBS)

			Independent-samples t-tests for equality of means		
Group	**Mean IL-12 titre ± 1SE**	**Standard Deviation**	**t statistic**	**p value**	**df**
Echinacea Triplex™	109.6 ± 8.5	19.0	4.132	0.002	8
Echinacea purpurea 1:1	88.8 ± 11.1	24.8	2.074	0.037	8
Echinacea angustifolia 1:1	98.8 ± 12.8	28.5	2.588	0.016	8
PBS (Placebo)* control	58.4 ± 9.0	20.1	n/a	n/a	n/a

SE = standard error
df = degrees of freedom
n/a = not applicable since comparison of means is made to this group
p value is one tailed (equal variance assumed)
* Placebo (PBS) = mice vaccinated but not given the herbal extract

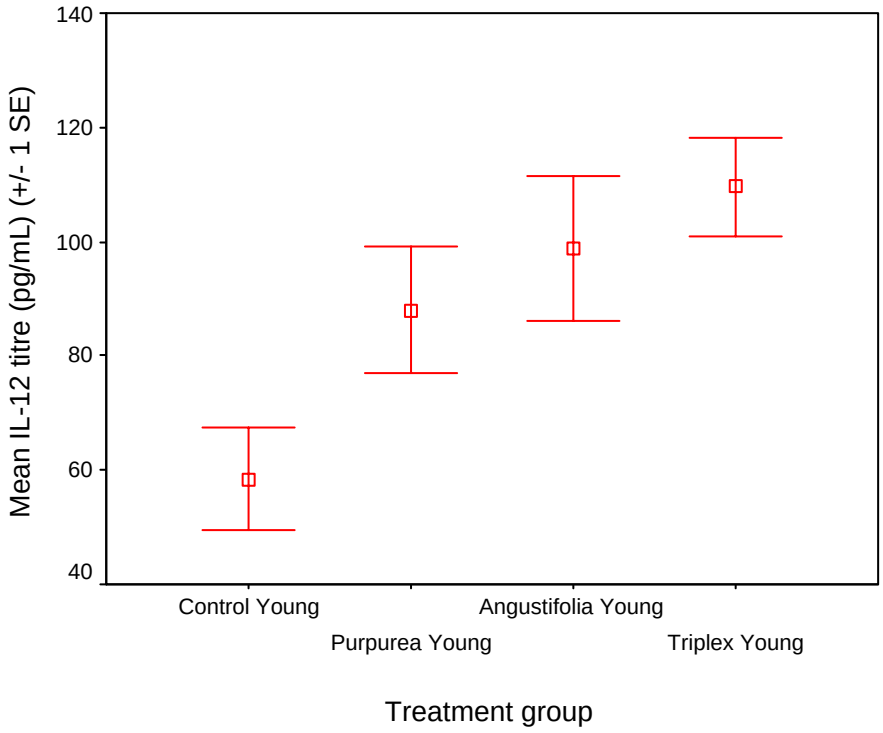

Figure 15: Comparison of mean interleukin-12 titres (± 1SE) between young treatment groups vaccinated with DTaP treated with daily oral doses of either Echinacea Triplex™, *Echinacea purpurea* 1:1, *Echinacea angustifolia* 1:1 or placebo (PBS) control

The probability that the differences in mean interleukin-12 levels observed between young Echinacea Triplex™ and young placebo (PBS) control groups was due to chance alone is 0.2% (t=4.132, df=8, p=0.002). Statistically significant results were also obtained for other young treatment groups: purpurea (t=2.074, df=8, p=0.037, one-tailed) and angustifolia (t=2.588, df=8, p=0.016).

4.7.1.4 Tracheal wash total (IgG + IgA + IgM) antibody titres

Total antibody (IgG + IgA + IgM) titres were also determined (by ELISA) for tracheal secretions of young mice vaccinated with DTaP and treated with either *Echinacea purpurea* 1:1, *Echinacea angustifolia* 1:1, Echinacea Triplex™ or placebo (PBS) (see Figure 16 for summary of these results; Appendix G, Figure 7 for curves from which this data was determined).

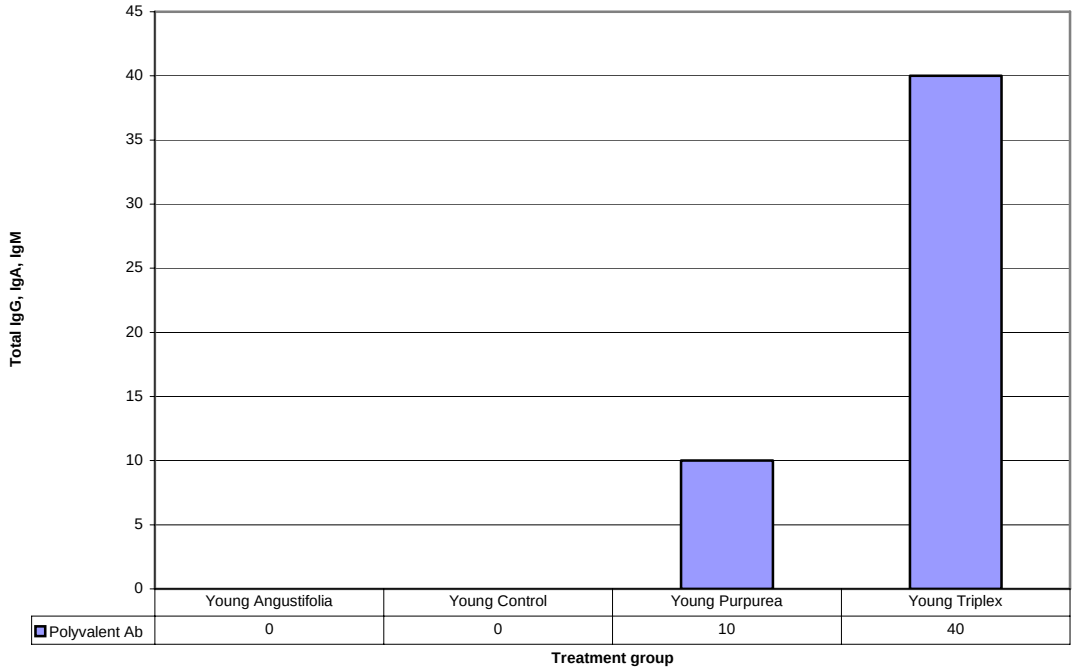

Figure 16: Comparison of total antibody (IgG + IgA + IgM) titres in pooled tracheal secretions between young DTaP-vaccinated mice treated with oral doses of either Echinacea Triplex™, *Echinacea purpurea* 1:1, *Echinacea angustifolia* 1:1 or placebo (PBS)

The titres were very low and also a high level of non-specific binding was observed in the series dilutions of the pooled tracheal secretions of young DTaP-vaccinated mice (Appendix D, Charts 13 and 14).

4.7.2 Old DTaP-vaccinated mice

4.7.2.1 Total serum antibody titres

The total anti-*B pertussis* antibody (IgG + IgA + IgM) titres determined by ELISA in the old mice receiving daily doses of either *Echinacea purpurea* 1:1, *Echinacea angustifolia* 1:1, Echinacea Triplex™ or placebo (PBS) are summarized in Table 7, (see also Figure 17 for a graphic representation of this data, see Appendix D, Table 3c for individual titres, see Appendix G, Figures 8-11 for curves used to determine titres).

Table 7: Serum anti-*B pertussis* antibody titres of DTaP-vaccinated mice given oral doses of either *Echinacea angustifolia* 1:1, Echinacea Triplex™, *Echinacea purpurea* 1:1, or placebo (PBS)

			Independent-samples t-tests for equality of means		
Group	**Mean antibody titre ± 1SE**	**Standard Deviation**	**t statistic**	**p value**	**df**
Echinacea Triplex™	390.0 ± 53.4	119.4	1.844	0.054	7
Echinacea purpurea 1:1	220.0 ± 127.1	284.2	-0.032	0.976	7
Echinacea angustifolia 1:1	30.0 ± 5.0	11.1	-2.949	0.011	7
Placebo (PBS)* control	225.0 ± 75.0	150.0	n/a	n/a	n/a

SE = standard error
df = degrees of freedom
n/a = not applicable since comparison of means is made to this group
p value is one tailed (equal variance not assumed)
* Placebo (PBS) = mice vaccinated but not given the herbal extract

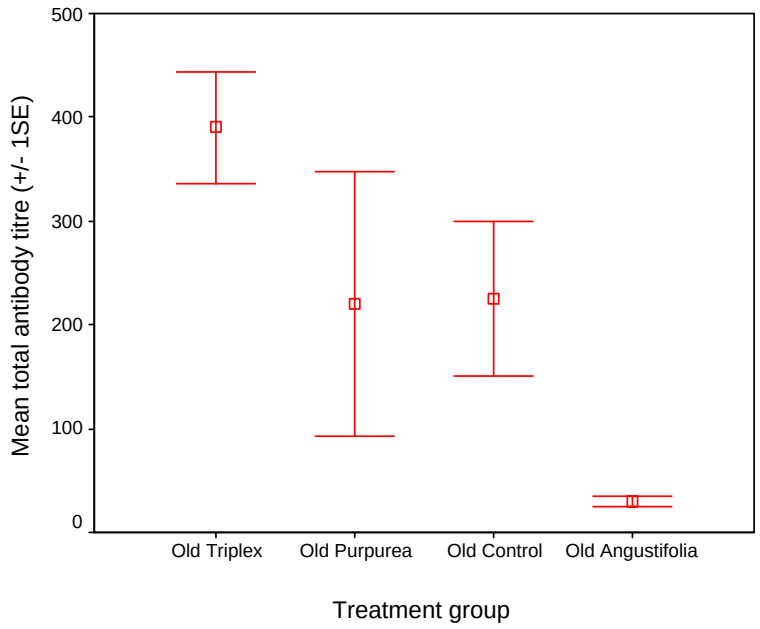

Figure 17: Comparison of total antibody (IgG + IgA + IgM) titres (±1SE) between old mice vaccinated with DTaP and treated with oral doses of either Echinacea Triplex™, *Echinacea purpurea* 1:1, *Echinacea angustifolia* 1:1 or placebo (PBS) control

The Echinacea Triplex™ treated group of mice gave a mean antibody titres that were significantly greater than those recorded for placebo (PBS) control. The difference in mean antibody titre between these two groups was not likely to be due to chance alone. (t=1.844, p=0.054, df=7). The *Echinacea purpurea* 1:1 treated group, on the other hand, did not display any statistically significant difference in mean antibody titre compared to that of the placebo control (t=-0.032, p=0.976, df=7). The *Echinacea angustifolia* 1:1 treated group had a mean antibody titre that was actually statistically significantly less than that of the placebo control group (t=-2.949, p=0.011, df=7). It must be stated that one outlier was omitted from the old control group (for individual titres see Appendix D, Table 3c), as the antibody titre was higher than the hyperimmune titre. The reason for this unexpectedly high value (1 out of 5 mice) is not known.

4.7.2.2 Antibody isotype titres

The antibody isotypes (IgG and IgA) were also quantified for the pooled serum samples from groups of old DTaP-vaccinated mice receiving either *Echinacea purpurea* 1:1, *Echinacea angustifolia* 1:1, Echinacea Triplex™ or placebo (PBS) (see Figure 18 for summary of data; Appendix G, Figures 12 and 13 for curves used to determine titres).

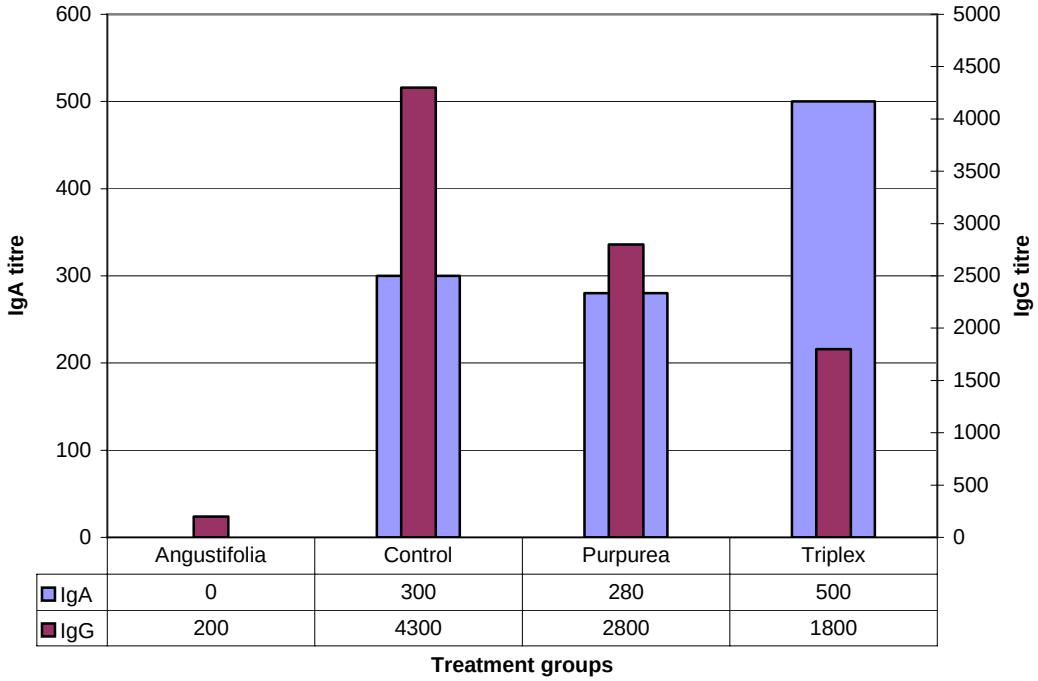

Figure 18: Comparison of antibody isotypes IgG and IgA in pooled sera between old DTaP-vaccinated mice treated with oral doses of either Echinacea Triplex™, *Echinacea purpurea* 1:1, *Echinacea angustifolia* 1:1 or placebo (PBS) control

The placebo control and *Echinacea purpurea* 1:1 treated groups display very similar results for both IgG and IgA. IgA antibody levels in the Echinacea Triplex™ group, on the other hand, were greater than those observed in the other groups. However, an

unexpected finding was the highest IgG titre observed in the pooled sample of the control group compared to the other treatment groups.

4.7.2.3 Interleukin-12 (IL-12) levels

Interleukin-12 levels in serum obtained from old mice vaccinated with DTaP and treated with either Echinacea *purpurea* 1:1, *Echinacea angustifolia* 1:1, Echinacea Triplex™ or placebo (PBS) were also quantified. These results are summarized in Table 8 (see also Figure 19 for graphic representation of this data, see Appendix D, Table 3d for individual titres, see Appendix G, Figure 15 standard curve used to determine titres).

Table 8: Serum IL-12 titres of old DTaP-vaccinated mice given oral doses of either *Echinacea angustifolia* 1:1, Echinacea Triplex™, *Echinacea purpurea* 1:1, or placebo (PBS)

			Independent-samples t-tests for equality of means		
Group	**Mean IL-12 titre ± 1SE**	**Standard Deviation**	**t statistic**	**p value**	**df**
Echinacea Triplex™	126.4 ± 9.4	21.1	2.951	0.009	8
Echinacea purpurea 1:1	83.6 ± 8.0	17.9	0.526	0.307	8
Echinacea angustifolia 1:1	72.4 ± 6.7	14.9	1.632	0.141	8
Placebo (PBS)* control	89.6 ± 8.2	18.2	n/a	n/a	n/a

SE = standard error
df = degrees of freedom
n/a = not applicable since comparison of means is made to this group
p value is one tailed (equal variance assumed)
* Placebo (PBS) = mice vaccinated but not given the herbal extract

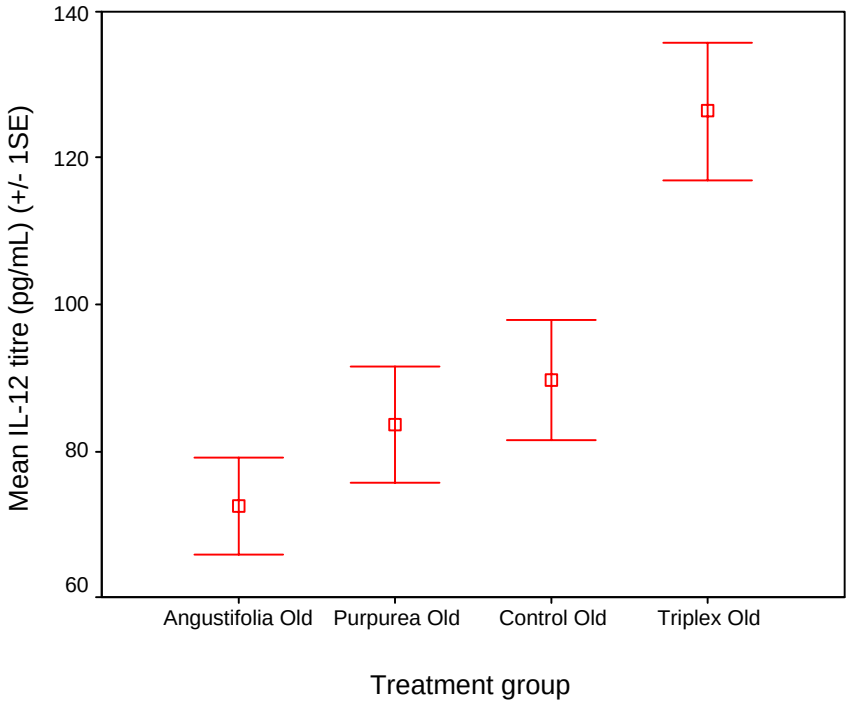

Figure 19: Comparison of mean interleukin-12 titres between old treatment groups vaccinated with DTaP and given oral doses of either Echinacea Triplex™, *Echinacea purpurea* 1:1, *Echinacea angustifolia* 1:1 or placebo (PBS, control)

The only old treatment group whose difference in mean serum interleukin-12 titre from the old control group was unlikely to have been due to chance alone was the Echinacea Triplex™ old group (t=2.951, df=8, p=0.009, one tailed). Both *Echinacea purpurea* 1:1, and *Echinacea angustifolia* 1:1 treated mice had mean serum interleukin-12 titres whose difference from the mean placebo serum interleukin-12 titre could be attributed to chance alone (*E purpurea* t=0.526, p=0.307, df=8; *E angustifolia* t=1.632, p=0.141, df=8).

4.7.2.4 Tracheal wash total antibody (IgG + IgA + IgM) titres

Total antibody (IgG + IgA + IgM) titres were determined (by ELISA) for tracheal secretions of old mice vaccinated with DTaP and treated with either Echinacea *purpurea* 1:1, *Echinacea angustifolia* 1:1, Echinacea Triplex™ or placebo (PBS). These results are summarized in Figure 20 (see Appendix G, Figure 14 for curves from which this data was determined).

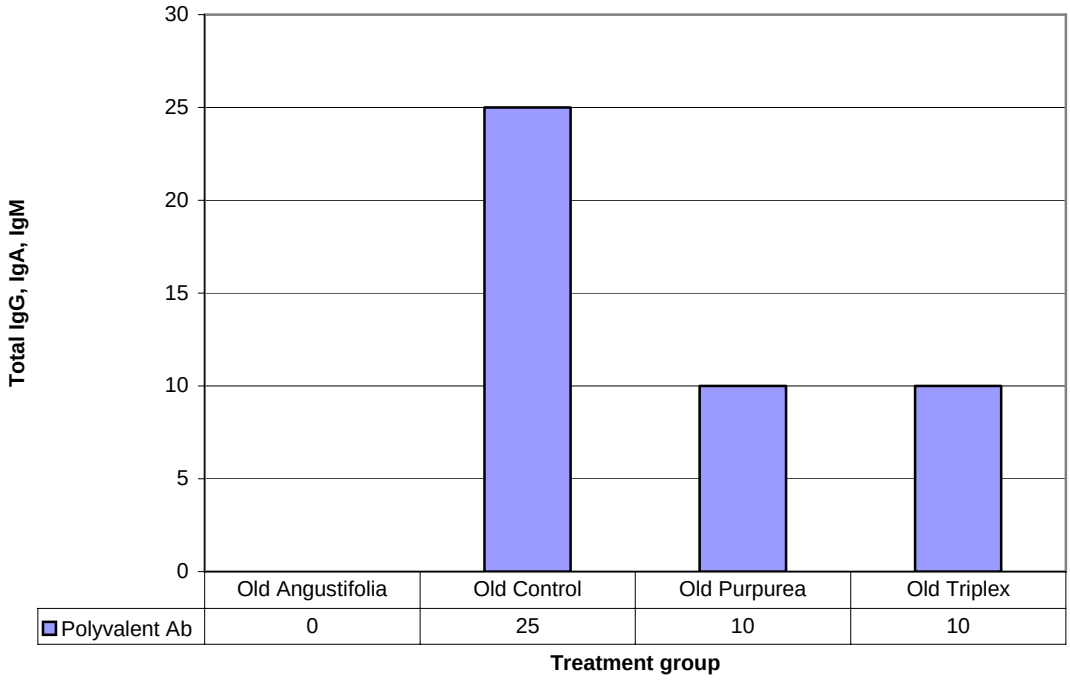

Figure 20: Comparison of total antibody (IgG + IgA + IgM) titres in pooled tracheal secretions between old DTaP-vaccinated mice treated with oral doses of either Echinacea Triplex™, *Echinacea purpurea* 1:1, *Echinacea angustifolia* 1:1 or placebo (PBS, control)

No antibodies were detected in the *E angustifolia* treatment group, whereas in the other treatment groups very low antibody levels were detected. The old mice vaccinated with DTaP show no increased antibody response in any of the treatment groups compared to the placebo control.

Chapter 5: Discussion

Knowledge of the traditional uses of some *Echinacea* species as immunostimulants and the methods used for preparation of *Echinacea* extracts provides invaluable information for contemporary research. However the lack of enthusiasm to exploit the anecdotal evidence has resulted in an incredible hiatus in the acquisition of knowledge that could be of significant value for promotion of the health of our community. While it is only reasonable to assume that compounds isolated from *Echinacea* may have similar biological activity when applied by non-traditional methods, it is important to understand the traditional applications of *Echinacea* extracts with the aim of developing and exploring strategies to make use of the traditional applications of *Echinacea* (Bauer, 2000).

A major criticism put forward by many contemporary researchers regards the lack of characterization of the bioactive principles responsible for the medicinal properties claimed for the traditional medicines. In some cases the even the phytochemical profile for the extracts being tested has not even been obtained or reported. In addition, persisting occurrence of adulterants, such as *Parthenium integrifolium,* in commercially available extracts of "*Echinacea* species" emphasizes the need for accurate identification of extracts used for characterization of biological activity (Bauer, 2000). In some studies the phytochemistry of the extract is complicated by the addition of more than one species of *Echinacea* or other herbal extracts (Bodinet and Freudenstein, 1999). Hence it is clear that the focus now needs to be concentrated on isolating biologically active compounds for two reasons. Firstly, the extracts can be standardized based on the presence of pharmacologically active

compound(s) and secondly, an opportunity is provided for the evaluation of the pharmacologically active principle for use in contemporary medicine.

5.1 Rationale underpinning this study

The studies published thus far on the stimulatory effect of *Echinacea* extracts on the non-specific and specific immune systems has been conflicting and as such have not been convincing. The study described in this thesis was aimed at addressing some of the shortcomings associated with the previous studies as highlighted below:

Firstly, the phytochemical profiles of the extracts used in this project were obtained, the exception being the initial study, which was conducted using extracts that had not been analysed by HPLC. This approach was taken in the first instance to determine if the extract did indeed have biological activity. The search for the active principle(s) was carried out in a step-wise process, from extract, to crude fractions, to purified fractions and compounds, which ensured a logical and progressive elimination of compounds and isolation and identification of all biologically active fractions, as assessed by induction/promotion/enhancement of specific antibody, interleukin-12 and interferon-γ (IFN-γ) responses. Secondly, the herbal extracts and the isolated compounds were administered daily by oral gavage for a prolonged period. This facilitated accurate dosing in a regime similar to that prescribed by many commercial preparations for use in humans by the oral route. Thirdly, the vaccination of mice with bacterial vaccines that have potential application or are already used against infectious diseases of significance to animal and/or human health is novel in this study. It is known that the killed *Salmonella typhimurium* vaccine does not induce cell-mediated immunity (CMI) (Harrison et al., 1997; Mukkur et al., 1987; Mukkur et

al., 1995; Mukkur et al., 1991b), and as such provided a model for the assessment of the CMI-inducing potential of the *Echinacea* extracts and bioactive principles. If the CMI was promoted by the administration of *Echinacea* species by the oral route, mice vaccinated with the killed *Salmonella typhimurium* vaccine should emulate the highly effective CMI response generated by live attenuated *Salmonella* vaccines (Harrison et al., 1997; Mukkur et al., 1987; Mukkur et al., 1995; Mukkur et al., 1991b). Similarly, the acellular pertussis vaccine (DTaP) is generally accepted to stimulate only antibody responses but not CMI (Mills, 2001; Cornford-Nairn, personal communication, manuscript in prep). The reason for using the DTaP as a vaccine in this project was to determine if oral administration of *Echinacea* extracts was capable of promoting one or both effector arms of the immune response viz., the humoral and/or CMI.

5.2 Problems arising during the execution of this project

Several problems arose during this investigation that needed resolution. The high non-specific binding apparent in the first study (see Appendix E, Figures 1-9) was attributed to the poor quality of the ELISA plates (ELISA tray, Disposable Products, Technology Park, SA), however the problem was resolved with the use of new plates, which were used for subsequent antibody analysis.

Although the polysaccharide fraction in the second study "the *in vivo* evaluation of fractions isolated from *Echinacea purpurea* extract for specific immunostimulatory function following vaccination with the killed whole-cell *Salmonella typhimurium* vaccine" contained low levels of phenolics, when evaluated by thin-layer chromatography at very high concentrations of polysaccharide (17 mg polysaccharide/mL) the phenolic concentration present in the immunizing dose

(which is equivalent to 24 μg polysaccharide/mL) was considered to be of little significance.

5.3 Humoral antibody response

5.3.1 Total specific antibody response

From the results of the initial study involving the use of *S typhimurium* as vaccine, it appeared that oral administration of the ethanolic extracts of *Echinacea* species had an immunostimulatory effect on the total specific antibody response. Of the three medicinal species of *Echinacea, E purpurea* had the greatest effect on specific antibody response in the first trial. However, as seen also in the DTaP study, oral administration Echinacea Triplex™ had an even greater stimulatory effect on total specific antibody response. On the other hand, "Triplex innovation" did not have a statistically significant effect on specific antibody response. This apparent anomaly is most likely due to the differences in extraction procedures between the two preparations (see Section 3.5.5), since both were made from the same herbs but extracted by different methods (see below).

There is one major component that differs markedly between the two mixtures that is likely to explain the differences in biological activity, while the other two components are very similar. The *Echinacea pallida* 1:1 and the *Echinacea angustifolia* 1:1 extracts were extracted according to the same procedures in both mixtures, although in differing concentrations (*E pallida* 1:1 was dried roots extracted in 45% ethanol; *E angustifolia* was dried roots extracted in 60% ethanol). However, the *Echinacea purpurea* extracts differed markedly between the two mixtures. The *Echinacea*

purpurea extract in *Echinacea Triplex*™ was a combination of *Echinacea purpurea* FP 1:2 (fresh aerial parts extracted in 25% then 60% ethanol combined with fresh root extracted in 50% ethanol in a 9:1 ratio) and *Echinacea purpurea* 3:1 bolster (dried aerial parts extracted in 95% ethanol). The *Echinacea purpurea* extract in "Triplex innovation," was *Echinacea purpurea* WP 1:1 (whole-plant extracted in 65% ethanol). It was likely that the *Echinacea purpurea* component of Echinacea Triplex™ contained higher amounts of polysaccharides (because of the extraction in 25% ethanol) and of alkylamides (because of the extraction in 95% ethanol) than that contained in the *Echinacea purpurea* component of "Triplex innovation." Unfortunately, the phytochemical profile of the latter was not recorded, and as such the stated assumption has not been verified. The state (fresh/dried) of the herb, as well as plant part (aerial vs roots) used for preparation of the extract was likely to affect the phytochemical profile as well.

We can deduce, however, that *E. pallida* extract was not likely to contain active biological principle(s) since the *E pallida* 1:1 root extract was not found to have a significant positive effect on specific antibody response in the initial study (see Section 4.5). (It was for this reason that *E pallida* 1:1 roots was omitted from the third study involving the use of DTaP vaccine). While the *E angustifolia* 1:1 treatment group in Study 1 displayed a significant increase in mean antibody response, the increase was not as great as that observed for the *Echinacea purpurea* 1:1 treatment group. Since it was only rational to fractionate the most promising extracts for further analysis in this investigation, and given the insignificant immunostimulation obtained in the *E. angustifolia*-treated group in the DTaP study (Study 3), it was decided to concentrate only on determining the bioactive principles

of the *E. purpurea,* component of Echinacea Triplex™, which yielded the most significant increase in serological specific antibody response in both the initial *S typhimurium* and DTaP studies compared to the other *Echinacea* species (see Section 4.5).

In Study 2, "the *in vivo* evaluation of fractions isolated from *Echinacea purpurea* extract for specific immunostimulatory function following vaccination with the killed whole-cell *Salmonella typhimurium* vaccine," the polysaccharide, phenolic and alkylamide groups all yielded statistically significant immunostimulation, as determined by the increase of total specific antibody response. However, if polysaccharide, phenolic and alkylamide fractions were all indeed promoting an increase in the level of specific anti-*S typhimurium* antibody formation, the effect did not appear to be a cumulative or synergistic one, since the increase observed in mean antibody titre of the Echinacea Triplex™ group was comparable to the levels observed in both the alkylamide and polysaccharide groups. Of course, this study could not rule out the presence of inhibitory bioactive principles present in the Echinacea Triplex™, which is a mixture of the non-fractionated extracts of the three species of *Echinacea* viz. *Echinacea purpurea, Echinacea angustifolia* and *Echinacea pallida.*

5.3.2 Antibody isotype

The antibody isotype titres were obtained for pooled serum samples from treatment and control groups for both Study 2 and Study 3. The reason for using the pooled sera

was the shortage of sera after using individual mouse sera for determination of total specific antibody and IL-12 levels (see Sections 4.7.1.2 and 4.7.2.2).

In Study 2, the only treatment group that affected an apparent increase in IgA was the Echinacea Triplex™ group. This was interesting given the statistically significant increases in total antibody titres seen in all treatment groups (polysaccharide, phenolic, alkylamide, and Echinacea Triplex™). This apparent difference may be due to the following reasons: (a) unrepresentative dosages of the purified compounds, leading to a diminished biological activity, despite every effort being made to ensure that dosage rates of fractions was comparable to that of the crude extracts, (b) the fractions did have biological activity but the effect observed in the IgA response was a synergistic one. No apparent effect, however, was observed in the IgG levels of any of the treatment groups for this study. This was an interesting albeit unexpected result given the statistically significant results of the total antibody titre, and hence needs to be confirmed in a repeat experiment.

In Study 3 similar differences were apparent. Again, the only group that displayed an apparent increase in IgA levels for both young and old treatment mice was Echinacea Triplex™. Interestingly, the *Echinacea purpurea* 1:1 treatment group displayed an apparent increase in IgG level compared to the placebo group in the young treatment group, but not in the old treatment group. This trend was also observed in the total antibody titre, with the young *Echinacea purpurea* 1:1 treatment group yielding a significant increase, while the old treatment group did not. Although these differences could be due to statistical anomalies, the study needs to be repeated with larger groups in order to draw definitive conclusions. Also the antibody isotypes

titres should be determined using individual mouse sera, rather than pooled group sera, to obtain a more accurate representation of changes in the antibody isotype levels. Further work into the effect of age on specific antibody response in mice fed *Echinacea* extracts and immunised with microbial vaccines needs to be carried out, as there has been no investigations published on this subject.

5.3.3 Comparison of immunomodulatory properties of *Echinacea* species with previously published studies

There is conflicting evidence on the efficacy of *Echinacea* extracts to stimulate specific antibody formation. Rehman et al. (1999) showed that the oral treatment of rats with a commercially available aqueous-ethanolic extract of *Echinacea angustifolia* root (Eclectic Institute, Inc, Sandy) significantly increased serological primary and secondary IgG response to a high molecular weight protein, keyhole limpet hemocyanin. While the extract was administered orally, it was presented in the drinking water, and as such exact quantity of the extract consumed could not be calculated. Furthermore, unlike the present study, the phytochemical profile of the extract was not determined, and the extraction procedure not specified. Similarly, Bodinet and Freudenstein (1999) showed that a mixture of aqueous-ethanolic extracts of *Thuja occidentalis* herba, *Baptisie tinctoriae* radix, *Echinacea purpurea* radix and *Echinacea pallidae* radix augmented the specific antibody response to sheep blood cells in mice. Again, determination of the quantity of the extract consumed was difficult as the extracts were administered via the drinking water; and the phytochemical profile, which was not elucidated, was complicated by the presence of multiple herbs. In another study, chickens immunised with human serum and fed *Echinacea angustifolia* extract were reported to develop a significant increase in three

classes of antibody (IgG, IgA, IgM) (Schranner et al., 1989). However, the phytochemical profile of the extract was not presented. On the other hand, a recent study by South and Exon (2001) however, found no evidence of altered specific antibody response in rats fed a commercial *Echinacea* preparation.

Hence, the studies presented in this thesis are novel for the following reasons: (a) doses representing standardised quantities of the extracts were administered by the oral route; (b) phytochemcial profiles of the extracts or fractions used were elucidated; (c) it is the first time, to the best of my knowledge, that vaccines against diseases of significance to animals or humans have been used in the elucidation of the immunostimulatory properties of *Echinacea* species.

5.4 Cell-mediated immunity

Interleukin (IL)-12 is an important cytokine, produced by macrophages and B lymphoblastoid cells, that is involved in the inflammatory response and induces naïve T cells to differentiate into interferon- (IFN)-γ producing T helper $T_H1$cells (Trinchieri, 1994). Interferon-γ contributes to resistance to infection by increasing expression of MHC class II antigens on a variety of cell types resulting in an increase in antigen presentation to T cells, and by inducing activation and proliferation of natural killer (NK) and cytotoxic T (Tc) cells. This study has shown a statistically significant increase in IL-12 production (1) in the serum of young mice vaccinated with the DTaP vaccine following oral application of ethanolic extracts of *E. angustifolia*, *E. purpurea*, and Echinacea Triplex™, (2) in old mice vaccinated with the DTaP vaccine receiving oral doses of Echinacea Triplex™ and (3) in serum of mice vaccinated with killed *Salmonella typhimurium* vaccine following oral

application of Echinacea Triplex™ and phenolic compounds isolated from *E. purpurea* (fresh plant) extracts.

In the second study "the *in vivo* evaluation of fractions isolated from *Echinacea purpurea* extract for specific immunostimulatory function following vaccination with killed whole-cell *Salmonella typhimurium* vaccine," the pooled splenocyte culture supernatant was found to contain comparatively high levels of IFN-γ in the phenolic and polysaccharide treatment groups (see Figure 12), however these results also need to be repeated for confirmation because of the high variability of hyperimmune and alkylamide replicates. Another reason for recommending repetition of this experiment is the low yield of IFN-γ produced by splenocytes of mice in the Echinacea Triplex™ treatment group considering the relatively high titres obtained in the phenolic and polysaccharide groups (fractions found in Echinacea Triplex™). The low IFN-γ titre obtained for the hyperimmune group (which received three vaccinations) confirms that vaccination with a killed *Salmonella typhimurium* vaccine does not induce CMI (Harrison et al., 1997; Mukkur et al., 1987; Mukkur et al., 1995; Mukkur et al., 1991b).

The ultimate proof of the effectiveness of the induced CMI by extracts of *Echinacea* species will be the demonstration of protection against challenge infection with virulent *S typhimurium* in mice vaccinated with the killed vaccine and fed extracts of *Echinacea* species. However, this experiment was not carried out in this project because of time constraints.

5.4.1 Comparison of cell-mediated immunity results with previous studies

Since IL-12 is known to cause the activation of natural killer (NK) cells, which are responsible for the spontaneous lysis of tumor cells and antibody-dependent cellular cytotoxicity, an extract or compound that was able to stimulate IL-12 production could find application in the treatment of cancer. Currier and Miller (2000, 2002) demonstrated that an extract of the roots of *E purpurea* stimulated NK cell production, both in aging mice and also in leukemic mice immunised with killed erythroleukemia cells. However, neither the phytochemical profile of the extracts nor determination of IL-12 levels were carried out. On the other hand, South and Exon (2001) found no evidence of the enhancement of NK cell production or T-cell mediated delayed-type hypersensitivity by commercial *Echinacea* products consisting of either glycerol-extracted (10%) *Echinacea purpurea*, or in ethanolic extracts (60%) of *Echinacea angustifolia, E pallida* or *E purpurea,* however the phytochemical profiles were also not presented. One possible reason for the failure of these preparations could be the absence of alkylamides in the glycerol extracts and the absence of polysaccharides in the ethanolic extracts, in some way resembling the process used for the preparation of "Triplex innovation" in this project.

Hayashi et al. (2001) reported an increase in the production of endogenous interferon-γ (IFN-γ) in mice given oral doses of powdered *Echinacea purpurea* leaves (prepared by drying a 70% ethanol extract), however, levels of interleukin-12 were unaffected, which is surprising because of the well-accepted role of this cytokine in the production of IFN-γ by T_H1 and NK cells (Trinchieri, 1994). Goel et al (2002), on the other hand, found that components isolated from *Echinacea purpurea* (cichoric acid,

polysaccharides and alkylamides) had had no effect on the release of endogenous IFN-γ or IL-2 by the splenocytes in male Sprague-Dawley rats. However, in this experiment the immune system of the rats was not challenged by vaccination, nor were the splenocytes stimulated with antigen. Studies presented in this thesis clearly demonstrated the potential of *Echinacea* products or extracts (Echinacea Triplex™, *Echinacea purpurea, Echinacea pallida, Echinacea angustifolia* and phenolic fraction obtained from *Echinacea purpurea* extract) in the stimulation of IL-12 with potential implications for intracellular microbial (bacterial and viral) infections and cancers.

5.5 Future research directions

Considering the knowledge gained in this project on the immunostimulatory properties of Echinacea Triplex™ and *Echinacea purpurea,* further confirmatory and exploratory investigations using the microbial vaccine models used in this project are warranted. This is important because IL-12 production promoted by these respective extracts, or fractions thereof have been documented to promote the replication of NK and Tc cells and could play an important role in the treatment of many disease conditions particularly intracellular infectious diseases such as salmonellosis (Mastroeni et al., 1998). Possible synergistic effect of active fractions/compounds on the immune system also requires exploration.

The immunomodulatory properties of polysaccharide and alkylamide fractions should also be re-assessed using a similar experimental design including administration via invasive routes such as intraperitoneal, intramuscular or subcutaneous routes. This suggestion is considered to be rational since many of the currently used regimes, for example in the treatment of cancer, are administered by one or more of these routes.

This will be particularly relevant if the levels of IL-12 and IFN-γ are found to be promoted using one or more of these modes of administration. This is not withstanding the fact that in addition to specific antibodies, induction of IL-12 and IFN-γ following vaccination are considered to play a significant role in long-term protection against whooping cough (Mills, 2001).

From the perspective of herbal medicine formulations, isolation of compounds with specific immunostimulatory function also has significant implications. Knowledge of active principle(s) would also enable standardization of commercial extracts currently sold in the marketplace and could also be of assistance in the determination of the shelf-life of these preparations. Finally, this knowledge will also provide opportunity for the plant biotechnologists to produce transgenic medicinal plants producing large amounts of the active principle(s).

Chapter 6: Summary and Conclusions

In this project the suggested applications of the currently marketed commercial products of Greenridge Botanicals, a Division of Thursday Plantations Pty, Ltd, and the available literature on contemporary research were taken into consideration. Based on this information, it was decided to adopt a dosing regime involving oral gavage, at doses calculated on a body weight basis, over a definitive period of time. A daily oral treatment regime for a period of 35 days was chosen and calculated doses administered by oral gavage, ensuring delivery of the accurate doses. It was also considered important to fully describe the procedures used for preparation of the extracts and characterisation of their phytochemical profiles where considered appropriate.

The decision to use the bacterial vaccines in this study was novel. Two vaccines were used in this study, namely, a killed *Salmonella typhimurium* vaccine and an acellular pertussis vaccine (DTaP). The former vaccine was chosen because it is known that the killed *Salmonella typhimurium* vaccine does not induce cell-mediated immunity (CMI) and as such provided a model in which induction of CMI by the extracts used would be measurable. If CMI was induced by the administration of *Echinacea* species by the oral route, mice vaccinated with the killed *Salmonella typhimurium* vaccine should emulate the highly effective CMI response generated by live attenuated *Salmonella* vaccines, for which investigation is currently in progress in Professor Mukkur's laboratory. The decision to use the DTaP vaccine, selected as a human vaccine representative, was also based upon the consensus opinion in the literature about its inability to stimulate the induction of CMI. Since both the above-

mentioned vaccines also induce specific antibody responses, the use of these two vaccines should also permit measurement of an enhanced humoral immune responses following oral administration of the *Echinacea* extracts.

Given the substantial conflicting evidence on the immunostimulatory potential of *Echinacea* species extracts to stimulate either one or both arms of the immune response, that is, the humoral and cell-mediated immune response, results obtained in this study supported some and contradicted the findings published by other investigators. In Studies 1 and 2, oral administration of Echinacea Triplex™, *Echinacea purpurea,* and *Echinacea angustifolia* as well as polysaccharide, alkylamide and phenolic fractions prepared from *Echinacea purpurea* to mice vaccinated with killed *Salmonella typhimurium* caused a significant enhancement of specific antibody levels. On the other hand, "Triplex innovation," which represented a mixture of equal volumes of the three *Echinacea* species extracts, was found to be ineffective in enhancing the antibody levels against *Salmonella typhimurium* in Study 1. The observed difference in the immunostimulating property of Echinacea Triplex™ vs "Triplex innovation" could be due to the differences in the physical state of the herbs (fresh vs dried) as well as the differing percentages of ethanol used in the extraction processes.

In the second study involving the use of killed *Salmonella typhimurium* as vaccine, it was discovered that the polysaccharide and alkylamide fractions significantly enhanced the production of specific antibodies. The extraction process used in the preparation of Echinacea Triplex™ involves, firstly, the extraction of fresh (not dried) *Echinacea purpurea* with 25% ethanol, which ensures extraction of the polar

polysaccharides, and secondly, extraction of dried *Echinacea purpurea* with 95% ethanol, which ensures extraction non-polar alkylamides. This is in contrast to the single-step extraction procedure used in the preparation of "Triplex innovation" which involved extraction of *Echinacea purpurea, E angustifolia* and *E pallida* in 65%, 60%, and 45% ethanol respectively.

The results for the antibody isotype titres carried out using pooled sera yielded variable results and as such need to be repeated using individual serum samples. However several observations were made. In both Studies 2 and 3 (antibody isotypes were not determined for Study 1) the only treatment group that displayed an obvious increase the IgA titre compared to the placebo (PBS) control was the Echinacea Triplex™ group. However, these titres cannot be subjected to statistical analysis because of the use of pooled sera. This result was unexpected, particularly for the polysaccharide and alkylamide fraction groups, in which significant increases total antibody titre had been observed. Whether any synergism between two bioactive fractions may have contributed to observed enhancement of IgA antibody isotype titres in the Echinacea Triplex™ group was not investigated. The IgG isotype displayed an apparent increase in only the young *Echinacea purpurea* 1:1 treatment group in Study 3. On the other hand, the old *Echinacea purpurea* 1:1 treatment group did not display any increase in IgG response in Study 3, nor did fractions prepared from *Echinacea purpurea* display any increase in IgG response in Study 2. It is thus clear that there is a need to repeat the experiments on the generation of antibody isotype specificity using individual serum samples from vaccinated mice at different ages and subjected to treatments with the *Echinacea* extracts as per the design used in this project.

Mice immunised with the DTaP vaccine and given oral gavage with *E. angustifolia*, *E. purpurea* or Echinacea Triplex™ showed significant increases in IL-12 production in serum of young mice. These results were not confirmed in the old mice receiving identical treatments, except in the Echinacea Triplex™ group, which displayed a statistically significant increase compared to the placebo control. The Echinacea Triplex™ group, as well as the phenolic fraction treatment group, also displayed significant increases in the mean IL-12 titre in Study 2, in which mice were vaccinated with the killed *Salmonella typhimurium* vaccine. These results are indeed exciting but need to be confirmed in repeat experiments.

Overall it is clear that the experiments particularly relating to the DTaP as a vaccine need to be carried out using mice of different age groups because of the differences in the results obtained in the young versus older mice. The information to be gained is likely to be particularly interesting since there is no published comparative data on the immunostimulatory properties of Echinacea extracts in young versus older mice.

Another reason for suggesting repetition of studies, particularly on the promotion of the CMI-indicator cytokines, is the terribly low IFN-γ titres obtained in Study 2 for all the treatment groups. Of particular concern was the Echinacea Triplex™ treatment group, which displayed very low IFN-γ titres despite displaying significant serum IL-12 titres. While on the other hand, the IFN-γ titres in the phenolic and polysaccharide fractions treatment groups (fractions present in Echinacea Triplex™) although also low in magnitude had higher mean values than those in the Echinacea Triplex™ treatment group.

Further studies particularly on the CMI-inducing or promoting properties of the purified bioactive polysaccharide, alkylamide and phenolic compounds are necessary and crucial for determination of their potential therapeutic value against intracellular microbial infections and in cancer patients being subjected to radiation or chemotherapy. The possible synergistic effect of the immune-enhancing fractions/compounds also deserves to be explored, testing for determination of optimal dosage rates for maximal enhancement of the immune functions.

APPENDIX A: Reagents

(in alphabetical order)

ANISALDEHYDE-SULPHURIC SPRAY REAGENT (For detection of carbohydrates)

Conc. H_2SO_4	0.5 mL
95% EtOH	9.0 mL
Anisaldehyde	0.5 mL
Glacial CH_2COOH	Few drops

Add the sulphuric to the ethanol, allow to cool. Add anisaldehyde, and acetic acid, mix gently and thoroughly.

COUPLING BUFFER (0.05M CARBONATE BUFFER, pH 9.6)

Na_2CO_3	1.18 g
$NaHCO_3$	3.47 g

Dissolve in 1 litre of water and adjust pH to 9.6. Store at 4°C. Make fresh solution every fortnight.

MOTILITY-TEST AGAR, pH 7.0

Peptone	10 g
Sodium Chloride	5 g
Agar	3.5 g

Suspend in 1 L of water by shaking vigorously. Boil until agar melts then autoclave at 121ºC for 15 minutes.

20 mM PHOSPHATE BUFFER, pH 7.5

K_2HPO_4	2.30 g
KH_2PO4	0.92 g

Dissolve the above in 975 mL water an adjust pH to 7.5 with 1 M phosphoric acid; add water to final volume of 1 l.

PHOSPHATE BUFFERED SALINE (PBS)

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

Dissolve the above in 975 mL water and adjust pH to 7.2 with 1M phosphoric acid. Add water to final volume of 1 l. Make fresh solution every week.

Composition of SIGMA-FAST™ TABLETS (o-phenylenediamine dihydrochloride substrate tablet sets)

When dissolved in 20 mL water:

o-phenylenediamine dihydrochloride (OPD)	0.4 mg/ml
Hydrogen peroxide	0.4 mg/ml
Phosphate-citrate buffer	0.05 M

TISSUE CULTURE MEDIA

(a) WITHOUT FETAL BOVINE SERUM (FBS) (used for washing cells)

To Dulbecco's Modified Eagle Media (Gibco, Invitrogen Corporation, Auckland, NZ) add 25 mN HEPES, 12 uM 2-mecaptoethanol and 100 units/ml penicillin-steptomycin (all final concentration).

(b) WITH 10% FBS (used for culturing cells)

To the tissue culture media (a) add 10% fetal bovine serum.

WASH BUFFER

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

APPENDIX B: Chemical analytical techniques

Contents:

PHENOL-SULPHURIC TOTAL CARBOHYDRATE ASSAY

Glucose (Sigma, St Louis, MO) standards were accurately prepared in 10 µg intervals from 0 µg/mL to 100 µg/ml. The sample was diluted 1/400, and 1/800. 150 µL of each standard and sample was aliquoted into 1.5 mL microfuge tubes (Axygen Scientific, Union City, CA). 150 µL of 5% phenol (Sigma, St Louis, MO) was added to each tube, mixed and stood at room temperature for 5 minutes. 750 µL of concentrated sulphuric acid (Sigma, St Louis, MO) was added rapidly to each tube, the tubes were sealed, vortexed, and placed in a boiling water bath for 10 minutes. The tubes were allowed to cool to room temperature, and the absorbance was measured at 490 nm, correcting against the reagent blank. A standard curve was plotted, and values for the unknown samples extrapolated (see Appendix C, Chart 1).

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

The method used to determine the presence and concentration of phenolics was a 10-15% acetonitrile gradient over 15 minutes on a Waters Symmetry C_8 column (150 x 3.9 mm) with 0.1% phosphoric acid added to both water and acetonitrile using a Waters photo-diode array (PDA) detector (Waters 600 controller, Waters Australia, Rydalmere, NSW).

A 50-70% acetonitrile gradient over 15 minutes on a Phenomenex (Pennant Hills, NSW) Luna 5 µm $C_{18}(2)$ (150 x 2.0 mm) with 0.1% acetonitrile added to both water and acetonitrile was used to quantify the alkylamides in the fractions. The instrument and detector was the same as for phenolics method.

THIN-LAYER CHROMATOGRAPHY

Method 1. CONFIRMATION OF AUTHENTICITY OF HERBS/PURITY OF POLYSACCHARIDE FRACTION

The mobile phase was acetic acid (Sigma, St Louis, MO), formic acid (Sigma, St Louis, MO), water, and ethyl acetate (Sigma, St Louis, MO) in the ratio 11:11:27:100. The stationary phase was a glass-backed silica-60 plate, F_{254} (EM Science). Standards were prepared from 1 g certified authenticated herb sample (authenticated by Greenridge Botanicals) sonicated for 10 minutes in 5 mL methanol (Merck, Kilsyth, Vic), then filtered. 5 microlitres of the herbal extracts, and 10 microlitres standards were spotted onto the plate in 1 cm by 1 mm bands, allowed to dry for 15 minutes, and run for 30 minutes in the pre-equilibrated tank. The plate was observed and photographed under 254nm. The plate was then sprayed with 1% w/v diphenylboric acid, beta-ethylamino ester (Sigma, St Louis, MO) in methanol (Sigma, St Louis, MO), followed by 5% w/v polyethylene glycol-4000 (Sigma, St Louis, MO) in ethanol (Recochem, Lytton), allowed to dry and photographed under 365 nm.

Method 2. ANALYSIS OF FRACTIONATED POLYSACCHARIDE EXTRACT

The stationary phase was a glass-backed silica-60 plate, F_{254} (EM Science), and the mobile phase was methanol only (Sigma, St Louis, MO). 10 µl of undiluted fractions were spotted onto the plate 2 cm from the edge of the plate, in 1 x 10 mm bands. The plate was allowed to dry, run for 25 minutes in the pre-equilibrated tank, and allowed to dry. The plate was sprayed with Anisaldehye-Sulphuric reagent (Appendix A), heated at 100ºC for 10 minutes and photographed under natural light. The photographs were enhanced in by increasing the contrasts.

DETERMINATION OF PROTEIN CONCENTRATION (Coomassie® Plus Protein Assay Reagent Kit, supplied by Progen, Richlands Qld, Bradford Assay)

Briefly, standards of known protein concentration were prepared, and the sample diluted ½. 150 µL of each standard and sample was aliquoted into separated wells of a 96 well plate (ELISA Plate, Disposable Products, Technology Park, SA). 150 µL of the Coomassie® Plus Reagent was added to each well, mixed for 30 seconds. The absorbance was measured at 595 nm, minus the reagent blank (Biorad Microplate reader, Model 550, Hercules, CA). A standard curve was plotted, from which the unknown protein concentrations were determined (See Appendix C, Chart 2).

REFERENCES

Bauer, R. (2000). Chemistry, pharmacology and clinical applications of *Echinacea* products. In Herbs, Botanicals and Teas, O. B. Mazza G, ed. (Lancaster, Technomic Publishing Co., Inc).

Bauer, R., and Foster, S. (1991). Analysis of alkamides and caffeic acid derivatives from *Echinacea simulata* and *E. paradoxa* roots. Planta Medica *57*, 447-449.

Bauer, R., Khan, I., Wray, V., and Wagner, H. (1985). Structure and stereochemistry of new sesquiterpene esters from *Echinacea purpurea* (L) Moench. Helv Chim Acta *68*, 2355-2358.

Bauer, R., and Remiger, P. (1989). TLC and HPLC analysis of alkamides in *Echinacea* drugs. Planta Medica *55*, 367-371.

Bauer, R., Remiger, P., and Wagner, H. (1988a). Alkamides from the roots of *Echinacea purpurea*. Phytochemistry *22*, 2339-2342.

Bauer, R., Remiger, P., and Wagner, H. (1989). Alkamides from the roots of *Echinacea angustifolia*. Phytochemistry *28*, 505-508.

Bauer, R., and Wagner, H. (1991). *Echinacea* species as potential immunostimulatory drugs. In Economic and Medicinal Plant Research, F. R. Wagner H, ed. (London, Academic Press), pp. 253-321.

Bauer, V. R., Jurcic, K., Puhlmann, J., and Wagner, H. (1988b). Immunologic *in vivo* and *in vitro* studies on Echinacea extracts. Arzneimittelforshung *38*, 276-281.

Begg, A. P., Walker, K. H., Love, D. N., and Mukkur, T. K. (1990). Evaluation of protection against experimental salmonellosis in sheep immunised with 1 or 2 doses of live aromatic-dependent *Salmonella typhimurium*. Australian Veterinary Journal *67*, 294-298.

Bodinet, C., and Freudenstein, J. (1999). Effects of an orally applied aqueous-ethanolic extract of a mixture of *Thuja occidentails* herba, *Baptisia tinctoriae* radix, *Echinacea purpurea* radix and *Echinacea pallidae* radix on antibody response against sheep red blood cells in mice. Planta Medica *65*, 695-699.

Bonadeo, I., Bottazzi, G., and Lavazza, M. (1971). Echinacina B: polisaccaride attivo dell' Echinacea. Riv Ital Essenze Profumi *53*, 281-295.

Brevort, P. (1998). The booming US botanical market: a new overview. Herbalgram *44*, 33-46.

Church, M., and Robinson, C. (1988). Advances in Eicosanoid Research: Eicosanoids in Inflammatory Conditions of the Lung (Lancaster, MTP Press Limited).

Coeugiet, E. G., and Elek, E. (1987). Immunomodulation with Viscum album and *Echinacea purpurea* extracts. Onkologie *10*, 27-33.

Currier, N. L., and Miller, S. C. (2000). Natural killer cells from aging mice treated with extracts from *Echinacea purpurea* are quantitatively and functionally rejuvenated. Experimental Gerontology *35*, 627-639.

Currier, N. L., and Miller, S. C. (2002). The effect of immunization with killed tumor cells, with/without feeding of *Echinacea purpurea* in an erythroleukemic mouse model. Journal of Alternative Complementary Medicine *8*, 49-58.

Egert, D., and Beuscher, N. (1992). Studies on antigen specificity of immunoreactive arabinogalactan proteins extracted from *Baptisia tinctoria* and *Echinacea purpurea*. Planta Medica *58*, 163-165.

Eisenberg, D. M., David, R. B., and Ettner, S. L. (1998). Trends in alternative medicine use in the United States 1990-1993. JAMA *280*, 1569-1575.

Eisenberg, D. M., Kessler, R. C., and Foster, C. (1993). Unconventional medicine in the United States. N Engl J Med *328*, 246-252.

Elsasser-Beile, U., Willenbacher, W., Bartsch, H. H., Gallati, H., Schulte, J., Monting, J., and von Kleist, S. (1996). Cytokine production in leukocyte cultures during therapy with Echinacea extract. J Clin Lab Anal *10*, 441-445.

Facino, R. M., Carini, M., Aldini, G., Saibene, L., Pietta, P., and Mauri, P. (1995). Echinacoside and caffeoyl conjugates protect collagen from radical-induced degradation: a potential use of *Echinacea* extracts in the prevention of skin phytodamage. Planta Medica *61*, 510-514.

Facino, R. M., Sparatore, A., Carini, M., Gioia, B., Arlandini, E., and Franzoi, L. (1991). Field desorption mass spectrometry, fast atom bombardment mass spectrometry and fast atom bombardment tandem mass spectrometry of Echinacoside, the main caffeoyl-glycoside from *Echinacea angustifolia* roots (Asteraceae). *26*, 951-955.

Giles, J. T., Palat, C. T., Chien, S. H., Chang, Z. G., and Kennedy, D. T. (2000). Evaluation of Echinacea for treatment of the common cold. Pharmacotherapy *20*, 690-697.

Goel, V., Chang, C., Slama, J. V., Barton, R., Bauer, R., Gahler, R., and Basu, T. K. (2002). Alkylamides of *Echinacea purpurea* stimulate alveolar macrophage function in normal rats. International Journal of Immunopharmacology *2*, 381-387.

Harrison, J. A., Villarreal-Ramos, B., Mastroeni, P., Demarco de Hormaeche, R., and Hormaeche, C. E. (1997). Correlatesof protection induced by live Aro- *Salmonella typhimurium* vaccines in the murine typhoid model. Immunology *90*, 618-625.

Hayashi, I., Ohotsuki, M., Suzuki, I., and Watanabe, T. (2001). Effects of oral administration of *Echinacea purpurea* (American herb) on incidence of spontaneous leukemia caused by recombinant leukemia virusis in AKR/J mice. Japanese Journal of Clinical Immunology *24*, 10-20.

He, X., Lin, L., Bernart, M., and Lian, L. (1998). Analysis of alkamides in roots and achenes of *Echinacea purpurea* by liquid chromatography - electrospray mass spectrometry. Journal of Chromatography A *815*, 205-211.

Kim, L. S., Waters, R. F., and Burkholder, P. M. (2002). Immunological activity of larch arabinogalactan and Echinacea: A preliminary, randomized, double-blind, placebo-controlled trial. Alternative Medicine Review *7*, 138-149.

Leuttig, B., Steinmuller, C., Gifford, G. E., Wagner, H., and Lohmann-Matthes, M. (1989). Macrophage activation by the polysaccharide arabinogalactan isolated from plant cell cultures of *Echinacea purpurea*. Journal of the National Cancer Institute *3*, 669-675.

Lindenmuth, G. F., and Lindenmuth, E. B. (2000). The efficacy of Echinacea compound herbal tea preparation on the severity and duration of upper respiratory and flu symptoms: a randomized, double-blind placebo-controlled study. Journal of Alternative Complementary Medicine *6*, 327-334.

Lloyd, J. U. (1904). History of *Echinacea angustifolia*. Pharm Review *22*, 9-14.

Maclennan, A. H., Wilson, D. H., and Taylor, A. W. (1996). Prevalence and cost of alternative medicine in Australia. Lancet *347*, 569-573.

Mastroeni, P., Harrison, J. A., Robinson, J. H., Clare, S., Khan, S., Maskell, D. J., Dougan, G., and Hormaeche, C. E. (1998). Interleukin-12 is required for control of the growth of attenuated aromatic-compound-dependent salmonellae in BALB/c mice: role of gamma interferon and macrophage activation. Infect Immun *66*, 4767-4776.

Melchart, D., Linde, K., and Fischer, P. (1999). Echinacea for the prevention and treatment of the common cold. The Cochrane Library.

Mengs, U., Clare, C. B., and Poiley, J. A. (1991). Toxicity of *Echinacea purpurea*. Acute, subacute and genotoxicity studies. Arzneimittelforshung *41*, 1076-1081.

Mills, K. H. (2001). Immunity to *Bordatella pertussis*. Microbes Infect *3*, 655-677.

Mills, K. H., Ryan, M., Ryan, E., and Mahon, B. P. (1998). A murine model in which protection correlates with pertussis vaccine efficacy in children reveals complementary roles for humoral and cell-mediated immunity in protection against *Bordatella pertussis*. Infect Immun *66*, 594-602.

Moreman, D. E. (1998). Native American Ethnobotany (Portland, Timber Press).

Mukkur, T. K., McDowell, G. H., Stocker, B. A., and Lascelles, A. K. (1987). Protection against experimental salmonellosis in mice and sheep by immunisation with aromatic-dependent *Salmonella typhimurium*. Journal of Medical Microbiology *24*, 11-19.

Mukkur, T. K., Stocker, B. A., and Walker, K. H. (1991a). Genetic manipulation of Salmonella serotype Bovismorbificans to aromatic-dependence and evaluation of its vaccine potential in mice. Journal of Medical Microbiology *34*, 57-62.

Mukkur, T. K., and Walker, K. H. (1992). Development and duration of protection against salmonellosis in mice and sheep immunised with live aromatic-dependent *Salmonella typhimurium*. Res Vet Sci *52*, 147-153.

Mukkur, T. K., Walker, K. H., Baker, P., and Jones, D. (1995). Systemic and mucosal intestinal antibody response of sheep immunized with aromatic-dependent live or killed *Salmonella typhimurium*. Comp Immunol Microbiol Infect Dis *18*, 27-39.

Mukkur, T. K., Walker, K. H., Jones, D., Wronski, E., and Love, D. N. (1991b). Immunizing efficacy of aromatic-dependent *Salmonella dublin* in mice and calves. Comp Immunol Microbiol Infect Dis *14*, 243-256.

Muller-Jakic, B., Breu, W., Probstle, A., Redl, K., Greger, H., and Bauer, R. (1994). *In vitro* inhibition of cyclooxygenase and 5-lipoxygenase by alkamides from *Echinacea* and *Achillea* species. Planta Medica *60*, 37-40.

Mullins, R. J., and Heddle, R. (2002). Adverse reactions associated with echinacea: the Australian experience. Annals of Allergy, Asthma and Immunology *88*, 42-51.

Pham, N. H., and Baldo, B. A. (1995). Allergenic relationship between taxonomically diverse pollens. Clinical and Experimental Allergy *25*, 599-606.

Pinn, G. (2001). Herbal medicine: What is the evidence? Australian Family Physician *30*, 1154-1159.

Pomponio, R., Gotti, R., Hudaib, M., and Cavrini, V. (2002). Analysis of phenolic acids by micellar electrokinetic chromatography: application to *Echinacea purpurea* plant extracts. Journal of Chromatography A *945*, 239-247.

Proksch, A., and Wagner, H. (1987). Structural analysis of a 4-*O*-Methyl-Glucuronoarabinoxylan with immuno-stimulating activity from *Echinacea purpurea*. Phytochemistry *26*, 1989-1993.

Rehman, J., Dillow, J. M., Carter, S. M., Chou, J., Le, B., and Maisel, A. S. (1999). Increased production of antigen-specific immunoglobulins G and M following in vivo treatment with the medicinal plants *Echinacea angustifolia* and *Hydrastis canadensis*. Immunology Letters *68*, 391-395.

Rennels, M. B., Deloria, M. A., Pichichero, M. E., Losonsky, G. A., Englund, J. A., Meade, B. D., Anderson, E. L., Steinhoff, M. C., and Edwards, K. M. (2000). Extensive swelling after booster doses of acellular pertussis-tetanus-diphtheria vaccines. Pediatrics *105*, e12.

Rininger, J. A., Kickner, S., Chigurupati, P., McLean, A., and Franck, Z. (2000). Immunopharmacological activity of Echinacea preparations following simulated digestion on murine macrophages and human peripheral blood mononuclear cells. Journal of Leukocyte Biology *68*, 503-510.

Roesler, J., Emmendorffer, A., Steinmuller, C., Leuttig, B., Wagner, H., and Lohmann-Matthes, M. L. (1991a). Application of purified polysaccharides from cell cultures of the plant *Echinacea purpurea* to test subjects mediates activation of the phagocyte system. International Journal of Immunopharmacology *13*, 931-941.

Roesler, J., Steinmuller, C., Kiderlen, A., Emmendorffer, A., Wagner, H., and Lohmann-Matthes, M. L. (1991b). Application of purified polysaccharides from cell cultures of the plant *Echinacea purpurea* to mice mediates protection against systemic infections with *Listeria monocytogenes* and *Candida albicans*. International Journal of Immunopharmacology *13*, 27-37.

Schindler, H. (1940). Geschichte, Systematik und Verbreitung der therapeutisch wichtigen *Echinacea* Arten: *E angustifolia* DC, *E pallida* Nutt und *E purpurea* Moench. Pharm Zentrahalle *81*, 579-583.

Schranner, I., Wurdinger, M., Klumpp, N., Losch, U., and Okpanyi, S. N. (1989). Modification of avian humoral immunoreactions by Influex(TM) and *Echinacea angustifolia* extract. Zentralbl Veterinarmed *36*, 353-364.

Schulten, B., Bulitta, M., Ballering-Bruhl, B., Koster, U., and Schafer, M. (2001). Efficacy of *Echinacea purpurea* in patients with a common cold. A placebo-controlled, randomised, double-blind clinical trial. Arzneimittelforshung *51*, 563-568.

Schulthess, B. H., Giger, E., and Baumann, T. W. (1991). Echinacea: anatomy, phytochemical pattern, and germination of the Achene. Planta Medica *57*, 384-388.

Schumacher, A., and Friedberg, K. D. (1991). The effect of Echinacea angustifolia on non-specific cellular immunity in the mouse. Arzneimittelforshung *41*, 141-147.

See, D. M., Broumand, N., Sahl, L., and G, T. J. (1997). *In vitro* effects of *Echinacea* and *Ginseng* on natural killer and antibody-dependent cell cytotoxicity in healthy subjects and chronic fatigue syndrome or acquired immunodeficiency syndrome patients. Immunopharmacology *35*, 229-235.

South, E. H., and Exon, J. H. (2001). Multiple immune functions in rats fed Echinacea extracts. Immunopharmacology and immunotoxicology *23*, 411-421.

Steinmuller, C., Roesler, J., Grottrup, E., Franke, G., Wagner, H., and Lohmann-Matthes, M. L. (1993). Polysaccharides isolated from plant cell cultures of Echinacea purpurea enhance the resistance of immunosuppressed mice agains systemic infections with *Candida albicans* and *Listeria monocytogenes*. International Journal of Immunopharmacology *15*, 605-614.

Stimpel, M., Proksch, A., Wagner, H., and Lohmann-Matthes, M. L. (1984). Macrophage activation and induction of macrophage cytotoxicity by purified polysaccharide fractions from the plant *Echinacea purpurea*. Infection and Immunity *46*, 845-849.

Sun, L. Z., Currier, N. L., and Miller, S. C. (1999). The American coneflower: a prophylactic role involving nonspecific immunity. J Altern Complement Med *5*, 437-446.

Tragni, E., Tubaro, A., Melis, S., and Galli, C. L. (1985). Evidence from two classic irritation tests for an anti-inflammatory action of a natural extract, Echinacina B. Food and Chemical Toxicology *23*, 317-319.

Trinchieri, G. (1994). Interleukin-12: A cytokine produced by antigen-presenting cells with immunoregulatory functions in the generation of T-helper cells Type1 and cytotoxic lymphocytes. Blood *84*, 4008-4027.

Tubaro, A., Tragni, E., Del Negro, P., Galli, C. L., and Della Loggia, R. (1987). Anti-inflammatory activity of a polysaccharidic fraction of *Echinacea angustifolia*. J Pharm Pharmacol *39*, 567-569.

Turner, R., Riker, D., and Gangemi, D. (2000). Ineffectiveness of *Echinacea* for prevention of experimental rhinovirus colds. Antimicrobial Agents and Chemotherapy *44*, 1708-1709.

Wagner, H. (1997). *Echinacea*-polysaccharide und immunologische Wirkung. *Phytoctherapy 18*.

Wagner, H., Bladt, S., and Zgainski, E. M. (1984a). Plant Drug Analysis - A Thin Layer Chromatography Atlas (Berlin, Springer-Verlag).

Wagner, H., and Juric, K. (1991). Immunologic studies of plant combination preparations. *In vitro* and *in vivo* studies on the stimulation of phagocytosis. Arzneimittelforshung *41*, 1072-1076.

Wagner, H., Proksch, A., Riess-Maurer, I., Vollmar, A., Odenthal, S., Stuppner, H., Jurcic, K., Le Turdu, M., and Fang, J. N. (1985). Immunostimulating action of polysaccharides (heteroglycans) from higher plants. Arzneimittelforshung *35*, 1069-1075.

Wagner, H., Proksch, A., Riess-Maurer, I., Vollmar, A., Odenthal, S., Stuppner, H., Jurcic, K., Le Turdu, M., and Heur, Y. H. (1984b). Immunostimulant action of polysaccharides (heteroglycans) from higher plants. Arzneimittelforshung *34*, 659-661.

Wagner, H., Stuppner, H., Schafer, W., and Zenk, M. (1988). Immunologically active polysaccharides of *Echinacea purpurea* cell cultures. Phytochemistry *27*, 119-126.

Ward, P., Fasitsas, S., and Katz, S. E. (2002). Inhibition, resistance development, and increased antibiotic and microbial resistance caused by nutraceuticals. Journal of Food Protection *65*, 528-533.