Investigation of anti-inflammatory compounds from food and plant libraries for use in Alzheimer's disease

Thesis submitted by

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Abbreviations

Aβ	β-amyloid
AChEIs	acetyl-choline esterase inhibitors
AD	Alzheimer's disease
ADAS	Alzheimer's disease Assessment Scale
APP	Amyloid precursor protein
ARE	antioxidant response element
ASE	accelerated solvent extraction
ATP	adenosine triphosphate
BACE	β site cleaving enzyme
BBB	Blood-brain barrier
CAC	Citric acid cycle
CDR	Clinical Dementia Rating
CNS	central nervous system
COX	Cycloxygenase
CSF	cerebral spinal fluid
DMEM	Dulbeccos's Modified Eagle Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DPPH	1,1-Diphenyl-2-picryl-hydrazyl
IC_{50}	50% Inhibitory concentration
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent sandwich assay
FBS	foetal bovine serum
GABA	γ-aminobutyric acid
GAG	glycosaminoglycan
GC	Gas chromatography
GSH	glutathione
H_2O_2	hydrogen peroxide
HCl	hydrochloric acid
HPLC	high pressure liquid chromatography
IL	interleukin
INF-γ	interferon-γ
LPS	Lipopolysaccharide
MAPs	microtubule-associated proteins
MS	mass spectroscopy
MT	Melatonin
NAD(P)H	reduced nicotinamide adenine dinucleotide (phosphate)
NFκB	nuclear factor kappa B
NFT	neurofibrillary tangles
NMDA	N-methyl-D-aspartate
NO	nitric oxide
Nrf2	nuclear factor erythroid-2-related factor 2
NSAID	non-steroidal anti-inflammatory drugs
PBS	phosphate buffered saline
PD	Parkinson's disease

PKA	protein kinase A
PKC	protein kinase C
PPARγ	peroxisome proliferator-activated receptor-γ
PPP	pentose phosphate pathway
PVDF	polyvinylidene
ROS	reactive oxygen species
RNS	reactive nitrogen species
SD	standard deviation
SEM	standard error of the mean
TCA	tricarboxylic acid
TMB	Tetra methyl benzidine
TNF-α	tumour necrosis factor-α

Abstract

In many chronic neurodegenerative diseases including Alzheimer's disease (AD), chronic activation of microglia can be observed. Microglia, the resident macrophages of the central nervous system, are found in increased numbers surrounding senile amyloid plaques which play a central role in the inflammatory cascade. Macrophages were reported to contribute amyloid angiopathy and massive neuronal tissue destruction is also reported due to high macrophage number. It is also evidenced that COX-2-positive macrophages infiltrate into AD brain damage the blood-brain barrier. In autoimmune animal models, these two related cell types, microglia and macrophages were involved in brain pathology in multiple sclerosis and experimental allergic encephalomyelitis. When activated both secrete a variety of cytokines, including interleukin-1, interleukin-6, and tumor necrosis factor as well as reactive oxygen and nitrogen species (ROS/RNS). Since ROS act as signaling molecules in pro-inflammatory redox-active signal transduction pathways, it is likely that intracellularly acting plant derived antioxidants, including polyphenols, have been shown to scavenge these "signaling" reactive oxygen species, and thus perform in an anti-inflammatory capacity. Also opportunities exist via diet and lifestyle for contributing to chronic inflammation or alternatively exert anti-inflammatory activity.

A selection of Chinese medicinal plants and a sample library from the CSIRO including plant and fungal material such as fruit, leaves, stems, roots, tubers, seeds, juice and pulp, as well as food processing co-products or waste material, for example spent grain from brewing, were tested for ability to attenuate NO and TNF-α production, *in vitro*. The most potent of them was selected by a high through put screening procedure, involving murine microglia and macrophages. Considering the stability of activity during processing stages (patented processing methods of the CSIRO plant and food library), activity on both the cell lines and suppression of both NO and TNF-α without cytotoxicity,

C.zeylanicum has been selected as the lead candidate for further chemical analysis. Though a variety of cinnamon species were extensively studied for the anti-oxidant, anti-diabetic, anti-microbial, anti-cancer and anti-arthritis properties, there was not much evidence in support of anti-inflammatory properties especially relevant to the species *C.zeylanicum*.

We have identified the presence of 9 bioactives in dried powder of *C.zeylanicum* bark, namely β-caryophyllene, p-cymene, cinnamaldehyde, 2-methoxy cinnamaldehyde, α-amyl cinnamaldehyde, citral, benzyl benzoate, furfural, cinnamyl alcohol. β-caryophyllene, p-cymene, cinnamaldehyde and its derivatives 2-methoxy cinnamaldehyde and α-amyl cinnamaldehyde, citral, benzyl benzoate and furfural have shown both NO and TNF-α inhibitory activity while cinnamyl alcohol and eugenol have shown NO inhibitory activity but not TNF-α inhibition. Neither of these constituents showed significant levels of cytotoxicity at doses inhibiting NO (percentage cell viability <80%), but citral, p-cymene and α-amyl cinnamaldehyde showed cell death at IC_{50} doses of TNF-α inhibition.

In conclusion, the compounds β-caryophyllene, cinnamaldehyde, 2-methoxy cinnamaldehyde, benzyl benzoate and furfural may be promising as leads in the development of anti-inflammatory treatments in diseases of pathological inflammation, including AD.

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Statement of Sources

I declare that this thesis is my own work and has not been submitted in any form for another degree or diploma at any university or other institutions of tertiary education. Information derived from the published or unpublished work of others has been acknowledged in the text and a list of references is given.

Statement of Contribution of Others

I declare that this thesis is my own work and was supported by the following organisations and people. The work was generously supported by a scholarship from the CSIRO Preventative Health Flagship (2008-2010) and James Cook University provided financial support through a Postgraduate Research scholarship (Jan 2011 – June 2011).

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Declaration of Ethics

The research presented and reported in this thesis was conducted within the guidelines for research ethics outlined in the *James Cook University Policy on Experimentation Ethics, Standard Practices and Guidelines (2001)* and *James Cook University Statement and Guidelines on Research Practice (2001)*.

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CHAPTER 1

INTRODUCTION

1.1 Alzheimers disease

Diseases such as cardiovascular disease, cancer, diabetes and neurodegenerative diseases, which are often described as lifestyle diseases, are responsible for around 70% of the total burden of illness and injury experienced by the Australian population. This proportion is expected to increase to 80% by 2020 (National Public Health Partnership, 2001). Continuous improvement in health care and life style making people live longer and appear to increase in frequency of these 'life style diseases'. Alzheimer's disease (AD) is the most common age-related neurodegenerative disorder, which currently affects 20 to 30 million people worldwide (1). Diet, lifestyle and environment are among the factors thought to influence susceptibility to these diseases. Over the last few decades, there is increase in interest to correlate certain diets and the incidence of disease. Diets such as the Mediterranean diet are suggested to be associated with a decreased risk in developing disease (2,3). Diet has been accepted to influence the risk of developing certain diseases, but exactly which foods, and by what mechanisms this occurs, is largely debateable. Some interesting findings were shown in connecting diet with disease risk, particularly in AD research. Numerous studies have emerged reputedly linking AD with various aspects of diet, including intake of dietary antioxidants and polyphenols, polyunsaturated fatty acids, folic acid, cholesterol and overall caloric intake (4).

1.2 Pathophysiology of AD, disease modification and treatment approaches

The brain pathology in AD that ultimately leads to cognitive decline and profound dementia is characterized by extensive neuronal loss, formation of neurofibrillary tangles (NFTs) and extracellular deposition of β-amyloid (Aβ) plaques in susceptible regions of the brain (5). This is accompanied by a chronic inflammatory response and extensive oxidative damage, most probably as a reaction to Aβ deposition (5).

Table 1.1: Treatment of Alzheimer's Disease (6)

Variable	Target/Mechanism of Action	Benefit
Current treatment		
Tacrine (Cognex, Warner-Lambert, Morris Plains, New Jersey)	Cholinesterase inhibitor	2.4-point improvement in ADAS*****-Cognitive score compared with placebo (7)
Donepezil (Aricept, Eisai, Inc., Teaneck, New Jersey, and Pfizer, Inc., New York)	Cholinesterase inhibitor	2.9-point improvement in ADAS*****-Cognitive score compared with placebo (8)
Rivastigmine (Exelon, Novartis, Basel, Switzerland)	Cholinesterase inhibitor	2.6-point improvement in ADAS*****-Cognitive score compared with placebo (9)
Galantamine (Reminyl, Janssen, Titusville, New Jersey)	Cholinesterase inhibitor	3.6-point improvement in ADAS*****-Cognitive score compared with placebo (10)
Vitamin E	Antioxidant	Delay in time to institutionalization, death, loss of >/-2 activities of daily living, or progression from CDR score of 2 to 3 (11)
Therapy under development		
Immunotherapy	Amyloid plaques	Halt progression by clearing existing amyloid plaques
Secretase inhibitors	β-secretase, γ-secretase	Reduce formation of β-amyloid by inhibiting an enzymatic step in the intracellular process of APP†
NSAIDs†	Inflammatory response	Slow progression by reducing amyloid plaque–associated inflammation

* ADAS = Alzheimer's Disease Assessment Scale; CDR = Clinical Dementia Rating;

† Includes both nonspecific cyclooxygenase and selective cyclooxygenase-2 inhibitors.

There are several medications currently used to treat AD, and many therapies under development (Table 1.1). A separate but important issue involves the treatment decisions that are made during the final stages of AD. Treating this disease is one of the important, but unmet needs in medicine. Current drugs improve symptoms, but do not have any profound disease-modifying effects, so they are of limited benefit to most patients. Other drugs are used to manage mood disorder, agitation

and psychosis in later stages of the disease, but there is no treatment with a strong disease-modifying effect currently available. Three main classes of disease-modification approaches may be defined: those that target specific aspects of AD pathology; those that aim for neuroprotection, and those based on epidemiological observation.

1.2.1 Aβ in AD pathology

Aβ is the main constituent of senile plaques and these form one of the key pathological features of AD. Although the initial cause of sporadic AD is still debated, the 'amyloid cascade hypothesis' states that the aberrant production, aggregation and deposition of Aβ is a causative process in the pathogenesis of AD (12). Aβ is a proteolytic fragment of the amyloid precursor protein (APP), which is a ubiquitously expressed type I transmembrane protein. There are two proteolytic processing pathways of APP and its metabolic derivatives *in vivo*. In the non-amyloidogenic pathway, APP is cleaved by α-secretases of the ADAM define family of proteases, releasing a large soluble amino (N)-terminal ectodomain, APPs-α, and this has been proposed to exert neurotrophic and neuroprotective functions (13). This cleavage event occurs in the Aβ domain and precludes formation of full-length pathogenic Aβ peptides. In the amyloidogenic pathway, APP molecules are sequentially processed by two aspartyl proteases (Figure 1.1). APP is first cut by β-secretase (BACE1) to generate a slightly shorter N-terminal ectodomain, APPs-β. This is then cleaved by γ-secretase within the transmembrane domain to generate the carboxyl terminus of the Aβ peptide (5,14). The γ-secretase also cleaves near the cytosolic border of the membrane (ε-cleavage), liberating the APP intracellular domain (AICD), which is a soluble proteolytic fragment with potential signaling functions, which include transcriptional regulation (15). Aβ is a peptide product of a secretase soluble cellular metabolite (16) comprising two predominant forms with different COOH-termini, $A\beta_{40}$ and $A\beta_{42}$. Other similar intracellular domains

(ICDs) are also generated by γ-sectrease, from other known substrates, such as NOTCH and ErbB-4 (14).

The γ-secretase exists in a multiprotein complex containing presenilin, a family of related multi-pass transmembrane proteins that play a key role in the modulation of intracellular Ca^{2+} involved in presynaptic neurotransmitter release and long-term potentiation induction. During APP proteolysis, it generates Aβ peptides of variable length (14,17). Of these peptides, the 40- and 42-amino acid Aβ species are those most commonly deposited in the brain, and the longer $A\beta_{42}$ isoform is thought to be the key pathogenic species (5). The view that $A\beta_{42}$ plays a central role has developed from observations that mutations in the APP and presenilin genes invariably increase production of the longer $A\beta_{42}$ species and thus higher the $A\beta_{42}/A\beta_{40}$ ratio in primary fibroblasts and plasma of individuals affected with familial AD and also in transfected cells and transgenic animals (5). $A\beta_{42}$ is the first species to be deposited in the brain of individuals with AD (5), despite the fact that $A\beta_{40}$ is the principal species produced in humans. $A\beta_{42}$, is thought to help initiate amyloid deposition in the brains of patients with Alzheimer's disease (18–20). $A\beta_{42}$ is particularly prone to aggregation *in vitro* and aggregated $A\beta_{42}$ peptide is toxic to various cell types in culture (5). $A\beta_{42}$ forms aggregates that are thought to initiate the pathogenic cascade, leading ultimately to neuronal loss and dementia (21).

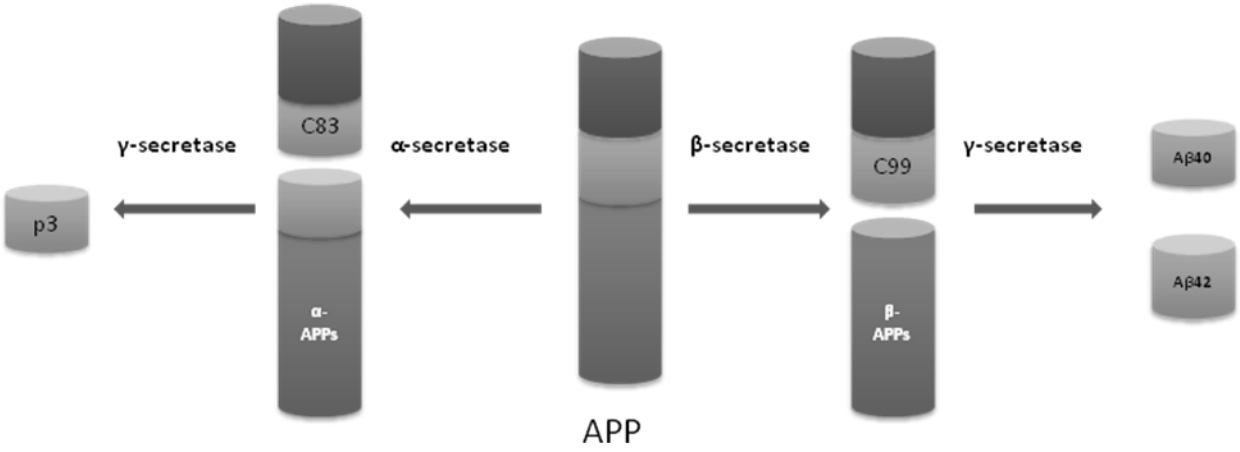


Figure 1.1 | Action of Secretases. Cleavage of APP with γ and β secretases results in the 40-42 amino acid Aβ peptide, which aggregates to form insoluble extracellular plaques, typical in AD.

Some researchers have found evidence that Aβ fibrils form pores in neurons, leading to calcium influx and the neuron death associated with AD (22,23) (Figure 1.2). Apart from the direct role in cell death, Aβ-mediated glutamate receptor modifications can lead to synaptic dysfunction, resulting in excitotoxic neurodegeneration during the progression of AD. Cognitive deficits could be caused by Aβ-induced changes in GABAergic and NMDA receptor functions, involving metabotropic glutamate receptor (mGluR) signaling. The mGluRs are located in the presynaptic membrane and regulate glutamate release, thereby optimizing synaptic transmission (24). They are inhibited by Aβ, probably through inhibiting mGluR activation of PKC (25).

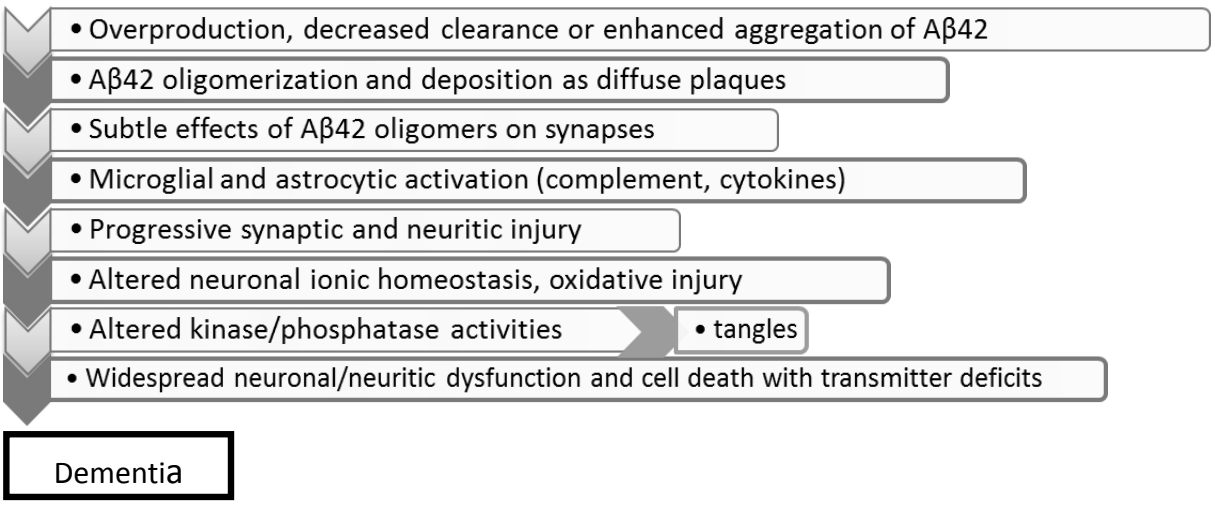


Figure 1.2 | Amyloid cascade hypothesis. The sequence of pathogenic events that are thought to lead to Alzheimer's disease (AD) is shown (21,21).

1.2.1.1. Targeting Aβ

There are many aspects of AD pathology that may be targeted. Treatment approaches that fall into this class include the classic targets of Aβ and NFTs, as well as the more recently studied target of synaptic loss. These treatment approaches may be further classified by target and action: blocking Aβ aggregation; anti-amyloid immunotherapy; lowering Aβ production; targeting tau; and targeting synaptic loss.

Blocking Aβ aggregation

Aggregation of Aβ could be blocked by using small drugs capable of penetrating the brain that may interfere in Aβ-Aβ peptide interactions. The glycosaminoglycan (GAG) mimetic, Tramiprosate (ALZHEMED^{TM}), is a small, orally-administered molecule known to be an Aβ antangonist. It crosses the blood brain barrier (BBB) and binds to soluble Aβ peptide, interfering with the amyloid cascade that is associated with amyloid deposition and the toxic effects of Aβ peptide in the brain. Phase III clinical trials of Tramiprosate, however, have failed in both North America and Europe (Neurochem, unpublished data). Tramiprosate failed to demonstrate efficacy in long-term clinical testing of cognitive improvement.

Homocysteic acid (HA) seems to induce amyloid effects on intracellular $A\beta_{42}$ accumulation. In the presence of Aβ, HA accumulates $A\beta_{42}$ into neuronal cell, which induces α-synuclein and consequently induces tau aggregation. Then neuronal degeneration will be induced by lower level of HA (26). Higher level of HA also induces the neuronal degeneration in the absence of amyloid by

oxidative stress and mitochondrial inhibition. In the 3xTg-AD mouse model, the anti-HA polyclonal antibody inhibited the disease process (26).

Anti-amyloid immunotherapy

In a recent study, active Aβ vaccination reduced the plaque burden in aged Caribbean vervet monkeys and stabilised or improved cognitive impairment. This supports the potential of active Aβ immunotherapy as a prevention or treatment approach for early AD. In 1999, Elan Corporation's Schenk et al. study reported that amyloid pathology was reduced in an APP transgenic mouse model, following vaccination with aggregated $A\beta_{42}$. $A\beta_{42}$ immunisation augmented a highly specific immune response to clear Aβ, markedly reducing the pathology in the animal model (27). Elan's immunotherapy, Bapineuzumab, a fully humanised, monoclonal antibody, raised against the N-terminus of Aβ, is currently in Phase III clinical trials. It has shown, preclinically, that it can reduce amyloid burden, and has favourable effects on synaptic density and memory. When mouse splenocytes, however, were examined *in vitro*, no T-cell proliferative response to Aβ was seen, indicating that a T-cell response is not required for amyloid plaque reductions. These findings have led to three different, but not mutually exclusive, hypotheses to explain the antibody mediated effects: microglial activation and phagocytosis of amyloid; direct resolution of deposits; and peripheral sink – capture of Aβ.

Microglial activation and phagocytosis of amyloid

A small proportion of peripherally administered antibody reached the CNS and binds to amyloid deposits, triggering endogenous microglia to phagocytose the amyloid. This mechanism requires antibodies to reach parenchymal deposits, and there is evidence that this occurs. Peripherally administered anti-amyloid antibodies have been shown to directly bind to amyloid deposits in the brain (28).

Direct resolution of deposits

Antibodies restricted to amino acids 3-6 of the Aβ peptide are able to resolve, *in vitro*, aggregated Aβ fibrils (29). It has been proposed that this direct resolution of amyloid deposits may underlie its therapeutic effects (29). The mechanism, however, of the dissolution of an existing, insoluble fibril by an antibody, is still not understood. It is also documented *in vitro* efficacy of antibodies to different Aβ epitopes, indicating that the effect cannot be indisputably linked to amino acids 3-6 *in vivo* (29).

Peripheral sink – Aβ capture

An unusually high (picomolar) affinity for soluble Aβ is shown by the monoclonal antibody 266. It is efficient in amyloid reduction on passive administration, but does not bind to plaques. It was proposed that at concentrations sufficient to produce detecTable levels in the CSF, the antibody captures the serum soluble Aβ and produces a net flux of Aβ from the CNS, to the periphery, down the concentration gradient of soluble Aβ. Over an extended time period, this would lead to decreased parenchymal amyloid load (30). Antibody 266 is unique in the scientific literature, because its efficacy does not depend on binding to brain amyloid deposits. Several (lower affinity) antibodies that recognise only soluble Aβ did not show efficacy in the Elan Corporation's studies (28,31), while a vaccination with peptides that reflect the 266 epitope was also not efficacious (31). Chronic capture of soluble Aβ might contribute to the observed amyloid reduction, but very high affinity to soluble Aβ may be necessary to reduce parenchymal amyloid in the absence of other mechanisms (32). Clinical trials are currently being conducted by Elan Corporation for Bapineuzumab (Phase III North American studies) and ELND005 (Phase II study).

Lowering Aβ production

This is the most direct approach in anti-amyloid therapy. Selectively lowering production of $A\beta_{42}$ could be a promising strategy for prevention or treatment of this disease (33). The three potential strategies to reduce Aβ production are: inhibition of γ-secretase; inhibition of β-secretase; and stimulation of α-secretase.

Inhibition of γ-secretase

The γ-secretase is an unusual transmembrane protease complex, consisting of at least four proteins – presenilin, nicastrin, anterior pharynx (APH1) and presenilin enhancer 2 (PEN2) (17)– with a still unknown active site structure. The identification of several γ-secretase components, and the generation of gene targeted models, lead to the rapid identification of several γ-secretase substrates other than APP. These include the notch receptor 1 (NOTCH1) (34), the notch ligands delta-like protein 1 (DELTA1) and jagged 1 (JAG2), v-erb-a erythroblastic leukaemia viral oncogene homologue 4 (ERBB4) and others (35). As a result of these other substrates, γ-secretase inhibitors have lead to a number of detrimental effects, including a reduction of thymocyte numbers, thymocyte differentiation blockade (36), splenic B-cell maturation and profound changes in the gastrointestinal tract (37).

In 2001, Bristol-Myers Squibb announced that the first γ-secretase inhibitor had entered phase II clinical trials. The results have not been published. Lilly published a 6-week phase II trial of a functional γ-secretase inhibitor. This demonstrated a significant decrease in Aβ concentration in plasma, but not CSF, using a dose that was well tolerated during the short trial (38). Tarenflurbil (*Flurizan*, Myriad Genetics), γ-secretase activity modulator that lowers $A\beta_{42}$ selectively, in an earlier phase 2 study (39) suggested that patients with mild AD had a dose-related slower rate of decline than those treated with placebo and that the drug was well tolerated. But in the succeeded multi-centre phase

III clinical trial it failed to show effects on cognition or activities of daily living after 18 months of treatment in patients with mild AD.

Semagacestat, a γ-secretase inhibitor from Eli Lilly was compared with placebo in two pivotal Phase III trials, in more than 2,600 patients with mild-to-moderate Alzheimer's disease. Lilly has now reviewed data from a pre-planned interim analysis of semagacestat studies that has showed, as expected, cognition and the ability to complete activities of daily living of placebo-treated patients worsened. However, by these same measures, patients treated with semagacestat worsened to a statistically significantly greater degree than those treated with placebo. In addition, data showed semagacestat is associated with an increased risk of skin cancer compared with those who received placebo. This was the latest in a long line of setbacks for novel Alzheimer's therapies.

Inhibition of β-secretase

The β-secretase, also known as the transmembrane aspartic protease β site cleaving enzyme 1 (BACE1), has met a renewal of interest following the realisation that presenilin might be a γ-secretase component (40) and the subsequent problems with inhibition of notch cleavage. The physiological role of the homologue, BACE2, and its substrates, are unknown. This enzyme does not seem to have a key role in APP processing, however, so it is not considered to be a drug target for AD (41). Serious challenges, with respect to BBB penetration and selectivity of inhibitors are expected. It is also currently unclear whether cross-inhibition of BACE2 is accepTable or whether one has to create exclusive specificity for BACE1 (41). An unclear and less exploratory pattern of behaviour, associated with increased 5-hydroxytryptamine (serotonin) turnover in the hippocampus, was reported in BACE1-knockout mice. This raises the possibility that BACE1 could play a role in neurotransmitter turnover or release (42). The effect of BACE1 knockout on memory function has been analysed in a study using one of the standard APP transgenic mouse models of amyloid function, Tg2576 (43), where BACE1 deficiency was found to rescue memory deficits and cholinergic dysfunction (44). Altogether, the absence of Aβ production and distinct pathology in BACE1-knockout mice is quite encouraging for β-secretase drug development. Inhibitor development is, however, proving to be challenging (41). Usually potent and specific peptidic inhibitors can be identified for aspartic proteases, but it is more difficult to generate small molecules with the desired pharmacokinetic properties (45). X-ray crystal structure reveals a large active site for β-secretase and this poses an additional hurdle in drug development (46). Novel drugs that simultaneously modulate both β- and γ-secretase cleavage of APP are also under development.

Stimulation of α-secretase

To reduce APP substrate, the α-secretase pathway might be stimulated through cell-surface receptors (47). The potential side effects of this approach, however, remain unknown. The stimulation of α-secretase by M1 muscarinic receptor agonists, which could function as cognition enhancers, has been explored in depth. M1-agonist treatment patients with AD have shown decreased CSF levels of $A\beta_{42}$ (48). Research is needed, however to establish the side effects caused by activation of other muscarinic receptors. EHT0202 is a potential oral drug that acts by exerting a specific regulation on $GABA_A$ receptors and links APP processing to $GABA_A$ signalling (49). It redirects APP processing towards the α-secretase pathway. Phase IIa safety results and the potential efficacy signals observed in AD patients support the advancement of EHT0202 into later stage clinical development, to collect further evidence about safety and efficacy, using a larger number of patients over a longer trial period. EHT0202 treated patients have shown encouraging signs of cognitive improvement, as measured by ADAS-Cog, the gold standard test for cognition. These results build on earlier observations based on EHT0202 use in animal models of AD. It was also observed, in some assessments, including ADAS-Cog that the ApoE4-positive subpopulation (patients with one or two ApoE4 alleles) tends to respond to EHT0202 treatment better than those patients with no ApoE4 allele. These first patient data support the progression of EHT0202 into phase IIb trials, in order to establish the cognitive benefits of EHT0202 in a longer and larger clinical trial, to further explore the benefit of EHT0202 in the ApoE4 positive population and to identify the optimal therapeutic dosage for EHT0202.

1.1.1. Tau in AD pathology

Tau is one of the microtubule-associated proteins (MAPs) that stabilize neuronal microtubules for their role in the development of cell processes, establishment of cell polarity and intracellular transport (50). Neurofibrillary protein aggregates are one of the major hallmarks of AD. These

aggregates, also known as neurofibrillary tangles (NFTs), are formed as a result of abnormal hyperphosphorylation of tau protein, aggregation into 'paired helical filaments' (PHFs), and loss of ability to maintain the microtubule tracks. Despite a lack of understanding of the intermediate steps involved, NFT formation seems to cause inappropriate signals that lead to an imbalance of kinases/phosphatases, resulting in hyperphosphorylation of tau and its detachment from microtubules. Microtubule breakdown follows, and aggregation of tau into PHFs, which in turn bundle into NFTs of 'neuropril threads' that lead to the breakdown of intracellular transport and neuronal degeneration.

The pathological cascade for the disease process is most likely to be: Aβ deposition leading to tau phosphorylation and tangle formation eventually leading to neuronal death (12). Specific kinases responsible for the hyperphosphorylation are not yet known, but Aβ may facilitate Ca^{2+} entry into neurons, causing calcium-activated kinases to excessively phosphorylate tau protein thereby leading to NFTs. Fibrillar Aβ can induce MAPK, leading to tau phosphorylation and hence to NFTs, but inhibition of protein phosphatases, especially PP2A, is reportedly more responsible for hyperphosphorylated tau than kinases (51,52). Tangles are harmful to neuronal processes but tangles alone may not be toxic to the cell until they become modified by truncation (53). Studies also suggest that the amino terminal region of tau may be a primary site of toxicity in neurodegenerative disease (53). Glycation of N-terminus of tau is a consequence of oxidative damage and cross-linking, which accumulates once tangles have formed (54). There is an increase of tau in the cerebrospinal fluid (from ~200 pg ml^{-1} to ~600 pg ml^{-1}), which is thought to arise from dying neurons (55).

1.2.2 Targeting Tau

Hyperphosphorylation of microtubule-associated protein tau at specific sites is a recognized pathological process in AD. The concept of designing medicines to stop abnormal tau protein formation is another promising approach in the AD drug development process (56). The new drug candidate developed by Canada-based Allon Therapeutics Inc., AL-108, has passed phase II clinical trials in U.S.F.D.A. approved clinical trials. Rember (methylthioninium chloride), a potential disease-modifying oral drug under development by TauRx, a Singapore-based company, acts by targeting the tau aggregation pathway. This has the potential to prevent NFT development in the brain

(56). It has also been shown to reverse tau pathology in mouse models of AD. Peripheral administration of a novel Hsp90 inhibitor (EC102), has promoted selective decreases in toxic forms of phosphorylated tau, using a mouse model of tauopathy (57).

Protein kinase A (PKA) is a crucial kinase in AD-like tau hyperphosphorylation. In a study, tau hyperphosphorylation was induced by injecting bilaterally a specific PKA activator, isoproterenol (ISO) into hippocampus of rat brain and melatonin (MT) was shown to protect against ISO-induced tau hyperphosphorylation (58). MT also attenuates tau hyperphosphorylation in Tg2576 transgenic mice (59).

1.2.3. Involvement of cholinergic system in AD

The pathogenesis of AD has been linked to a deficiency in the brain neurotransmitter, acetylcholine, based on observations that there is a correlation between cholinergic system abnormalities and intellectual impairment (60). As a result, the current standard of care for mild to moderate AD is to improve cognitive function by treatment with acetylcholinesterase inhibitors

(AChEIs). Acetylcholine dysfunction, however, is not a primary pathological cause for AD, but a consequence of the disease (61). In the UK, three AChEIs (donepezil, rivastigmine and galantamine) are licensed as medications for symptomatic treatment of AD. Apart from their use in stabilisation of cognitive decline, there is also evidence linking these agents with improvement in behavioural and psychological symptoms of dementia (62). The symptomatic efficacy of AChEIs is attained through their augmentation of acetylcholine-mediated neurotransmission.

There is also evidence that AChEIs may slow disease progression and may have disease modifying effects (63,64). To establish AChEIs as anti-inflammatory agents, research is required to emphasise the direct link between the cholinergic system and inflammation, as well as the effects of AChEIs on inflammatory mediators. Acetylcholine has been shown to suppress the pro-inflammatory cytokine release from peripheral tissue-activated macrophages, in an animal model of toxaemia, through its action of specific nicotinic receptors (65). There is also evidence of cholinergic modulation of microglial activation by α7 nicotinic receptors, through murine primary cell cultures (66). Data has also shown that the AChEIs, Huperzine A and tacrine, both directly attenuate Aβ peptide-induced oxidative injury (67). The protective effects of donepezil against the toxicity of Aβ, has also recently been shown in rat septal neuronal cells (68). Despite the evidence mounting in favour of AChEIs as anti-inflammatory agents, more research is now needed to clarify their anti-inflammatory role in AD patients, and to define the mechanisms involved (Table 1.2).

Tacrine (an acridine) produces hepatotoxicity and must be administered four times daily and is consequently no longer marketed in the United States. As a result of this toxicity, tacrine derivatives are being examined for efficacy and safety, as possible alternatives. Velnacrine, one of these derivates, has recently been examined in the Cochrane review, which concluded that it has a similar side effect

profile to tacrine and should therefore not be recommended for use in AD (69). Acridines are likely to be discontinued as a treatment strategy for AD.

Donepezil (a piperidine) has a very low incidence of nausea and diarrhoea at the 10 mg dose (the higher of the two available doses) and is administered once daily. Trials suggest that the incidence of nausea and diarrhoea is greater with the other AChEIs, rivastigmine (a carbamate) and galantamine (a phenanthrene alkaloid), than with donepezil, especially at the higher doses; both are administered twice daily (70). Studies have suggested that rivastigmine may be a suiTable alternative for patients who are unresponsive to donepezil or galantamine (71). Transdermal patches of rivastigmine existing as a 24 hour patch have also been shown to improve cognitive function, memory, lead to reduced incidences of nausea and vomiting compared to capsules, allowing for higher doses and therefore better efficacy in some patients (72). One study has found that switching from donepezil to rivastigmine patches did not result in any significant reduction in drug function (71), suggesting that it may be a suiTable alternative to donepezil. Galantamine has a dual mechanism of action, combining allosteric modulation of nicotinic acetylcholine receptors with reversible, competitive inhibition of acetylcholinesterase. There are no published clinical trials, however, to compare galantamine to other AChEIs to determine whether this pharmacologic property is associated with any additional clinical benefit.

Another AChEI, physostigmine was shown to have some shown some promise in AD treatment research by benefitting memory improvement more than two decades ago, but was dropped as a target because it has a very short half-life, which would require frequent administration (73). Recent studies have looked to overcome this shortfall by trialling alternative drug formulations and routes of administration. At present however, there is limited evidence to suggest that physostigmine is effective

in any of the forms that have been tested, including controlled release oral formula, IV infusion, and transdermal patches. Data also suggests that this drug has a poor side effect profile, with a number of adverse effects causing more people to withdraw from trials. As such, a Cochrane review has concluded that physostigmine shows no benefit over other AChEI drugs, and therefore should not be recommended for AD treatment and does not require further testing.

A long-acting irreversible AChEI, metrifonate, has entered clinical trials as another potential treatment of AD (74). Metrifonate has been used in the treatment of schistosomiasis, but not for any long-term uses. While it shows some positive effects on cognition, it was also unfortunately shown to lead to severe and serious side effects with long-term use in some study participants, with patients suffering from respiratory paralysis and neuromuscular difficulties. As a result of this, AD trials of metrifonate have ceased. The irreversible nature of this drug is the likely cause of the side-effects and unfortunately means that similar long-acting AChEIs may be more likely to harm participants than will make up for the reduced dose frequency.

1.2.4 Loss of synapses in AD

Among the pathologic hallmarks of AD neurodegeneration, only synaptic loss in the brains of AD patients closely correlates with the degree of dementia *in vivo*. Recent research has shown that reduction of synaptogenic protein kinase C (PKC) isozymes and their downstream synaptogenic substrates, such as brain-derived neurotrophic factor occurs in association with elevation of soluble Aβ, but synapses are actually lost before Aβ plaques and NFTs even develop, and that synaptic loss provides the strongest correlation with the extent of AD progression of all currently known disease markers (75). Some reports suggest that prevention of synapse loss also prevents the other pathological features of AD, including high levels of Aβ accumulation, neuronal loss, plaque formation and even the

hallmark symptom of reduced cognitive function. Through the use of mice genetically engineered to reproduce the pathology and symptoms of human AD, researchers have shown that cognitive dysfunction actually develops before plaques and tangles appear (76). This may suggest that plaques and tangles are not the cause of AD, but rather a consequence of it.

Targeting synaptic loss

This is a new and alternate treatment approach to AD, where synaptogenesis is targeted in an attempt to slow the progression and development of AD and to treat AD that is symptomatic. As mentioned earlier, the PKC enzymes are known to play a role in synapse maintenance and have been shown to exist in lower than normal levels in AD patients (75). A reduction in PKCα and PKCε appear to be most strongly associated with Aβ production, with PKCε also playing a key role in synapse formation (75). As a result, a recent study examined the effects of Bryostatin, a drug synthesised to target PKCε in particular, in mice models of AD (75). Evidence from this study suggests that Bryostatin was associated with the growth of new synapses, as well as the preservation of existing ones. In the mice treated with Bryostatin there was also a decrease in PKCε loss and soluble Aβ levels. This has led to the argument that Bryostatin may prevent the hallmark pathology of AD, hence it is set to progress to Phase II clinical trials.

It has also been found that the cognitive dysfunction of AD is strongly related to loss of synpases in the hippocampus and cortex (77). Following from this, there have also been preclinical studies that have provided promising evidence to suggest that administration of the rate-limiting precursors of the phosphatides, including uridine, omega-3 polyunsaturated fatty acids and choline, may increase synapse production and repair by increasing the levels of phosphatides that that make up much of the synaptic membranes (78,79). Animal models have even shown improvement in cognitive

dysfunction with the administration of these precursors (80,81). These findings have led to the creation of Souvenaid, a medical drink containing precursors and nutrients that have been shown to be involved in synaptic membrane synthesis (82). In a recent double-blinded randomized clinical trial (RCT), Souvenaid was compared to placebo in patients with mild AD. The supplement was well-tolerated and has shown some promise in regards to AD treatment. The trial showed a significant improvement in memory for those patients taking Souvenaid, compared to placebo (82) and seems to add further human evidence in support of the results found by Honpaisan et al. in their mice models.

PKC activators in synapse maintenance

Regulation of potassium channels, calcium homeostasis, and protein kinase C (PKC) activation are molecular events that have been implicated during associative memory which are also altered or defective in AD. PKC is also involved in the processing of the amyloid precursor protein (APP), a central element in AD pathophysiology. Benzolactam (BL), a novel PKC activator reversed potassium channels defects, enhanced secretion of APPα in AD cells, and significantly increased the amount of sAPPα and reduced $A\beta_{40}$ in the brains of APP[V717I] transgenic mice (83). Another PKC activator, bryostatin 1, at subnanomolar concentrations dramatically enhanced the secretion of the α-secretase product sAPPα in fibroblasts from AD patients (83). In addition, in a more recently developed AD double-transgenic mouse, bryostatin was effective in reducing both brain $A\beta_{40}$ and $A\beta_{42}$ ameliorated the rate of premature death and improved behavioral outcomes (83). Collectively, these data corroborate PKC and its activation as a potentially important means of ameliorating AD pathophysiology and perhaps cognitive impairment, thus offering a promising target for drug development. Because bryostatin 1 is devoid of tumor-promoting activity and is undergoing numerous clinical studies for cancer treatment in humans, it might be readily tested in patients as a potential therapeutic agent for AD. Apart from these, PKC enzymes are known to control synaptogenesis and are

reduced in AD patients. It is therefore possible that PKC activators could be useful therapeutic targets in AD prevention and treatment.

1.2.5 Involvement of metals in AD

Various metals have been implicated as possible contributors in the development of AD. There is increasing evidence to support the argument that both APP and its proteolytic product Aβ play a role in metal ion homeostasis. Zinc and copper ions can interact with both APP and Aβ to potentiate AD, and do this by participating in their aggregation, and in the generation of ROS (84). A number of metal ions, including Fe^{2+}, Fe^{3+} , Al^{3+}, Zn^{2+}, and Cu^{2+}, are found co-localised with β-sheets of $A\beta_{42}$ in senile plaque cores in AD brains. Data from *in vitro* studies shows that Al^{3+} and Fe^{2+}/Fe^{3+} are directly involved in the precipitation of β-sheets of AB_{42} in senile plaque cores, while $Cu^{2+}$and Zn^{2+} induce similar assembly of transiently appearing Aβ oligomers at the early state (85). The co-deposition of Al^{3+}, Fe^{3+} and β-sheets of $A\beta_{42}$ may act as a source of ROS and begin to explain some of the oxidative damage found in the immediate vicinity of senile plaques (86). Whether such metal ion-$A\beta_{42}$ synergism is an integral part of the aetiology of AD or not, however, remains unconfirmed.

Metal chelators

Copper and zinc are both enriched in Aβ deposits in AD, and may enhance the Aβ aggregation. These aggregates could be solubilised by copper and zinc ion chelators *in vivo* (87), by preventing the ions from binding to Aβ and thus potentially reducing plaque formation. There are even animal model and *in vitro* studies that suggest that metal protein attenuating compounds (MPACs) can help in the dissolution and clearance of Aβ plaques (88). Zinc chelation, with the antibiotic clioquinol, was used by Prana Incorporated, in a small Phase II trial, in which 36 patients received 36 weeks of treatment. The treated group showed no significant cognition score differences, but *post hoc* stratification by illness severity revealed a significant clinical effect from the treatment in those more severely affected (41). On the other hand, only the less severely affected group showed any significant reduction in plasma $A\beta_{42}$. A Cochrane review (88), has also argued that clioquinol could have significant adverse effects (1 case in 36 participants). Further trials are planned (89). Though the theoretic principle seems attractive, a track record of failure when attempting to interfere specifically with $A\beta_{42}$ self-assembly using small molecules, has reduced the priority of this approach. The suggestion that Aβ upregulates glucose metabolism in neurons, arose from demonstrations that both heat shock and iron chelators protect cells from Aβ (90,91). Both of these conditions induce the transcription factor HIF-1, as does mild oxidative stress, a condition associated with AD. Since HIF-1 upregulates glycolysis in a manner similar to that seen in Aβ resistant cell lines, HIF-1 was studied in more detail and to be regulated by Aβ (92). As a result, therapeutics that elevate HIF-1, such as metal chelators, have the potential to reduce neuronal cell death caused by Aβ and other pathologies involving oxidative stress but remain unproven.

1.2.6 Cholesterol as a target in AD

Epidemiological studies support the potential of cholesterol-lowering 'statins' as a treatment for AD. This may reflect the fact that the main genetic factor for AD, ApoE4 (93), is associated with increased plasma cholesterol (94). Epidemiological studies support the potential of cholesterol-lowering 'statins' as treatment for AD (95,96).

Several *in vitro* and *in vivo* studies have shown that APP processing is cholesterol-dependent, and that cholesterol depletion leads to reduced Aβ production, though the mechanisms behind this are not completely understood. It remains unclear, however, if noTable changes in human brain Aβ metabolism will result from lowering cholesterol levels under physiological conditions, since several of the studies used severely perturbed systems (97). The hypothesis that physiological changes to cholesterol metabolism can reduce amyloid load is supported by the finding that Aβ production is reduced by inhibitors of acyl-co-enzyme A acyltransferase (ACAT), which catalyses the formation of cholesteryl-esters from cholesterol (98).

To examine the benefit of statins in AD, a recent RCT was conducted, comparing atorvostatin with placebo in a long-term trial (99). This study found no beneficial effects, though the statin was well-tolerated. The authors admit that a possible limitation of the study is that it was performed using participants with normal LDL levels. This means that it is possible that statins could show an effect on patients with hypercholesterolaemia, who are the population from which epidemiological data stems. The study also excluded patients with any cardiac or vascular disease which could be a possible target population that could benefit from statin use. Further studies could also show some role of statins in prevention of AD, as opposed to treatment of established AD that was examined in this study.

It is also possible, however, that the reported beneficial effects of statins actually result from their anti-inflammatory actions (100), as opposed to their direct effects on cholesterol metabolism. Prospective data on statin therapy for AD remain mixed (97). As a result, more prospective randomised treatment trials of statins in AD are still needed.

1.2.7 Inflammation and the immune response in AD

1.2.7.1 The role of microglia and astrocytes in chronic inflammation in AD

The two major types of brain cells that participate in the immune/inflammatory response are astrocytes and microglia. Microglia are abundant in senile plaques (101,102) and also surrounded by astrocytes (103). These cells function together in response to brain injury (104). This occurs through gliosis, one inflammatory response present in AD, where fibrous astrocytes become more numerous (105) and become activated and involved in prostaglandin/arachidonic acid-mediated inflammation (106). Microglial proliferation and activation is then thought to be further promoted, or has its cytotoxic activity modulated, by the growth factors produced by astrocyte reactions (107). Glial cells generate many molecules associated with inflammatory and immune functions. Inflammatory cytokines and other microglia-derived factors account for the neurotoxicity in gliosis, while reactive-astrocyte products such as IL-1 tend to be neuroprotective (108,109).

The inflammatory response at the site of injury, however, also represents a source of numerous growth factors and cytokines, with trophic, mitogenic, chemotactic and angiogenic activities (Table 1.2).

Table 1.2 | Secretory products of microglia and astrocytes that may have damaging or protective functions in AD (110).

Factor	Microglia	Astrocytes
Cytokines	IL-1α & β, IL-3, IL-5, IL-6, IL-8, INF-α, TNF-α	IL-1α & β, IL-3, IL-5, IL-6, IL-8, CSF-1, G-&CSF, TNF-α
Coagulation Factors	Plasminogen & Urokinase type Plasminogen Activator	Tissue Plasminogen & Urokinase type Plasminogen Activator
Complement Proteins	C1, C3, C4	C3, C4, C6, C7, C8, C9, Factor B, Factor I, Membrane Cofactor Protein, CD46, Clusterin, Vitrorectin
Eicosanoides	Prostaglandin D2, Leucotriene C4	
Growth Factors	Nerve Growth Factor, Transforming Growth Factor α & β, Basic Fibroblast Growth Factor	Nerve Growth Factor, Transforming Growth Factor α & β, Basic Fibroblast Growth Factor, Ciliary Neurotrophic Factor, Insulin-like Growth Factor I, Glia derived Growth Factor
Reactive N_2 Intermediaries	Nitric Oxide	Nitric Oxide
Reactive O_2 Intermediaries	Superoxide ions	
Proteases & Protease Inhibitors	Metalloproteinase Inhibitor TIMP-1 & TIMP-2	Protease Nexin 1, α-1-Antichymostrypsin, α-2-Macroglobulin, Cathepsin G
Transport proteins		Apolipoprotein D, Apolipoprotein B
Matrix proteins		Laminin, Fibronectin, Tenascin, Heparan-,Chondroitin- & Dermatin-Sulfate Proteoglycans
Adhesion factors		VCAM-1, NCAM, NCAM-1 & ICAM-2

1.2.7.2 Involvement of cytokines, chemokines and complement system in AD pathogenesis

Aβ activation of microglia causes production of inflammatory cytokines, including interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α). Aβ also activates the transcription factor nuclear factor-κB (NF-κB), which increases cytokine production by neurons and microglia (111). Microglia-induced enzymes, such as nitric oxide synthase, generate nitric oxide, leading to peroxynitrite formation and oxidative stress.

IL-1β further aggravates the inflammatory response by promoting more APP synthesis and by promoting the production of more Aβ-binding proteins by astrocytes (112). Over-expression of IL-1 near amyloid plaques may promote the phosphorylation of tau protein, leading to the formation of NFTs and neuron death (113).

Nearly all the cytokines and chemokines that have been studied in reference to AD, including IL-1β, IL-6, TNF-α, IL-8, transforming growth factor-β (TGF-β) and macrophage inflammatory protein-1α (MIP-1α), seem to be upregulated in individuals with AD when compared to levels in control individuals (114). An association between AD and polymorphisms in a number of pro-inflammatory genes has been described, including those that code for IL-1, (115), IL-6 (116), TNF-α (117,118), and α1-antichymotrypsin, an acute phase protein (119).

It has been shown that TGF-β treatment of human astrocytes markedly elevated APP mRNA levels and also increased the half-life of the APP message by at least five-fold (120). Rogers et al. (1999) also demonstrated that IL-1α and IL-1β increase APP synthesis by up to 6-fold in primary human astrocytes and by 15-fold in human astrocytoma cells without changing the steady-state levels of APP mRNA (121).

Animal models of AD, such as Tg2576 over-expressing APP carrying the Swedish mutation, also show enhanced levels of TNF-α, IL-1α, IL-1β, chemo-attractant protein-1, COX-2, and complement component 1q (122–124). In addition, it has also been reported that mice overexpressing the mutant human P301 tau protein have an increased immunoreactivity to IL-1 and COX-2 (125). The production of interleukins and other cytokines and chemokines may also lead to microglial activation, astrogliosis and further secretion of pro-inflammatory molecules and amyloid, thus perpetuating the pathological cascade of AD (126). It has been proposed that pro-inflammatory cytokines could affect Aβ formation by raising susceptibility to Aβ deposition or aggregation, or transcriptional upregulation of β-secretase and APP, as will be explained in detail in sections to follow.

Inflammation – susceptibility to Aβ deposition or aggregation

Studies performed with a transgenic amyloidosis animal model overexpressing V717F β-amyloid precursor protein showed that plaques did not develop unless inflammation was induced. This suggests that cerebral amyloid deposition increases under inflammatory conditions (127,128). Under non-inflammatory conditions, transgenic mice did not develop reactive amyloid or Amyloid A (AA) deposits in the brain. When a systemic acute-phase response was induced in transgenic mice, however, there was enhanced amyloid deposition. The deposition was preceded by an increase in cytokine levels in the brain, suggesting that systemic inflammation may be a contributing factor in the development of cerebral amyloid. Guo et al. have shown that the non-steroidal anti-inflammatory agent, indomethacin, reduced inflammation and cytokine expression and protected against the deposition of AA in the brain. These studies indicate that inflammation plays an important role in the process of amyloid deposition and inhibition of inflammatory cascades may attenuate amyloidogenic processes, such as AD (127,128). It has also been suggested that Aβ amyloidogenesis results from an IL-1/IL-6 mediated

acute phase reaction in the brain (129). Recent observations have shown elevated levels of other inflammatory proteins in AD brains. These include TNF-α, macrophage-colony stimulating factor (M-CSF), heme oxygenase-1 (HO-1), IL-1, and IL-6 (130–132).

Transcriptional upregulation of β-secretase

Cytokines are also able to upregulate β-secretase (BACE1) mRNA, protein and enzymatic activity (133). In neuronal Aβ formation, the enzymes BACE1 and presenilin-1 (PS1) play key role (Figure 1.1) since in their absence, Aβ synthesis is either abolished or considerably reduced (134). This is consistent with with data showing increased expression and activity of BACE1 in NT2 cells (from a Neuron-committed Teratocarcinoma cell line) exposed to oxidative stress (135), in experimental traumatic brain injury (136) and in reactive astrocytes in chronic models of gliosis (137).

Involvement of the complement system in AD

The complement system involves a collection of proteins including those designated C1 to C9, which participate in an amplifying cascade of enzymes, resulting in large numbers of downstream complement factors. The complement system participates in antigen-antibody reactions when outside the brain. Within the brain, however, complement protein C1b binds to Aβ fibrils, which are then phagocytosed by microglia. These fibrils may remain largely undegraded by the microglia, resulting in a "frustrated phagocytosis" that could lead to a worsened condition due to heightened microglia activation, if the toxic elements of the phagolysosome are released into the cell environment (138). *In vitro* fibrillar amyloid activates both the classical and alternative complement cascades (139), which could lead to further degeneration as a result of this possible frustrated phagocytosis. In line with this postulation, chronic treatment with a C5a antagonist, PMX205: hydrocinnamate-[Om-Pro-D-cyclohezylalanine-Trp-Arg], decreases AD pathology in two mouse models.

1.2.8 Anti-inflammatory drugs

Epidemiological studies suggest that non-steroidal anti-inflammatory drugs (NSAIDs) and cholesterol lowering agents (statins) are protective against AD but with U-shape dose response. There are many theories as to how these drug classes interfere with the disease cascade but the exact mechanism is still to be elucidated. Inflammation seems to play a clear role in AD pathology and this could explain the actions of both drug classes. Their use, however, stemmed from epidemiological studies, with understanding applied post hoc.

NSAIDs

Interest in the use of NSAIDs for AD prevention was sparked by a study that indicated that indomethacin, in doses of 100-150 mg/day, appeared to protect mild-to-moderately impaired AD patients from the degree of cognitive decline exhibited by well-matched controls (140). The pathology of AD clearly involves an inflammatory component (141) and a large body of epidemiological evidence indicates that use of a subset of NSAIDs is associated with a reduced risk of AD (142). The putative targets of NSAID actions is thought to be microglia associated with the senile plaques (143). Patients receiving long-term NSAID therapy have been shown to exhibit a 65% reduction in plaque-associated reactive microglia (144). The latest clinical trial results show reduced risk of AD in NSAID users, but only in association with patients with an ApoE4 allele, and show no advantage for $A\beta_{42}$-lowering NSAIDs (145).

The underlying mechanisms for the reduction of inflammation by NSAIDs in AD, remain unclear. Several mechanisms, however, have been proposed to explain the beneficial effects of

NSAIDs in AD: protection against Aβ aggregation and APP processing; COX-2 inhibition; and PPARγ activation.

Protection against Aβ aggregation and APP processing

The neurotoxic and proinflammatory actions of the Aβ peptide are dependent on its aggregation and β-sheet conformation. Certain NSAIDs may alter the β-sheet conformation of Aβ, with studies suggesting they affect the aggregation of Aβ peptides *in vitro* (146,147). Ibuprofen has been associated with the reduction of pro-amyloidogenic α1-antichymotrypsin (ACT), an effect likely mediated by a decrease in IL-1β. Chronic use of NSAIDs, such as aspirin for arthritis, has been shown to decrease the risk of developing AD, by as yet unknown mechanisms. These drugs inhibit human Aβ aggregation *in vitro*, and reversed the β-sheet conformation of preformed fibrils, at clinically relevant doses. Aspirin prevented enhanced Aβ aggregation by aluminium, an environmental risk factor for AD. This anti-aggregatory affect was restricted to NSAIDs, and not exhibited by other drugs used in AD therapy (146). The protective effect of NSAIDs has been associated with decreased secretion of Aβ peptides and soluble APP (APPs), though there is still much debate about the molecular mechanism involved (136,148–150). One hypothesis claims that a group of NSAIDs may directly affect the generation of $A\beta_{42}$, which is the most aggressive form of the amyloid peptide. It has been suggested that this subset of NSAIDs is able to shift the cleavage products of APP to shorter, less fibrillogenic forms (148), probably through an allosteric effect of γ-secretase by altering PS1 conformation (151).

COX-2 inhibition

The canonical targets of NSAIDs are cyclooxygenases (COXs) 1 and 2. The effect of NSAIDs on Aβ generation, however, has been proven to be independent of COX activity (152). This corroborates the recent failure of a clinical trial with a selective COX-2 inhibitor (153), suggesting that

another mechanism is behind the protective effect of NSAIDs in AD. Furthermore, recent data revealed that the effects of COX-2 inhibitors could be adverse, since many selective COX-2 NSAIDs are able to raise $A\beta_{42}$ levels (154).

A small trial of indomethacin hinted at an improvement (140) but large placebo-controlled trials of specific COX-2 inhibitors in AD have not been successful (153). This implies that inhibition of this target does not change the disease course. In a randomised controlled trial, a low dose of the traditional non-selective NSAID, naproxen, was also unable to slow cognitive decline in patients with mild to moderate AD (153). Some NSAIDs, as discussed above, are able to specifically lower $A\beta_{42}$ production at high doses, but this excludes the two COX-2 inhibitors and naproxen that failed in AD trials (148). Chronic administration of high doses of ibuprofen has also been shown to lead to a reduction in amyloid load and microglial activation in APP transgenic mice *in vivo* (131,155). The therapeutic effects of NSAIDs might therefore be mediated by direct or indirect effects on Aβ aggregation. A large phase III trial on indomethacin, however, has failed.

PPARγ activation

A potential downstream target of some NSAIDs is the peroxisome proliferator-activated receptor-γ (PPAR-γ) (156,157). The most prescribed NSAIDs that may potentially reduce the risk of AD (ibuprofen and indomethacin), have been shown to be effective PPAR-γ activators (156). Although PPAR-γ ligands have an anti-inflammatory effect *in vivo* and *in vitro*, the precise mechanism by which PPAR-γ activity is modulated by its ligands has not been fully elucidated. The major regulatory events identified in PPAR-γ function are ligand binding, co-activator docking, phosphorylation, and proteasome degradation (158) (Figure 1.3).

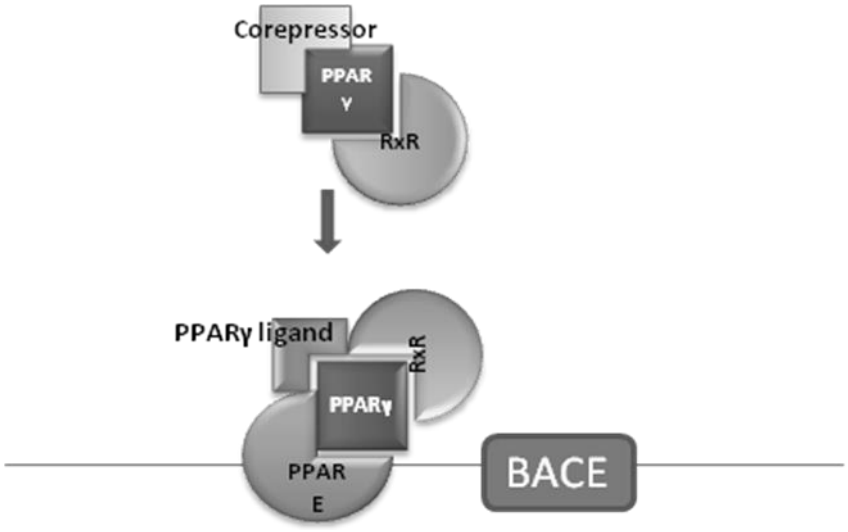


Figure 1.3 | BACE1 repression by PPAR

Incubation of N2a cells with NSAIDs that are PPAR-γ activators, showed a decrease in BACE1 gene promoter activity, an effect not produced by NSAIDs that are not PPAR-γ agonists, such as sulindac sulphide and aspirin (159). Overexpression of PPAR-γ1 and PPAR-γ2 also significantly reduced BACE1 promoter activity. The identification of a PPAR response element in the BACE1 promoter has also supported the possibility that PPAR-γ ligands may downregulate BACE1 transcription. This suggests that the potential effect of NSAIDs could be through regulation of BACE1 promoter activity (159) (Figure 1.3). At very high concentrations, NSAIDs can somehow modulate γ-secretase cleavage, such that $A\beta_{42}$ levels are reduced while the smaller isoform that is less prone to aggregation $A\beta_{38}$ is increased (160). Only extended clinical trials comparing several compounds, including $A\beta_{42}$-lowering NSAIDs, will conclusively establish whether or not NSAIDs can slow disease progression and if so whether this is related to the $A\beta_{42}$ –specific or via an alternative pathway.

Despite the beneficial effects, penetration of the BBB remains the greatest obstacle for NSAID use in AD. One study, examining NSAID use and risk in AD over a five-year period (the longest study

of this kind), produced mixed results for different types of NSAIDs, with ibuprofen showing the most benefit. Other NSAIDs, however, showed no benefit, with researchers noting that NSAID use may not have accounted for the reduced AD risk (161).

Nicotinic receptor agonists

Nicotinic receptor agonists, in relation to their anti-inflammatory action, suppress cytokine release and modulate microglial activation. In line with this, nicotine itself has been suggested as a possible protective agent against the development of AD (162). In animal models and observational studies, nicotine inhalation has been implicated as having a potentialpositive effect in AD. However, there have only been limited studies examining these effects, with only one randomised double-blind trial found for inclusion in a Cochrane review. To date, there is no real evidence to confirm the animal models or observational studies. This potential intervention needs more supportive evidence to show benefits that would exceed the adverse effects of nicotine.

A nicotinic receptor agonist called ABT-089 (2-methyl-3-(2-(S)-pyrrolidinylmethoxy)pyridine dihydrochloride; Abbott Laboratories) targets the α4β2 nicotinic receptor subtype (163) however, produced no statistically significant improvement in patients with Alzheimer disease in clinical trials (164). An α7 nicotinic receptor agonist, A-582941 (2-methyl-5-(6-phenyl-pyridazin-3-yl)-octahydro-pyrrolo[3,4-c]pyrrole; Abbott Laboratories), decreased hyperphosphorylation of tau protein in Tg2576-transgenic mice that overproduced APP (165). Another agonist, ABT-107 (5-(6-[(3R)-1-azabicyclo[2,2,2]oct-3-yloxy]pyridazin-3-yl)-1H-indole; Abbott Laboratories), improved cognition in monkeys, rats, and mice, and when administered with the acetylcholinesterase inhibitor donepezil hydrochloride it also improved short-term recognition memory (166). Spinal tau hyperphosphorylation was reduced by a continuous infusion of ABT-107 in tau/APP-double-transgenic mice, suggesting that

this approach may be useful in treating patients with AD (165). Recent testing in normal human controls, in whom this drug appeared to be well tolerated, with good pharmacokinetic findings and only mild adverse effects (167).

Another anti-inflammatory agent that does not belong to the NSAID class, but which may be useful in the treatment of AD, is propentofylline. Propentofylline is a phosphodiesterase inhibitor that also acts as an adenosine reuptake inhibitor. It readily crosses the BBB and appears to play a role in reducing inflammation by inhibiting free radical production and reducing microglial cell activation (168). A Cochrane review has found that evidence appears to suggest that propentofylline may be beneficial in many areas of cognitive function, but that this information is currently limited. The reason for this is that a pharmaceutical company producing propentofylline has been unwilling to release unpublished studies, suggesting that there could be a publication bias.

1.2.9 Oxidative damage in Alzheimer's disease

There is increasing evidence that free radical–induced oxidative damage may play a role in the pathogenesis of Alzheimer's disease. It could be an early event in the disease, even when there is no Aβ deposition (169). Free radicals are reactive oxygen compounds that include superoxide, hydrogen peroxide, and hydroxyl radicals that may attack and damage lipids, proteins, and DNA. Reactive oxygen species (ROS) are very small molecules or ions that are formed as natural byproducts of the normal metabolism of oxygen, and have important roles in cell signalling, but can also cause deleterious effects associated with cell damage. Oxidative stress results from the imbalance between the production of ROS and a biological system's ability to readily detoxify the reactive intermediates or easily repair the resulting damage. Increasing oxidative stress can cause cell apoptosis, cell death; and necrosis. The brain is especially sensitive to oxidative damage due to its high content of unsaturated

and oxidation-susceptible fatty acids, high use of oxygen, and low levels of antioxidants. Evidence for oxidative damage has been obtained from postmortem brain tissue as well as from living patients with Alzheimer's disease.

Peroxidation of polyunsaturated fatty acids, or lipid peroxidation, is especially important, because it is a self-propagating reaction that will continue until it is terminated by defences or until the substrate is exhausted. Free radical attack on polyunsaturated fatty acids leads to structural damage to membranes. It also generates several aldehyde byproducts, including malondialdehyde and C3-C10 straight chain aldehydes, as well as α,β-unsaturated aldehydes such as 4-hydroxy-2-nonenal (HNE) and acrolein. Of these, the α,β-unsaturated aldehydes in particular may be primary effectors of tissue damage (170). Acrolein is found to be increased levels in AD brain (171). They show high reactivity with nucleophiles, including sulfhydryl groups of cysteine, histidine, and lysine, as well as impairing key neuronal processes (172,173). Lipid peroxidation also produces oxidised and endocyclised products of arachidonic acid (F2-isoprostanes or F2-IsoPs) or docosohexaenoic acid (F4-neuroprostanes or F4-NeuroPs) that are quantitative *in vivo* biomarkers of free radical damage (174,175).

Free radicals, particularly the hydroxyl radicals, also attack nucleic acids. This leads to strand breaks, cross-linking and base modifications and these may contribute to alterations in protein production and propagate neuron dysfunction and death. Mitochondrial DNA (mtDNA) is more susceptible to free radical-mediated damage than nuclear DNA (nDNA), as a result of its proximity to the site of ROS production. It also lacks protective histones and significant noncoding sequences, and has limited repair capacity. As Dizdaroglu et al. argue in their review, DNA attack by ROS can lead to the generation of more than 20 oxidised base adducts, with 8-hydroxydeoxyguanine (8-OHdG) the

most prominent because of the relatively low oxidation potential of guanine (176). In addition to direct oxidation by ROS, DNA can also be modified by α,β-unsaturated aldehyde byproducts of lipid peroxidation through an initial addition of the exocyclic amino group, followed by ring closure of N-1 onto the aldehyde group to generate a bulky exocyclic 1-N2-propanodeoxyguanosine adduct (177). These adducts are potentially biologically relevant, because they may promote DNA-DNA and DNA-protein cross-linking that can limit transcription (178). Similar to the products of lipid peroxidation, protein oxidation is not simply a reflection of damaged tissue, but an effector of cellular dysfunction (179).

The brain is the organ with the highest basal rate of glucose consumption and most of the energy generated by the oxidation of glucose is used for the work necessary to maintain the ionic balances associated with synaptic transmission. When the nervous system is subjected to the oxidative stress of age-associated disease, there is a redistribution of glucose breakdown to pathways that more efficiently produce molecules involved in anti-oxidant metabolism.

Studies with Aβ-resistant CNS cell-lines (180,181) strongly support the suggestion that a change in ROS metabolism is a key component of Aβ toxicity. If oxidative stress is key to Aβ toxicity, then components of the cellular antioxidant machinery are also likely to be upregulated. One key component among these is the pathway that produces reducing equivalents to maintain antioxidant defences, such as glutathione and the thioredoxins. Within this pathway, NADPH is the primary reducing intermediate and is principally supplied by the pentose shunt. As a result, NADPH levels and the activity of the rate-limiting pentose shunt enzyme, glucose-6-phosphate dehydrogenase (G6PD), were examined in Aβ resistant clones of the neuroendocrine-like cell line, PC12. This showed that Aβ resistant cells have elevated levels of both G6PD and NADPH, and that the flux of glucose through the

shunt is also increased. Glycolysis is higher in Aβ resistant cells, as is the uptake of glucose. A variety of assays have clearly shown that Aβ resistant cells have elevated glycolytic and pentose shunt activities. These Aβ resistant clones also have greatly reduced levels of endogenous ROS (92).

Animal studies have shown that exposure of CNS tissue to low levels of stress, such as mild ischaemia or even dietary restriction, increases the resistance of neurons to more severe oxidative insults (182). This phenomenon has been studied most extensively with ischaemia and is thought to be mediated by the upregulation of a variety of defence mechanisms which then enable the nerve cells to protect themselves from a subsequent potentially lethal insult. The same phenomenon is seen with amyloid toxicity, where pretreatment with low levels of Aβ upregulates the pentose shunt and protects cells from high concentrations of Aβ (92). Observations of Aβ resistant cell lines in culture lead to the prediction that AD brain tissue will have elevated pentose shunt and glycolytic activities. There is a significant increase in CO_2 production from glucose, as well as O_2 uptake, in biopsied AD brain tissue, relative to age-matched controls. This demonstrates enhanced glycolysis and elevated respiration (183–185). With respect to pentose shunt activity, there is an approximately 50% increase in G6PD activity in AD brain (92). Other studies have shown a similar increase in G6PD, and another shunt enzyme, 3-phosphogluconate dehydrogenase, in AD brains (186), as well as an increased level of G6PD in surviving pyramidal neurons (187).

The production of free radicals in the brains of patients with AD comes from sources that are unique to the AD affected brain. Evidence has shown that the β-amyloid peptide and advanced glycation end products (AGEs) may be sources of free radical production (188,189). The β-amyloid peptide is the main component of the senile plaques that are the hallmark pathology of the AD-affected brain. Free radicals are produced by the β-amyloid peptide once it is formed outside the neurons, and

these free radicals were found to be neurotoxic to hippocampal cells and the synaptosomal membranes (188). AGEs are post-translationally modified proteins that are formed when the amino group of the protein reacts non-enzymatically with monosaccharides. The protein undergoes further modification via oxidation, condensation, and dehydration to produce the AGEs. This reaction, which is catalyzed by transition metals such as iron, also produces free radicals. AGEs may increase oxidative stress via either direct radical production by chemical oxidation and degradation of AGEs, indirect oxidative stress via AGE-receptor binding and activation of signaling pathways, or by interacting with microglia in an acute phase reaction that results in a respiratory burst and free radical production (190). AGEs have been found in the senile plaques of AD-affected brains (191). AGE modified β-amyloid accelerates the aggregation of soluble non-fibrillar β-amyloid *in vitro*, which suggests that this may also occur in vivo (192).

The production of ROS could activate NF-κB, the nuclear transcription factor, which is important in the expression of many genes whose proteins are involved in the control of apoptosis (cell suicide), in the development of B and T cells, in anti-viral and bacterial responses, in responses to multiple stresses, in embryonic development and in inflammatory responses. NF-κB exists in the cytosol as a pre-formed trimeric complex. The P50/P65 protein dimer is associated with an inhibitory protein known as I-κB. Oxidants trigger a change in the cell that results in phosphorylation of the I-κB subunit. After I-κB is phosphorylated, a process of the proteolytic digestion of this subunit is activated. When the inhibitor subunit is dislodged from the P60/P65 heterodimer the activator NF-κB can migrate to the nucleus and bind to DNA, thereby initiating transcription. Downstream products of NF-κB activation include inflammatory cytokines such as, TNF-α and nitric oxide synthase (NOS). Antioxidants such as the one present in plant, herbs and mushrooms could play an important role in preventing these diseases by counteracting these oxidants that modulate NF-κB activation.

Antioxidants

As explained in the previous section, oxidative damage plays a major role in AD and the agents that could prevent or minimize could be of therapeutic advantage. Several studies carried out on antioxidants showed their anti-inflammatory, anti-atherosclerotic, anti-mutagenic, anti-carcinogenic, anti-bacterial activities along with other potential health promoting properties (193). Currently there is an increase of interest in phytochemicals as potential sources of natural antioxidants with low cytotoxicity, which are candidates for the prevention of oxidative damage.

There is increasing evidence to suggest that omega-3 polyunsaturated fatty acids (PUFA) may help to prevent dementia as a result of their many positive effects (194). Omega-3 PUFA is thought to prevent atherogenesis and to have anti-inflammatory, anti-oxidant and anti-amyloid actions, as well as being neuroprotective. All of these may be involved in the prevention of dementia. At the moment however, available data is only observational or epidemiological, without any clinical trials to date. Animal models have shown positive effects, but have yet to be replicated in humans. As such, until trial data is available, omega-3 PUFA cannot be recommended specifically for dementia prevention. An RDI is defined for both CVD and cardio-matabolic disease. Protection is expected from proven protection of cardio risk factors of AD. Ongoing trials are investigating the effects of PUFAs on cognition in a wide range of conditions including *in-utero* and normal toddler development, pediatric bipolar disorder, autism, post-operative cognitive dysfunction, cardiovascular disease, septic encephalopathy, post traumatic stress disorder, intracTable partial seizures, and aging in the frail elderly. None are focused on assessing a selective response that may be dependent on ApoE status or involve AD directly, although the study of frail aging led by Bruno Vellas at the University Hospital in Toulouse, France could provide further insights into the cognitive decline associated with aging, mild cognitive impairment (MCI), or even early AD depending on how many subjects reach such study

endpoints by the end of the 36 month trial period. Additional trials are currently in the planning stages and so further insights into the potential benefits of omega-3 PUFA supplementation in AD may be years away, but nonetheless they are being relentlessly pursued by investigators across the globe that are not dismayed by clinical trial data presented to date (195).

The drug procaine has been used as an "over the counter" preparation in many countries (196). It has been promoted as an "anti-ageing" treatment but has also been said to prevent and even reverse the effects of dementia. A Cochrane review, however, has recently argued that there is more evidence to suggest that use of procaine is detrimental than beneficial; serious side effects such as bradycardia, dizziness, anxiety, nausea, conculsions occurred in a small number of participants. Studies of healthy patients suggest that it may have a positive effect on memory, however, in contrast, the results of a study on demented patients showed a negative effect of the drug. This suggests that, despite the numerous claims that abound, procaine should not be used in the treatment of AD.

1.3 Status of therapeutic strategies

There is an abundance of reported information describing AD biology and treatment. At the present time however, the gaps in understanding are still extensive. As the literature currently stands, however, the most promising therapies for AD are probably those that prevent formation of NFTs and senile plaques and thus reduce the apoptotic death of neurons. The most promising advances are the development of drugs that block the formation of the Aβ peptide (197), and a novel strategy to induce an immunologic response capable of clearing the amyloid plaques already formed (198). There are increasing levels of evidence becoming available to support this proposition with research continuing. But the succession of failures is frequent in clinical trial stages. One among the reasons would be that

the transgenic mouse models frequently used to select lead drug candidates are actually a poor substitute for the aged human brain. Another consideration may be that different states of microglial activation may have different impacts on the disease.

Drugs used to treat advanced AD may be efficacious at the pre-symptomatic stage but there is an ethical issue here. One of the potential pitfalls and areas of concern in the development and testing of AD treatments is the fact that side effects of the administered drugs may be more common in older patients (199). Older patients may also have multiple co-morbidities requiring multiple medications, leading to a greater potential for potential drug interactions (199). As a result of this, safety testing of new treatments is of great importance in the development and testing of AD treatments.

Despite the disappointing trial results with anti-inflammatory treatment in AD patients with clinically manifest dementia, the continuing accumulation of evidence of both epidemiological studies as well as laboratory experiments that suggest an early and pivotal role of inflammatory processes in the pathogenesis of AD offer a new and tempting perspective on the potential role of NSAIDs in the primary prevention of this disease. Randomised controlled trials will reveal whether this class of drugs, or alternative agents with different inflammatory targets and side effect profiles, can indeed delay or prevent AD (200). Until the results of these strategies in animals can be safely duplicated in humans, the anti-inflammatory treatment approach seems to stay.

The strategy of combining presymptomatic diagnosis with preventative medicine seems to be the most pragmatic approach in both medical and socio-economical terms. Also specific drugs are unavailable to treat inflammation associated with AD despite the fact that inflammation seems to play a clear role in AD pathology hence there is a need to discover them.

Hypothesis and aims

Inflammation, involving the innate and adaptive immune systems, is a normal response to infection. However, chronic inflammation may result in auto-immune or auto-inflammatory disorders, neurodegenerative diseases such as AD. AD therapy currently stands in addressing the symptoms of the disease and results in minor improvements in cognitive functions. The elucidation of the inflammatory processes responsible for AD has demonstrated similarities to other inflammation-associated diseases. It is therefore not surprising that therapies used for the treatment of other medical conditions, namely NSAIDs, statins and antioxidants, may be of benefit in AD. A variety of safe and effective anti-inflammatory agents are available, including aspirin and other non-steroidal anti-inflammatories such as statins. New therapeutics such as the new types of anti-inflammatory agents include "biologicals" such as anti-cytokine therapies and small molecules that block kinases, PPAR agonists and small RNAs under development.

Opportunities exist via diet and lifestyle for contributing to chronic inflammation or alternatively exert anti-inflammatory activity. A range of dietary plants and mushrooms and also non-dietary herbals have been reported to exhibit anti-inflammatory bioactivity, mainly through anti-oxidant mechanism. It is plausible to use a cellular model of inflammation involving the inducible NOS pathway to evaluate the potential anti-inflammatory properties of a selection of dietary plants and mushrooms and to select most potent plant extract for identifying the potent anti-inflammatory compounds present in them.

Hypothesis

1. Extracts from common dietary plants, herbs and mushrooms, contain anti-inflammatory compounds with a potential for treating inflammatory process in AD.
2. Most potent extract (lead) with anti-inflammatory properties can be identified by using cell-based anti-inflammatory assays.
3. Individual compounds with anti-inflammatory potential can be identified by bio-activity guided purification of the lead candidate or by comparison with identified constituents.

Aims

1. To select the most potent anti-inflammatory plant extract from plant, mushroom and herbal libraries by screening capacity to down-regulate the LPS, IFN-γ induced production of NO and TNF-α.
2. To fractionate the most potent plant extract, by preparative HPLC, followed by testing of the individual fractions for anti-inflammatory properties - and then use MS for chemical identification.
3. To identify potent compounds in the extract by solvent extraction and GC/MS, followed by purchasing these individual compounds, and testing them for anti-inflammatory properties.

CHAPTER 2

STUDIES ON ANTI-INFLAMMATORY PROPERTIES OF SELECTED PLANT EXTRACTS USED IN CHINESE TRADITIONAL MEDICINE

2.1 Introduction

There is growing evidence that reactive oxygen species (ROS) such as superoxide anion ($O_2^{\cdot -1}$, hydroxyl radicals, singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2) play an important role in many age-related diseases including cancer, cardiovascular diseases, diabetes, inflammation, degenerative diseases, anaemia, and ischemia(201). The production of ROS could activate NF-κB, the nuclear transcription factor, which is important in the expression of many genes whose proteins are involved in the control of apoptosis, in the development of B and T cells, in anti-viral and bacterial responses, in responses to multiple stresses, in embryonic development and in inflammatory responses. NF-κB is a ubiquitious transcription factor exists in the cytosol composed of two DNA binding sub-units, p50 and p65 (202). The P50-P65 protein dimer is associated with an inhibitory protein known as I-κB. Oxidants trigger a change in the cell that results in phosphorylation of the I-κB subunit (203). After I-κB is phosphorylated, a process of the proteolytic digestion of this subunit is activated. When the inhibitor subunit is dislodged from the P50-P65 heterodimer the activator NF-κB can migrate to the nucleus and bind to DNA, thereby initiating transcription. Downstream products of NF-κB activation include NO and inflammatory cytokines such as, TNF-α. Antioxidants are playing an important role in preventing these diseases by counteracting these oxidants that modulate NF-κB activation. Currently there is an increase of interest in phytochemicals as potential sources of natural antioxidants with low cytotoxicity, which are candidates for the prevention of oxidative damage.

Recent studies showed that a number of medicinal and dietary plants extracts containing polyphenols, flavonoids, and other compounds exhibited significant antioxidant activities (204). Anti-inflammatory activities was shown by alkaloids from *Forsythia suspensa* in rats (205), anti-inflammatory and antioxidant activities of the aerial part of *Helichrysum italicum* extracts have been

established in various *in vivo* and *in vitro* experimental models (206). A significant anti-inflammatory effect of aqueous extract of *Scrophularia frutescens L,* a anti-inflammatory drug in the folk medicine, was reported in carrageenan-induced oedema test on the rat paw (207).

Several studies carried out on antioxidants showed their anti-inflammatory, anti-atherosclerotic, anti-mutagenic, anti-carcinogenic, anti-bacterial activities along with other potential health promoting properties(193). For instance, 3-O-Methylquercetin (3-MQ), a flavanoid isolated from *Rhamnus nakaharai Hayata*, concentration-dependently inhibited LPS-induced NO production (IC_{50} = 4.23 µM) and significantly and concentration-dependently inhibited LPS-induced iNOS protein and mRNA expressions in RAW 264.7 cells (208).

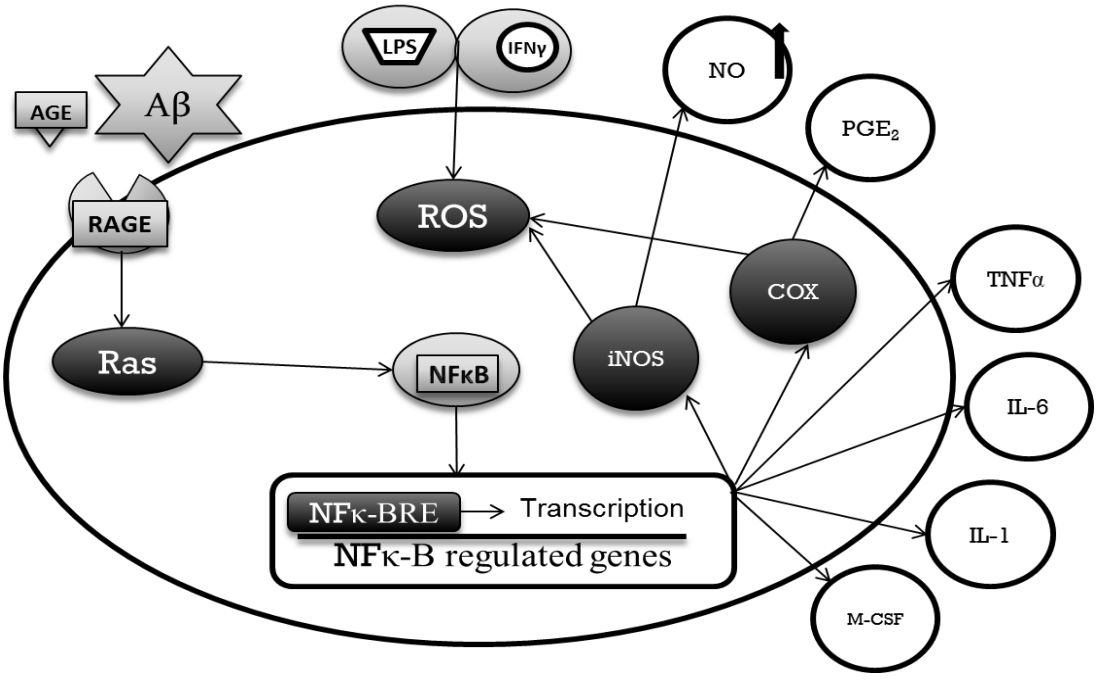


Figure 2.1 Molecular basis of inhibition of LPS and IFN-γ induced NO and TNF-α production. Activation of RAGE via AGES or beta-amyloid activates the transcription of various NFkB-regulated genes including iNOS and TNF-α.

The activation of macrophages leads to secretion of pro-inflammatory cytokines, apoptotic and inflammatory molecules such as TNF-α, nitric oxide (NO) which are believed to play an important role in modulating inflammation (209) (Figure 2.1). However, different pathological disorders, such as immune diseases, inflammation, cancer and other age related diseases, are closely associated with the production of excess amounts of NO and cytokines like TNF-α (210). Based on this fact, this study aims to evaluate anti-inflammatory activities of 58 species of Chinese medicinal herbs extracts selected based on phenolic & flavonoid contents, and to correlate these effects to their antioxidant potential.

2.2 Materials and methods

Materials

Chemicals, reagents and equipment

DMSO, sodium nitrate, hydrogen peroxide, 95% ethanol, Bovine serum albumin, Lipopolysaccharide (LPS) (*E.coli* serotype 0127:B8), N-(1-1-napthyl) ethylenediamine dihydrochloride, Ethylenediaminetetra acetic acid (EDTA), Penicillin G Sodium Benzyl, Resazurin Sodium 10%, Streptomycin, Sulfanilamide, Tetra methyl benzidine (TMB), Trypan blue 0.4% were purchased from Sigma-aldrich, Australia, Hydrochloric acid, Sodium hydrogen carbonate, Sodium chloride, Sodium di-hydrogen orthophosphate, Sodium hydroxide and Di-Sodium hydrogen orthophosphate from Univar. Antibiotics, Dulbecco's modified Eagle's medium (DMEM), Foetal bovine serum (FBS) and Glutamine were purchased from GIBCO. Interferon-γ (murine) and TNF-α – ELISA kits were purchased from Peprotech. 96-well flat bottom tissue culture plates and ELISA plates were purchased from BD (Greiner bio-one, Cellstar, BD), Cell scrapers from purchased from Sarstedt, Germany.

Plant material

Chinese herbs were selected by our collaborators Sundar Koyyalamudi initially for testing anti-oxidant content nad were obtained as the dried plant materials from Beijing Tong Ren Tang Chinese Herbal Medicine shop**,** Sydney, Australia. The scientific names and their medicinal use are listed in *Table 2.1*.

Table 2.1 List of the plants used in this study and their traditional medicinal usages

S.No	Medicinal Plant name	Chinese Name	Medicinal Use
1	*Viscum coloratum* (Komar.) Nakai	Hui ji sheng	Antiarrhythmic activity (211)
2	*Prunella vulgaris* L.	Xia ku cao	Antioxidant and anti inflammatory properties (212)
3	*Semen coicis* L.	Yi yi ren	Anti-diabetic (213)
4	*Pogostemon cablin* Benth.	Guang huo xiang	Anti-insecticidal, anti-fungal and bacteriostatic (214)
5	*Pseudostellaria heterophylla* (Miq.) Pax ex Pax et Hoffm.	Tai zi shen	Anti-oxidant activity (215)
6	*Paeouis suffuticosa* Sndr.	Mu dan pi	Anti-oxidant activity (216)
7	*Corydalis yanhusuo* W.	Yan hu suo	Improve blood circulation, treating headache (217)
8	*Hedyotis diffusa* Willd.	Bai hua she she cao	Antioxidant activity (218)
9	*Leonurus japonicus* Houtt.	Yi mu cao	Antipyretic (219)
10	*Andrographis paniculata* (Burm.f.) Wall. ex Nees	Shuan xin lian	Liver disorders, bowel complaints ofchildren, colic pain (220)
11	*Salvia miltiorrhiza* Bunge.	Dan shen	Anti-oxidant activity (221)
12	*Paeonia lactiflora* Pall.	Bbai shao	Antihyperlipidemic (216)
13	*Polygala tenuifolia* Willd.	Yuan zhi	Expectorant, tonic and sedative agent (222)
14	*Rehmannia glutinosa* (Gaertn.) Steud.	Sheng di huang	Anti-oxidant activity (223)
15	*Cyperus rotundus* L.	Xiang fu	Anti-oxidant activity (214)
16	Rhizoma *Alpinae officinarum*	Gao liang jiang	Gastric ailments (224)
17	*Lysinachia christinae* Hance.	Jin qian cao	Gallstones, urethra stones and rash (225)

18	*Ducheshea indica* (Andr.) Focke.	She mei	Anti-cancer (213)
19	*Asparagus cochinchinensis* (Lour.) Merr.	Tian men dong	Anti-oxidant activity (226)
20	*Schizandra chinensis* (Turcz.) Baill.	Wu wei zi	Anti-stress effect (227)
21	*Acanthopanax senticosus* Herms		Adaptogenic activity (228)
22	*Paris polyphylla* Smith	Qi ye yi zhi hua	Anti-cancer (213)
23	*Smilax glabra* Roxb.	Tu fu ling	Anti proliferative properties (229)
24	*Pleione bulbocadioides* (Franch.)Rolfe.	Shen ci gu	Anti-cancer (230)
25	*Tussilago farfara* L.	Kuan dong	Antitussive herbal medicine (231)
26	*Aster tataricus* L.	Zi wan	Anti tumor activity (232)
27	*Uncaria rhyncophylla* Miq	Gou Teng	Antioxidant activity and anti-inflammatory activity (233)
28	*Rabdosia rubescens*（Hamst.） Wuet.	Dong ling cao	Stomachache, pharyngitis, sore throat and cough (234)
29	*Platycodon grandiflorus* (Jacq.) A. DC.	Jie geng	Immunomodulation (235)
30	*Curcuma zedoaria* (Christm.) Roscoe	E zhu	Anti-oxidant acitivity, Anti-mutagenic activity and Anti-microbial activity (236)
31	*Artemisia vulgaris* L.	Ai ye	Hemodynamic actions, Anti-corrosive effects, Anti-oxidant and anti-fungal activity (237)
32	*Plantago asiatica* L.	Che qian cao	Chronic Liver Disease (238)
33	*Sarcandra glabre* (Thunb.) Nakai	Zhong jie feng	Antitumor and anti-bacterial (215)
34	*Solanum nigrum* L.	Long Kui	Hepatic cancer and several other cancers (239)
35	*Poria cocos* (Schw.) Wolf	Fu lin	Anti-rejection activity, Anti-inflammatory activity and Anti-oxidant activity (240)
36	*Codonopsis pilosula* Franch.	Dang shen	Immunomodulatory activity and Anti-oxidant properties (241)
37	*Curcuma aromatica* Salisb	Yu jin	Anti-inflammatory and gastrointestinal (242)
38	*Scutellaria baicalensis* Georgi.	Huang qi	Anti-inflammatory and anti-tumor (243)
39	*Actinidia arguta* (Sieb.et Zucc.)Flarich.ex Miq.	Teng li gen	Anti-cancer and anti-allergic (244)
40	*Taxillus chinensis* (DC.) Danser	Sang ji sheng	Obesity (245)
41	*Eucommia ulmoides* Oliv.	Du zhong	Neuroprotective, Anti-cancer and antifungal (246)
42	*Atractylodes macrocephala* Koidz.	Bai zhu	Anti-inflammatory activity (247)

43	*Rheum officinale* L.	Da huang	Digestive system diseases, treatment of various haemorrhages and trauma (248)
44	*Sophora japonica* (L.) Schott.	Gui hua	Anti-inflammatory activity (244)
45	*Polygonum cuspidatum* Houtt.	Gu zhang	Anti-oxidant activty and Anti-inflammatory activity (212)
46	*Ligustrum lucidum* Ait.	Nv zhen zi	Anti-oxidant activity (249)
47	*Polygonum aviculare* L.	Bian cun	Anti-oxidant activity, treatment of various inflammatory diseases, hepatitis, tumors and diarrhea (250)
48	*Solanum lyratum* Thunb.	Bai ying	Regulating body immune function and ability, anti-allergic (251)
49	*Akebia quinata* (Houtt.)Decne.	Ba yue zha	Anti-inflammatory, anti-cancer and anti-phlogistic (252)
50	*Sanguisorba officinalis* L.	Di yu	Anti-allergic (253)
51	*Spatholobus suberectus* Dunn.	Ji xie teng	Anti-tumour and rheumatic arthralgia (254)
52	*Saposhnikovia divaricata* (Turcz.)Schischk	Fang feng	Anti-inflammatory (255)
53	*Mahonia fortunei* (Lindl.)Fedde	Shi da gong lao	Anti-bacterial and anti-fungal (256)
54	*Cynanchum paniculatum* (Bge.) Kitag	Xu chang qing	Anti-tumor (257)
55	*Lobelia chinensis* Lour.	Ban bian lian	Antipyretic, anti-inflammatory, and antitoxic effects (258)
56	*Scutellaria barbata* Don.	Ban zhi lian	Anti-tumour and anti-bacterial (259)
57	*Sophora flavescens* Ait.	Ku shen	Antibacterial (260)
58	*Pinellia ternate* (Thunb.) Breit.	Ban xia	Antimicrobial and anti-inflammatory (248)

Stock solutions

A stock solution of 10mg/ml E. coli LPS (serotype O127:B8) in sterile PBS was used for cell assays with LPS. Concentrations of LPS in Dulbecco's modified Eagle's medium (DMEM) ranging from 0.1ug/ml to100μg/ml were used for the construction of a dose-dependent activation curve. A stock solution of 1000U/ml Murine IFN-γ (Lot# 10098) in DMEM was used to stimulate cells. A dose-dependent activation curve was constructed using concentrations of IFN-γ ranging from 0.1U/ml to

500U/ml.

Methods

Assays not mentioned

Assays not mentioned in this methods section (such as determination of the flavonoid and antioxidant content) are described the following publication: Ravipatiet al (2012) BMC Complementary and Alternative Medicine 12:173ff.

Preparation of the plant extract

Plant extracts were prepared and supplied by Anjaneya S. Ravipati, School of Natural Science, UWS. The plant materials were ground to a fine powder in a grinder and 3 g of each powdered plant material was taken and incubated in hot water at 121^0C for 1 h. Then transferred to centrifuge tubes and allowed to cool at room temperature. Then samples were centrifuged at 10,447 x *g* for 20 min), the supernatant was transferred into a 50mL volumetric flask adjusted the volume to 50 mL. The samples were stored at -20 ^{0}C until analysis.

Cell Maintenance

RAW 264.7 macrophages were individually grown in 175cm^2 flasks on DMEM containing 5% FBS respectively, supplemented with supplemented with penicillin (200 U/ml), streptomycin (200 µg/ml) and fungizone (2.6 µg/ml) and Glutamine (2 mM). The cell line was maintained in 5% CO_2 at 37°C, with media being replaced every 3-4 days.

Concentration of cells

Once cells had grown to confluence in the culture flask, they were removed using a rubber policeman, as opposed to using trypsin which can remove membrane-bound receptors such as RAGE.

The obtained cell suspension was concentrated by centrifugation for 3 minutes at 900rpm and resuspended in a small volume of fresh serum-free DMEM.

Cell count

Equal volumes of the resuspended cell solution and Trypan blue (0.1%) were mixed to give a total volume of 20µl. Half of this solution was placed onto a Neubuaer counting slide, which shows a large square divided into four smaller squares. Viewed under the microscope, the slide was used to calculate the number of cells per microliter of cell solution by counting cells in one of the small squares (equivalent to 0.1µl) and using the following equation - cell count x 2 (dilution factor for Trypan blue) x 10 (total dilution factor) = number of cells per microliter.

Activation of cells

For assays with extracts, 50µl volume of the dilutions (in media) were added an hour prior to addition of activator. Due to the often inconsistent nature of LPS at activating cells, a combination of 25µg/ml LPS and 10U/ml IFN-γ, both in DMEM, was used for activation. Usually a maximum dose of the extracts used was 2.5 mg/ml and a minimum of 6 doses made by serial dilution. Cells with media alone were used as negative control, while Cells with activator was used as a positive control. Then the cells were incubated for 24 hrs at 37^{o}C and 5% CO_2.

Determination of nitric oxide by Griess assay

Nitric oxide is determined by the Griess reagent quantification of nitrite, one of its stable reaction products. The Griess reagent is freshly made up of equal volumes of 1% sulfanilamide and 0.1% napthyethylene-diamine in 5% HCl, and in the presence of nitrite forms a violet colour. From

each well 80 µl of supernatant was transferred to a fresh 96-well plate and mixed with 80 µl of Griess reagent and after 30 minutes the colour produced was measured at 540 nm in a microplate reader (Bio-rad, Australia) and expressed as a percentage of that in control cells, after background fluorescence was subtracted. Remaining 20 µl of supernatent removed from each well was used for TNF-α assay using sandwich ELISA. Then the cell viabilty was assessed using Alamar Blue assay.

Determination of cell viability by Alamar Blue assay

Alamar Blue assay is a colorimetric assay involving the cellular reduction of resazurin to resorufin. Added 100ul of Alamar Blue solution (10 % Alamar Blue (Resazurin) in DMEM media) to each well and incubated at 37^0C for 1-2 hrs. After incubation, Fluorescence was measured with excitation at 530 nm and emission at 590 nm in a POLARstar Omega microplate reader (BMG Labtech, Mornington, Australia) and expressed as a percentage of that in control cells, after background fluorescence was subtracted.

TNF-α determination by ELISA

Capture antibody was diluted to 0.5µg/ml in PBS (1.9mM NaH_2PO_4, 8.1mM Na_2HPO_4, 154mM NaCl) (pH 7.4) and 100µl of diluted capture antibody was added to each well on a 96 well plate, sealed with parafilm and incubated overnight at room temperature. Then the plate was washed four times with wash buffer (0.05% Tween-20 in PBS). 250µl of block buffer (1% BSA in PBS) was added to each well, covered and incubated for 1 hour at room temperature. Then the plate was washed for 4 times, as before. Serial dilutions of TNF-α standard from 0 to 10000pg/ml in diluent (0.05% Tween-20, 0.1% BSA in PBS) were made. 50µl of cell supernatant or TNF-α standard in duplicate was added to each plate, covered and incubated for 2.5 hours. Then the plate was washed 4 times using wash buffer.Detection antibody was diluted to 0.125µg/ml in diluent and added 100µl of diluted detection

antibody to each well, covered and incubated for 2.5 hours. Then the plate was washed using wash buffer. Avidin Peroxidase conjugate was diluted in a ratio of 1:4000 in diluent and 100µl of diluted Avidin Peroxidase conjugate solution was added to each well, covered and incubated for 30 minutes. Then the plate was washed using wash buffer. 100µl of TMB Liquid Substrate Solution (0.5 mg TMB dissolved in 1 ml DMSO and made upto 10 ml using Phosphate-citrate buffer that has 30% H_2O_2 in concentration of 1 µl per 10 ml total volume) was added to each well. The colour development was monitored at 655nm, taking readings every 5 min. After 25 min the reaction was stopped using 0.5M Sulphuric acid and measured the absorbance at 455nm.

Data calculation and statistics

Two independent experiments were conducted with each experiment having samples in duplicates and dose-dependent curves were obtained for each read-out. Data calculations were done using MS-Excel 2007 & MS-Excel 2010 softwares and IC_{50} values were obtained by using the sigmoidal dose-response function in Graphpad Prism (v5.04). IC_{50} values from each experiment were averaged and standard deviation (SD) between these averages was calculated by using MS-Excel 2010. The results were expressed in mean ± standard deviation. Throughout our study, significant differences were assessed by one-way analysis of variance (ANOVA) with Dunnett's post hoc tests, unless otherwise mentioned. Correlation co-efficient was calculated by two tailed standard (Pearson) test using the GraphPad Prism (v5.04) and plotted using MS-Excel 2010.

2.3.Results

Screening for anti-inflammatory properties using RAW 264.7 macrophages

The plant extracts showed exhibited significant anti-inflammatory properties by inhibiting the production of NO and TNF-α in LPS-induced RAW 264.7 macrophages without affecting cell viability. The inhibitory activity of the extracts were expressed in IC_{50} values and presented in *Table 2.2*.

Table 2.2 Inhibitory activity of selected medicinal herbs against NO and TNF-α production and cell viability in the RAW 264.7 cell line

Sample no	IC_{50} for NO production inhibition (mg/ml) ± SD	Percentage Cell viability at IC_{50} (NO) ± SD	IC_{50} for TNF-α production inhibition (mg/ml) ± SD	Percentage Cell viability at IC_{50} (TNF-α) ± SD
1	0.43 ± 0.16	89.2 ± 8.2	2.46 ± 0.00	104.5 ± 6.4
2	0.12 ± 0.11	93.9 ± 6.6	2.38 ± 0.40	94.2 ± 4.0
3	0.63 ± 0.03	95.4 ± 5.7	1.17 ± 0.22	94.6 ± 5.7
4	0.07 ± 0.00	90.65 ± 9.3	0.42 ± 0.22	67.8 ± 0.6
5	1.06 ± 0.41	109 ± 12.7	>2.5	NA
6	0.09 ± 0.00	90.3 ± 5.7	0.64 ± 0.09	63.1 ± 3.1
7	0.10 ± 0.01	70.7 ± 9.8	0.92 ± 0.05	62.7 ± 1.6
8	0.05 ± 0.00	85.6 ± 0.0	0.70 ± 0.08	57.3 ± 3.1
9	0.04 ± 0.00	78.35 ± 6.2	0.18 ± 0.11	62 ± 2.5
10	0.05 ± 0.01	85.2 ± 6.6	0.10 ± 0.00	77.6 ± 7.2
11	0.20 ± 0.05	95.35 ± 4.6	0.90 ± 0.06	62 ± 7.6
12	0.30 ± 0.02	92.8 ± 1.0	1.07 ± 0.11	72.15 ± 0.5
13	0.10 ± 0.08	89.55 ± 13.8	0.67 ± 0.05	52.55 ± 12.8
14	1.05 ± 0.92	91.7 ± 11.7	>2.5	NA
15	0.35 ± 0.37	86.6 ± 19.0	2.39 ± 0.64	107.5 ± 10.6
16	0.12 ± 0.01	92.8 ± 5.1	0.50 ± 0.00	85.55 ± 13.4
17	0.06 ± 0.00	88.5 ± 0.0	0.85 ± 0.14	68.9 ± 3.1
18	0.04 ± 0.02	100.15 ± 1.2	0.38 ± 0.00	73.95 ± 2.1
19	1.49 ± 1.04	98.35 ± 3.7	>2.5	NA
20	0.27 ± 0.32	88.05 ± 16.9	2.31 ± 0.09	107.65 ± 11.8
21	0.12 ± 0.08	93.85 ± 8.7	1.23 ± 0.00	106.8 ± 11.6
22	0.13 ± 0.06	91.35 ± 6.2	0.30 ± 0.40	67.1 ± 1.6
23	0.36 ± 0.38	92.7 ± 13.2	2.35 ± 0.22	104.5 ± 6.4
24	0.58 ± 0.39	101.5 ± 0.7	1.59 ± 0.22	98.6 ± 2.0
25	0.33 ± 0.24	101 ± 1.4	>2.5	NA

26	0.14 ± 0.08	98.95 ± 1.5	2.3 ± 0.09	99.7 ± 0.5
27	0.05 ± 0.02	98.8 ± 1.7	0.45 ± 0.05	92.9 ± 11.5
28	0.05 ± 0.01	90.65 ± 2.1	0.36 ± 0.08	65.6 ± 2.5
29	0.43 ± 0.08	83.05 ± 3.6	1.79 ± 0.11	59.45 ± 2.1
30	0.25 ± 0.02	81.1 ± 8.0	1.79 ± 0.23	72.2 ± 1.5
31	0.05 ± 0.00	93.2 ± 6.6	0.21 ± 0.03	70.7 ± 0.6
32	0.05 ± 0.00	81.9 ± 0.0	0.10 ± 0.05	70.0 ± 8.7
33	0.11 ± 0.02	91.0 ± 8.8	0.40 ± 0.15	64.2 ± 1.6
34	2.50 ± 0.00	0.0 ± 0.0	1.24 ± 0.20	67.5 ± 2.1
35	0.34 ± 0.01	85.9 ± 9.8	>2.5	NA
36	0.62 ± 0.09	90.3 ± 10.7	>2.5	NA
37	0.46 ± 0.12	94.7 ± 0.5	>2.5	NA
38	0.04 ± 0.00	91.8 ± 4.6	0.18 ± 0.05	73.6 ± 0.6
39	0.07 ± 0.01	90.3 ± 1.6	0.73 ± 0.25	83.1 ± 4.6
40	0.05 ± 0.00	87.0 ± 0.0	0.14 ± 0.05	63.5 ± 7.7
41	0.07 ± 0.00	95.7 ± 0.0	0.80 ± 0.20	86.7 ± 0.5
42	1.09 ± 0.20	104.0 ± 5.7	>2.5	NA
43	0.15 ± 0.08	99.5 ± 2.2	0.58 ± 0.24	65.7 ± 3.6
44	0.06 ± 0.02	91.0 ± 2.5	0.18 ± 0.08	78.7 ± 0.5
45	0.12 ± 0.04	95.4 ± 2.6	0.55 ± 0.12	56.9 ± 5.7
46	0.14 ± 0.13	88.8 ± 15.8	0.30 ± 0.11	85.6 ± 20.4
47	0.23 ± 0.26	87.0 ± 9.2	>2.5	NA
48	0.49 ± 0.02	107.5 ± 10.6	1.68 ± 0.89	75.4 ± 7.2
49	0.43 ± 0.05	105.5 ± 7.8	1.63 ± 0.40	84.5 ± 19.0
50	0.03 ± 0.01	84.2 ± 5.2	0.07 ± 0.01	72.6 ± 2.1
51	0.06 ± 0.02	96.8 ± 1.6	0.20 ± 0.04	82.3 ± 15.8
52	0.42 ± 0.36	104.7 ± 7.6	>2.5	NA
53	0.09 ± 0.00	95.0 ± 0.0	0.17 ± 0.00	93.6 ± 1.1
54	0.24 ± 0.29	96.5 ± 1.1	>2.5	NA
55	0.36 ± 0.32	91.5 ± 13.5	2.44 ± 0.23	103.5 ± 4.9
56	0.05 ± 0.03	98.2 ± 2.5	0.60 ± 0.00	80.5 ± 20.5
57	0.13 ± 0.08	89.6 ± 6.7	0.08 ± 0.03	94.6 ± 6.7
58	1.05 ± 0.05	108.5 ± 9.2	>2.5	NA

Effect of plant extracts on LPS /IFN-γ induced NO production in RAW 264.7 cells

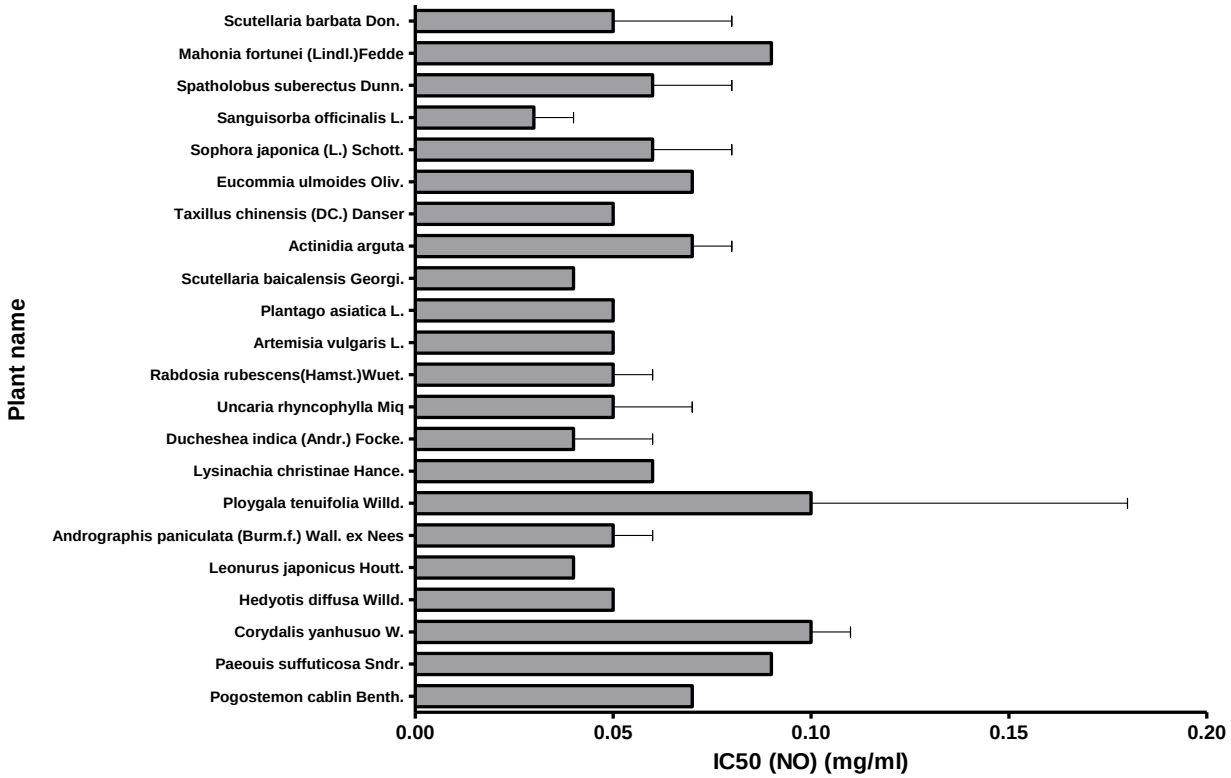


Figure 2.2: Concentration-dependent effect of potent plant extracts (with IC_{50} values less than or equal to 0.1 and CV >80%) in RAW264.7 macrophages, compared with activated cells as positive controls. Results are the mean of 2 independent experiments and error bars represent the SD.

All extracts, except *S. nigrum,* significantly suppressed NO production induced by LPS and IFN-γ in a dose-dependent manner and IC_{50} values were in the range of 0.04 to 1.49 mg/ml. The most potent inhibitory effect against NO production was shown by *S. officinalis L., L. japonicus* Houtt., *D. indica (Andr.)* Focke., *S. baicalensis Georgi., H. diffusa,* Willd., *A. paniculata (Burm.f.)* Wall. ex Nees,*U. rhyncophylla Miq, R. rubescens(Hamst.)*Wuet., *A. vulgaris L.,P. asiatica L.,T. chinensis (DC.)* Danser, *S. barbata Don., L. christinae* Hance., *S. japonica (L.)* Schott., *S. suberectus* ***Dunn****., P. cablin* Benth., *A. arguta, E. ulmoides Oliv., P. suffuticosa Sndr., M. fortunei (Lindl.)*Fedde, *C. yanhusuo W., and P. tenuifolia* Willd. with IC_{50} values less than or equal to 0.1 (Figure 2.2) and the cell viability of

these extracts was usually more than 80% at their IC_{50} concentration, which indicates their non-toxic nature on macrophage cells.

R. glutinosa (Gaertn.) Steud., *P. ternate (Thunb.)* Breit., *P. heterophylla (Miq.)* Pax ex Pax et Hoffm., *A. macrocephala* Koidz., *A. cochinchinensis (Lour.)* Merr. down regulated the NO production with IC_{50} values more than 1 mg/mL but to a maximum of 1.49 mg/ml. All the remaining extracts had IC_{50} values in between 0.1 and 1 mg/ml.

Effect of plant extracts on LPS /IFN-γ induced TNF-α production in RAW 264.7 cells

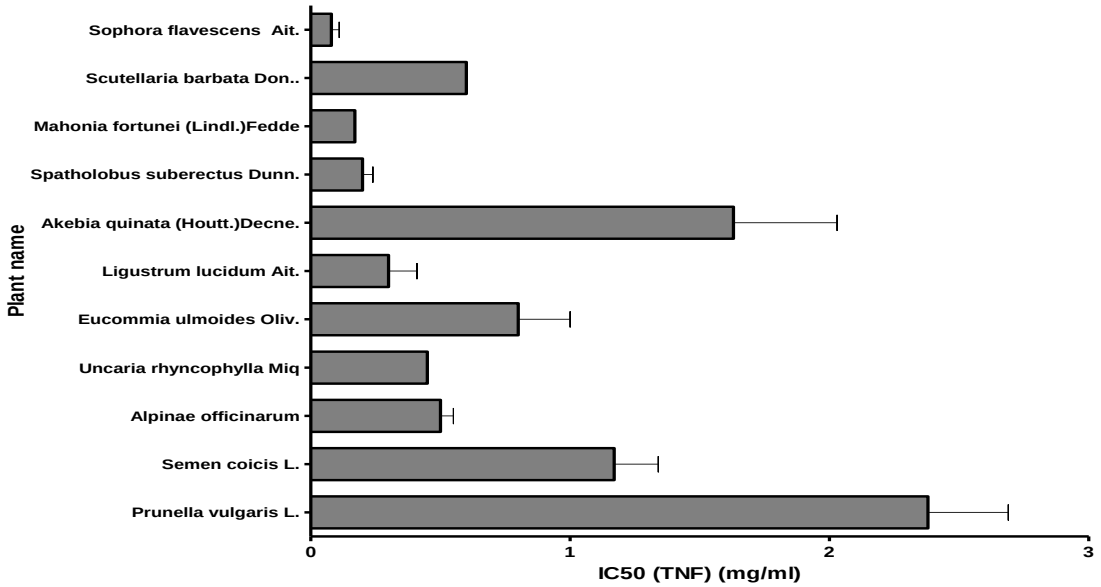


Figure 2.3: Concentration-dependent effect of potent plant extracts (with IC_{50} values less than or equal to 0.1 and CV >80%) in RAW264.7 macrophages, compared with activated cells as positive controls. Results are the mean of 2 independent experiments and error bars represent the SD.

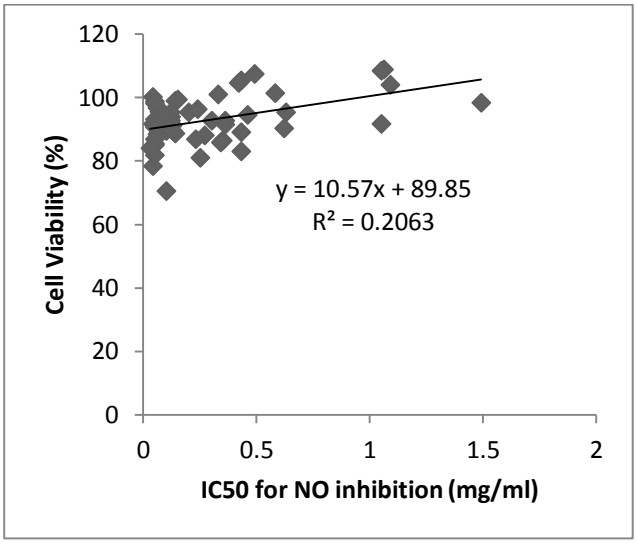
We also studied the effects of plant extracts on LPS and IFN-γ induced TNF-α production in RAW 264.7 cells. The inhibitory effect, expressed in the form of IC_{50} values, was in the range of 0.1 to 2.46 mg/ml. Considering cell viability of atleast 80% the effective inhibitory effect was shown by the following extacts: *S. chinensis (Turcz.)* Baill., *C. rotundus* L., *A. senticosus* Herms, *S. glabra* Roxb., *V.*

coloratum (Komar.) Nakai, *L. chinensis* Lour., *A. tataricus* L., *P. bulbocadioides (Franch.)* Rolfe., *S. flavescens* Ait., *S. coicis* L., *P. vulgaris* L., *M. fortunei (Lindl.)*Fedde, *U. rhyncophylla* Miq, *E. ulmoides* Oliv, *L. lucidum* Ait., *A. officinarum, A. quinata (Houtt.)*Decne., *A. arguta, S. suberectus* Dunn., *S. barbata* Don. Among them, *S. flavescens* Ait., *S. coicis* L., *P. vulgaris* L., *M. fortunei (Lindl.)*Fedde, *U. rhyncophylla Miq, E. ulmoides* Oliv., *L. lucidum Ait., Alpinae officinarum, A. quinata (Houtt.)*Decne., *A. arguta, S. suberectus* Dunn., *S. barbata* Don. showed significant inhibition of TNF-α production with IC_{50} values below 0.1 mg/ml concentration (Table 2.2).

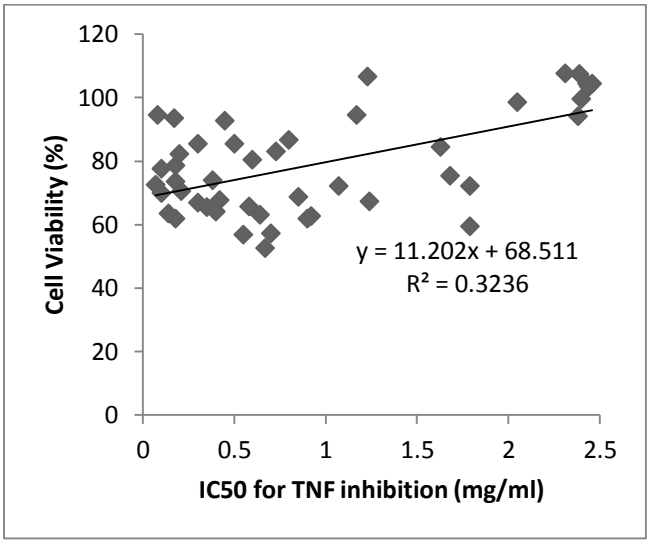
As presented in the *Table 2.2* most of the plants showed cell viability > 80% against the inhibition of NO production, while it ranged from 50 to 94.55% against the inhibition of TNF-α secretion. There was no TNF-α inhibition activity found with *P. heterophylla, R. glutinosa, A. cochinchinensis, T. farfara, P. cocos, Codonopsis pilosula, C. aromatic, Atractylodes macrocephala, P. aviculare, S. divaricate, C. paniculatum* and *Pinellia ternate* that required IC_{50} value more than 2.5 mg/ml concentration.

When considering inhibition of both NO and NO and TNF-α production, *S. flavescens Ait., S. coicis L., P. vulgaris L., M. fortunei (Lindl.)*Fedde, *U. rhyncophylla* Miq, *E. ulmoides* Oliv., *L. lucidum* Ait., *R. Alpinae officinarum, A. quinata (Houtt.)*Decne., *A. arguta, S. suberectus* Dunn., *S. barbata* Don. showed activity without affecting cell viability (>80% at corresponding IC_{50} values).

Correlation studies



A

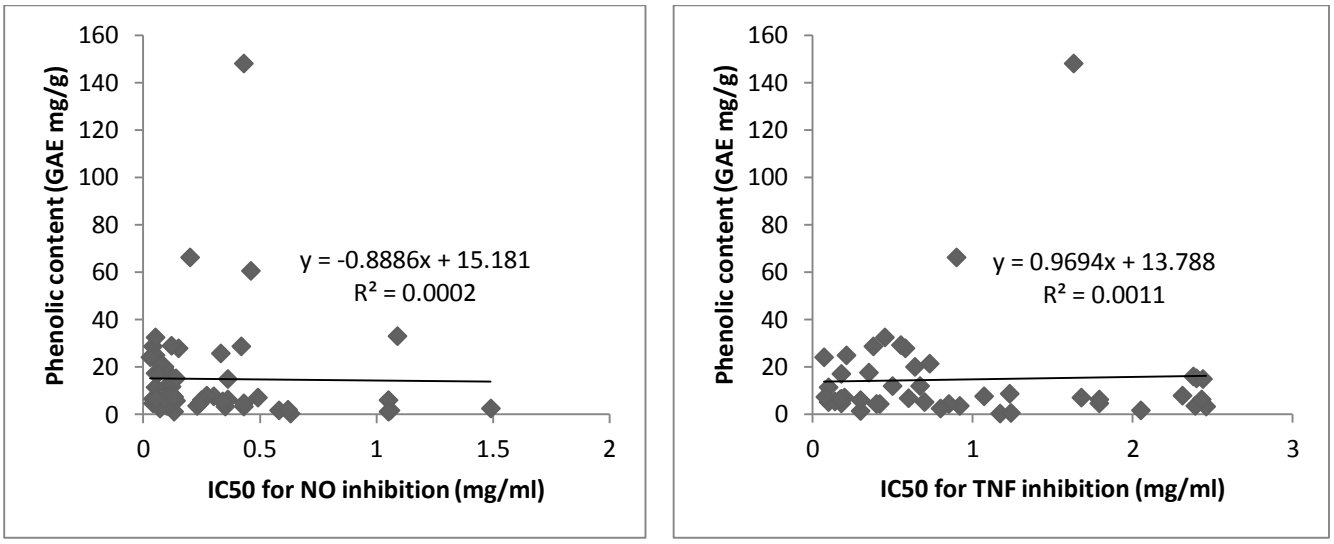


B

Figure 2.4: Correlation of cell viability with NO (R^2 = 0.2063) and TNF-α (R^2 = 0.3236) release was determined by using GraphPad prism (V5.04).

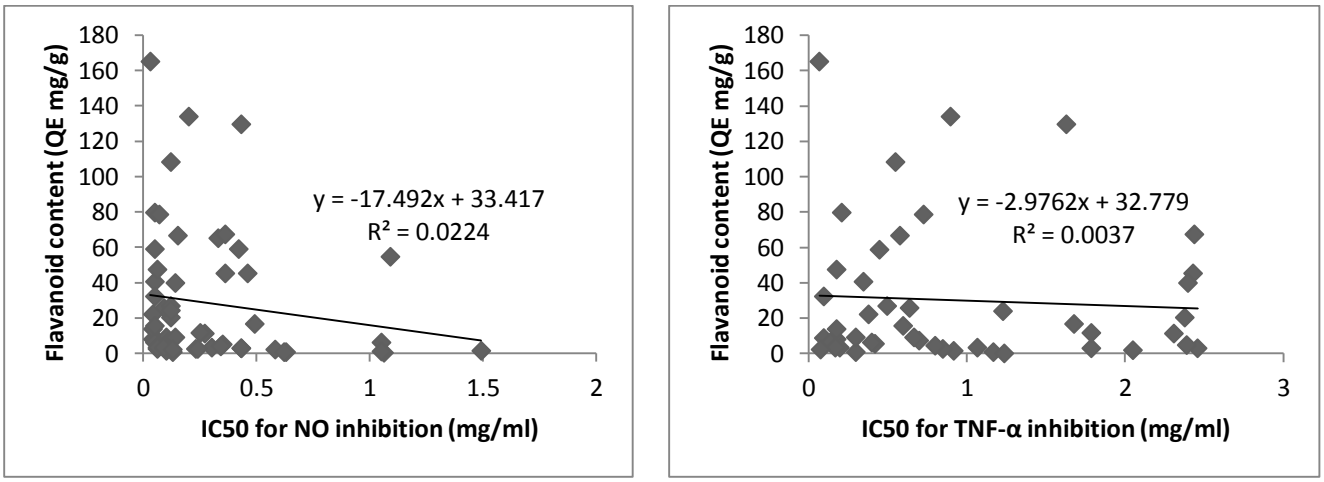
There was no significant correlation for cell viability with anti-inflammatory potency, measured as inhibition of NO (R^2 = 0.2063) and TNF-α production, respectively, (R^2 = 0.3236) as shown in Figure 2.4.

The anti-inflammatory properties were also compared to the anti-oxidant data from the collaboraters at UWS. Phenolic content flavonoid content, DPPH-scavenging activity and percentage yeast oxidative stress inhibition of these plant extracts were determined and correlated with the IC_{50} values of NO and TNF-α.



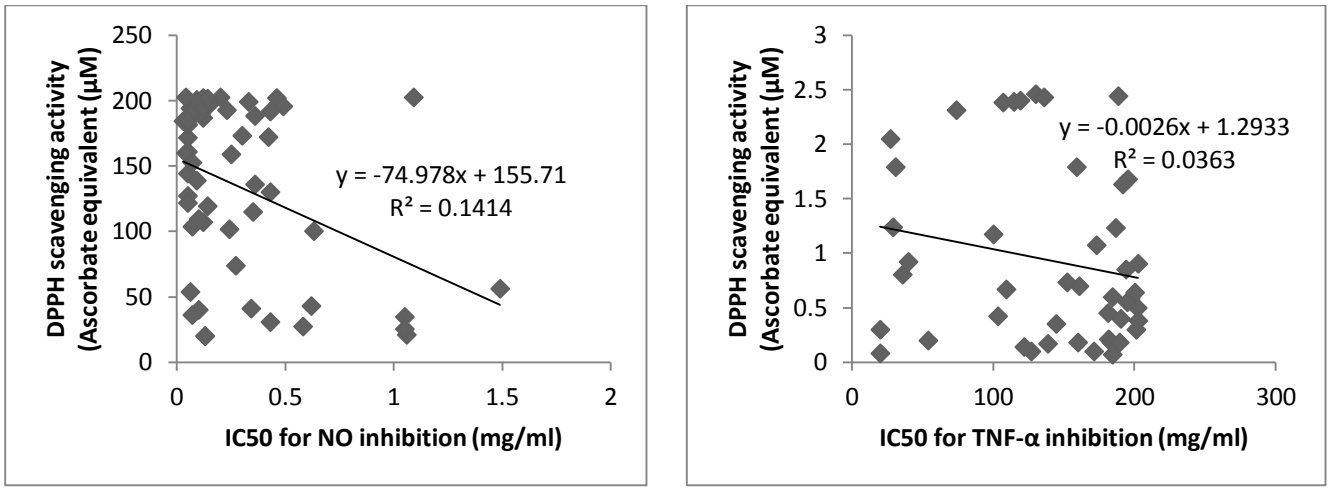
A B

Figure 2.5: Correlation of phenolic content with NO (R^2 = 0.0002) and TNF-α (R^2 = 0.0011) release was determined by using GraphPad prism (V5.04).



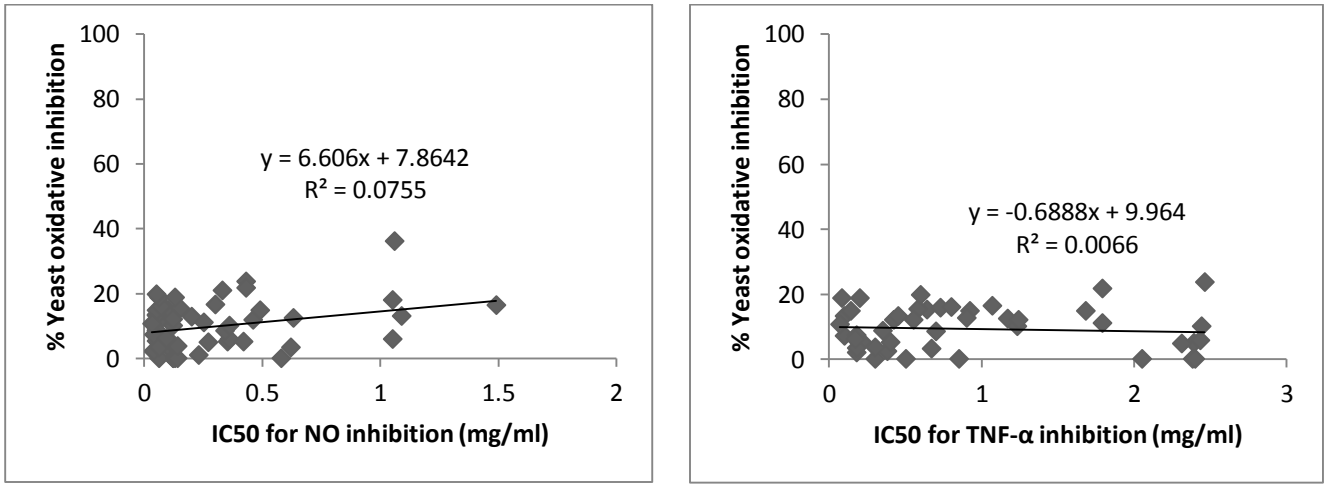
A B

Figure 2.6: Correlation of flavanoid content with NO (R^2 = 0.0224) and TNF-α (R^2 = 0.0037) release was determined by using GraphPad prism (V5.04).



A B

Figure 2.7: Correlation of DPPH-scavenging activity with NO (R^2 = 0.1414) and TNF-α (R^2 = 0.0363) release was determined by using GraphPad prism (V5.04).). Significance of correlation was checked by standard (Pearson) test with two tailed *P* values and 95% confidence interval (p <0.005).



A B

Figure 2.8: Correlation of yeast oxidative inhibition with NO (R^2 = 0.0755) and TNF-α (R^2 = 0.0066) release was determined by using GraphPad prism (V5.04).). Significance of correlation was checked by standard (Pearson) test with two tailed *P* values and 95% confidence interval (p <0.005).

Correlation of anti-inflammatory effects of the plant extracts against their phenolic and flavonoid content has shown to be statistically insignificant (p > 0.005). TNF-α inhibition was found to be insignificant statistically (p > 0.005) when correlated with DPPH-scavenging activity and yeast

oxidative inhibition. The was no evidence, that NO inhibition was significantly correlated to DPPH-scavenging activity ($R^2 = 0.1414$) and yeast oxidative inhibition ($R^2 = 0.07548$).

2.4 Discussion

After screening 58 Chinese medicinal plants, we have identified that all water extracts, except *S.nigrum* possessed potential anti-inflammatory properties. In detail, *S. flavescens* Ait., *S. coicis* L., *P. vulgaris* L., *M. fortunei (Lindl.)*Fedde, *U. rhyncophylla* Miq, *E. ulmoides* Oliv., *L. lucidum* Ait., *A. officinarum, A. quinata (Houtt.)*Decne., *A. arguta, S. suberectus* Dunn., *S. barbata* Don. inhibited both NO and TNF-α production with very low toxicity.

This study coincides with previously evidences of anti-inflammatory activity of *P. vulgaris* L. (212), *U. rhyncophylla* Miq (233) and A. quinata (Houtt.) Decne (249). Among these *P. vulgaris* L. (212) and *U. rhyncophylla* Miq (233) were also known to possess antioxidant activity. Anti-diabetic medicinal plant *S. coicis* L. (213), also had shown strong anti-inflammatory properties in our study .

S. flavescens Ait. (260), *M. fortunei* (Lindl.)Fedde (256), *E. ulmoides* Oliv. (246) and *S. barbata* Don (259) were known for their anti-microbial properties. *E. ulmoides* Oliv. (246) and *S. barbata* Don (259) also were shown to have anti-tumour effects. Neuroprotective properties of *E. ulmoides* Oliv also was known previously (246). *A. arguta* was also previously shown to possess anti-tumour effects and anti-allergic effects (244). *A. officinarum* was reported to have beneficial effects against gastric ailments (224). We had shown that they do possess anti-inflammatory properties in addition to all these properties.

These extracts were found be the most potent and could be promising candidates against inflammatory diseases. This study has established the anti-inflammatory properties of the whole herbal extracts rather than the individual constituents, therefore further systematic investigations are needed to characterise the bioactive compounds of these plant extracts. If bioactive properties can be verified in suiTable animal models and ultimately in humans, these plant extracts might represent useful dietary (or pharmaceutical) tools for use against inflammatory diseases including AD.

In addition, the findings from our correlation studies show that phenolics and flavonoids from these medicinal herbs might not be essential for the anti-inflammatory activities. This could imply that antioxidant activity might not be directly relevant for a herbs' potential to downregulate NO and TNF-α release.

CHAPTER 3

ANTI-INFLAMMATORY PROPERTIES OF WOOD EAR (*AURICULARIA POLYTRICHA*), OYSTER (*PLEUROTUS OSTREATUS*), HONEY BROWN (*Armillaria mellea*), WHITE BUTTON (*AGARICUS BISPORUS*), ENOKI (*FLAMMULINA VELUTIPES*)AND SHIITAKE (*LENTINUS EDODES*) MUSHROOMS

3.1 Introduction

The cerebral cortex, basal forebrain and hippocampal regions of the brains of AD patients exhibit numerous pathological hallmarks, including amyloid plaques, neurofibrillary tangles (NFTs), elevated levels of advanced glycation endproducts (AGEs) and their receptor (RAGE) (114). Amyloid plaques are surrounded by activated microglia and astrocytes, which drive the inflammatory response involving production of free radicals such as nitric oxide and superoxide as well as pro-apoptotic cytokines such as TNF-α. Amyloid plaques containing $A\beta_{42}$ have also been shown to activate micro- and astroglia. And increased levels of radicals and pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α) can be observed in microglial or astroglial cells adjacent to plaques (261). In cell culture experiments, fibrillar Aβ induces the expression of various cytokines and chemokines such as TNF-α or MCP-1 (262,263). A further study, using rat astrocyte cultures, suggested that $A\beta_{42}$ oligomers induced a strong early inflammatory response, whereas fibrillar Aβ induced a weaker pro-inflammatory response, consistent with a more chronic form of inflammation. IL-1, IL-6, TNF-α MIP-1α and MCP-1 increase in a dose-dependent manner after cultured microglia are incubated with Aβ (264–269). The pathology of AD clearly involves an inflammatory component (270), and a large body of epidemiological evidence indicates the use of a subset of NSAIDs is associated with reduced risk for AD (142). The putative target of NSAIDs actions is thought to be microglia associated with the senile plaques (271). Patients receiving long term NSAIDs therapy exhibit a 65% reduction in plaque-associated reactive microglia (144).

A range of dietary mushrooms have been shown to exhibit anti-inflammatory bioactivity by anti-oxidant and other mechanisms and some varieties have been assessed for neuroprotection in relation to cognitive function, with capacity for brain tissue regeneration shown for ‘Lions Mane’

mushrooms (272), however, potential protective relationship between mushrooms and cognitive health has not been fully explored.

In this study, we have evaluated the potential anti-inflammatory and neuroprotective properties of a selection of mushrooms by using *in vitro* assays modelling inhibition of RAGE receptor-mediated cellular inflammation. A generic food-compatible method for processing dietary plants was applied to a selection of mushrooms including: Honey Brown *(Armillaria mellea)*, Enoki (*Flammulina velutipes)* Wood Ear (*Auricularia polytricha*), White Button (*Agaricus bisporus*), Shiitake (*Lentinus edodes*) and Oyster mushrooms (*Pleurotus ostreatus*). Processing, which involved heating, physical and chemical treatments to disperse solids including ultrasonication, was intended to select for process-stable or process-modified products and also investigate the stability of bioactive species to processing conditions. The bioactivities detected thereby reflected process-stable components, concentrated approximately 10-fold by freeze-drying, and were systematically compared with unprocessed controls.

3.2 Materials and Methods

Materials

Mushrooms included in the study (White Button, Honey Brown (also called swiss brown), Wood Ear, Enoki, Shiitake and Oyster) and wheat-based cornflour (Home Brand) were obtained from retail suppliers in Melbourne, Australia. Regarding white button and honey brown mushrooms, both are taxonomically similar and called *Agaricus bisporus.* A young specimen with a closed cap and either pale white or light brown flesh is known as a button mushroom or white mushroom. In strains with darker flesh, the immature mushroom is variously marketed as a crimini mushroom, baby portobello, baby bella, mini bella, portabellini, Roman mushroom, Italian mushroom, or brown mushroom. L-ergothioneneine, Vitamin D2, iodo-acetic acid, mushroom polyphenol oxidsase (tyrosinase, EC1.14.18.1), EDTA, asorbic acid, lipopolysaccharide and phytohemeagglutinin were obtained from Sigma Aldrich (St Louis, Missouri USA). Clarase G Plus, Mannanase and Fungal Lipase 8000 were obtained from Enzyme Solutions (Victoria, Australia). Tetra sodium pyrophosphate was obtained from BDH, England); 1 methanol from Merck KGaA (Darmstadt, Germany); ethanol from CSR Distilleries (Victoria, Australia), sodium phosphate from Univar (NSW, Australia), Flavourzyme (EC 3.4.11.1; 1000L), and porcine pancreatic Trypsin (EC 3.4.21.4; Novo 6.0 S, Saltfree), were obtained from Novozymes (Bagsvaerd, Denmark).

Methods

Mushroom Processing

Sample processing methods designated unprocessed, Stage 1 and Stage 2 processing (273) were applied to mushrooms and the processing was done at CSIRO, Werribee. 'Unprocessed' forms of mushrooms were prepared by washing, drying and chopping fresh mushrooms and freeze drying. All dried samples were stored with desiccant at -18^{0}C and ground by mortar and pestle to fine powder

before analysis. Stage 1 and 2 processing involved heating (ie, 'cooking'), mechanical dispersion and treatments intended to solubilise both hydrophobic and hydrophilic solutes. Successive stages of processing (Stage 1, Stage 2) provided for progressive enrichment of bioactivity, and each represented a scalable, food regulation-compliant processing method, according to Australian standards, in terms of use of additives and technologies.

'Stage 1-processed' mushrooms were prepared by washing the raw, edible mushroom components, blending in a food processor with water (1:2 ratio w/v) before boiling by microwave heating (9000 W, 10 min). After cooling to room temperature, ascorbic acid (0.1% of solids) and ethanol (1% of solids) were added for anti-microbial stabilisation. Samples were ultrasonicated using a 400 W probe at 100% power for 2 minutes (Hielscher 400UPS, Hielscher, Germany) before freeze drying.

'Stage 2-processed' mushrooms were prepared by reconstituting the Stage 1 product at 2% total solids in de-ionised water and stirring for 2 hours. After addition of the following processing aids: wheat-based cornflour (0.01% w/v); Clarase G Plus (0.001% w/v); Fungal Lipase (0.01% w/v); tetra sodium pyrophosphate (0.01% w/v), the pH was adjusted to 5.5 using 1.0 M NaOH or 1.0 M HCl and samples were incubated at 45° C for 17 hours with agitation (Grant OLS 200 shaking waterbath at 100 rpm, Grant, England). Enzymes were subsequently inactivated by heating to 90°C for 30 min then cooled to room temperature before homogenisation for 2 min (T25 probe, Ultra-turrax, IKA, Germany). The sample was filtered (150 μM sieve) and the filtrate centrifuged (16900x *g* for 10 min at 20°C, Sorvall Centrifuge, SLA 3000 rotor 1 (Sorvall, Sweden). The supernatant was homogenised by passes through an in-house homogeniser (adapted from Milkscan, Foss, Denmark) before freeze drying.

Screening for bio-activity using RAW 264.7 macrophages

The samples were tested on RAW 264.7 macrophages, for the LPS and IFN-γ induced NO and TNF-α downregulating activity by employing a method which was described in Chapter 2. Usually the maximum dose of the extracts used was 2.5 mg/ml and a minimum of 6 concentrations were prepared by serial dilution. Apigenin is included in the assay as a reference standard. Two independent experiments were conducted with each experiment having samples in duplicates and dose-dependent curves were obtained for each read-out. IC_{50} values were obtained by obtained by using the sigmoidal dose-response function in Graphpad Prism (v5.01 – v5.04). IC_{50} values from each experiment were averaged and SD between these averages was calculated. The results were expressed in mean ± SD.

3.3 Results

Anti-inflammatory properties of mushrooms

Positive anti-inflammatory activity was identified in the murine macrophage assay by suppression of both NO and TNF-α in the absence of significant loss of cell toxicity (i.e., <20% at the IC_{50}), after taking into account contributions from reagent controls. Treatment with the positive control apigenin reduced both NO and TNF-α secretion with IC_{50} values of 0.042 mg/ml and 0.071 mg/ml, respectively (Table 3.1). L-ergothioneine also decreased NO secretion (IC_{50}: 0.109 mg/ml) but had a weak effect on TNF-a sectretion (IC_{50}: >2.5 mg.ml, Table 3.1). Of the 6 mushroom types tested, anti-inflammatory activity was detected in various Shiitake, Oyster, Wood Ear, Swiss Brown and White Button mushroom products (Table 3.1), with Oyster and Shiitake the focus of further studies.

Table 3.1. Summary of IC_{50} values for reference compounds and selected mushroom products exhibiting significant attenuation of NO and TNF-α, without evidence of cell toxicity, in RAW 264.7 macrophages.

Sample	Product Type	IC_{50} for NO production inhibition (mg/ml) ± SD	IC_{50} for TNF-α production inhibition (mg/ml) ± SD
Apigenin	none	0.04 ± 0.01	0.07 ± 0.00
L-ergothioneine	none	0.11 ± 0.01	>2.5
Wood Ear	Stage 1	0.15 ± 0.00	1.02 ± 0.05
	Stage 2	0.98 ± 0.25	2.23 ± 0.38
Oyster	Stage 1	0.26 ± 0.01	0.2 ± 0.11
	Stage 2	0.32 ± 0.35	0.30 ± 0.04
Honey brown	Stage 1	1.07 ± 0.22	>2.5
	Stage 2	1.04 ± 0.15	>2.5
Button	Stage 1	1.5 ± 0	>2.5
	Stage 2	1.25 ± 0.26	>2.5
Enoki	Stage 1	0.09 ± 0.07	>2.5
	Stage 2	0.75 ± 0.90	>2.5
Shiitake	Unprocessed	0.02 ± 0.00	0.02 ± 0.01
	Stage 1	0.10 ± 0.05	1.34 ± 0.16
	Stage 2	0.17 ± 0.11	2.11 ± 0.56

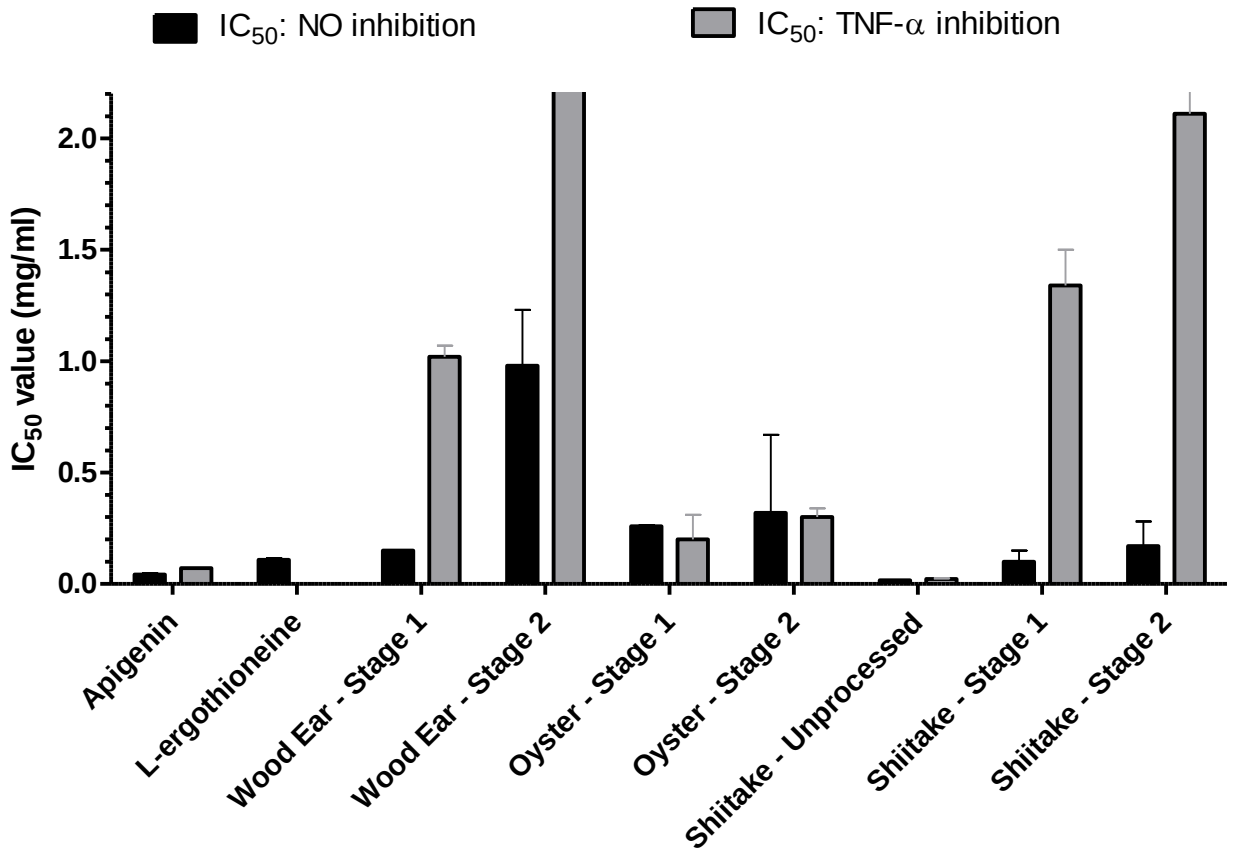


Figure 3.1. Comparison of IC_{50} values for reference compounds and selected mushroom products exhibiting significant attenuation of NO and TNF-α, without evidence of cell toxicity, in RAW264.7 macrophages, showing the mean of 2 independent experiments and error bars representing SD.

Shiitake mushrooms exhibited dose dependent suppression of both NO and TNF-α throughout the processing stages but the activity was found to be gradually reduced through the stages (Figure 3.1). (Table 3.1). This suggested that bioactive components in Shiitake mushroom may be unstable to heat or ultrasonics treatments, and also that bioactives responsible for NO and TNF-α suppression, were chemically distinct.

Similar effects were found with Oyster and Wood ear mushrooms. Honey brown, Button and Enoki mushrooms had shown NO suppression but no TNF-α suppression. Anti-inflammatory activity

of Stage 2 mushroom products was generally lower than or similar to Stage 1 products except for Honey brown (Table 3.1), suggesting that loss of activity was associated with Stage 1 processing conditions.

Figure a

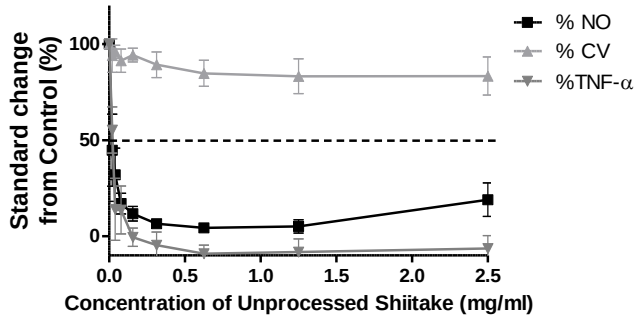


Figure b

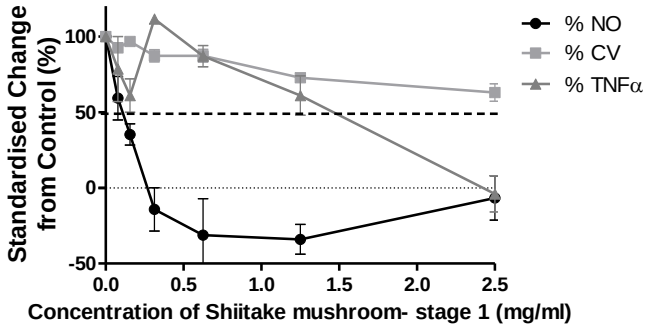


Figure c

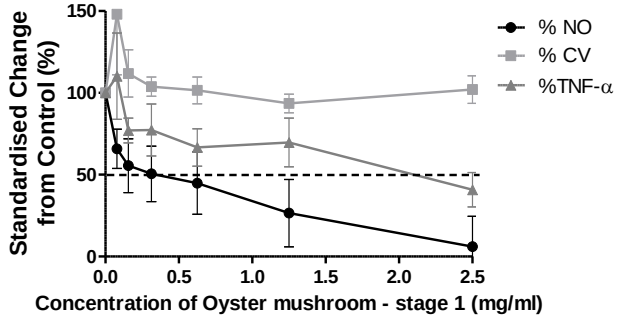


Figure d

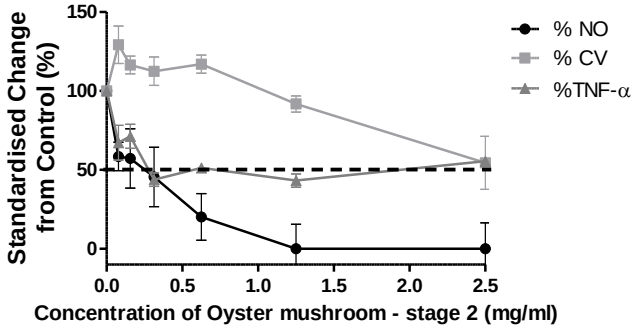


Figure 3.2. Concentration-dependent effects of mushrooms on inflammatory markers (NO and TNF-α production) as well as cell viability from RAW264.7 macrophages. Data show % change compared to activated cells (positive control) in the presence of (a) unprocessed, dried Shiitake, (b) Shiitake Stage 1, (c) Oyster Stage 1 products and (d) Oyster Stage 2 products showing the mean of 2 independent experiments and error bars representing SD ($p<0.001$).

Stage 1 and 2 Oyster mushroom products strongly reduced both TNF-α release (IC_{50}=0.20 mg/ml) in murine macrophages and lowered NO release (IC_{50}=0.259 mg/ml). Other mushroom products generally reduced NO strongly with moderate or no supression of TNF-α release and without cytotoxicity (Table 3.1).

3.4 Discussion

In general, edible mushroom consumption is associated with health benefits. In traditional Oriental therapies, mushrooms have a long history of medicinal use. Putative benefits of dried Yamabushitake mushroom (*Hericium Erinaceus*) has been reported in the treatment of mild cognitively-impaired 50-80 year-old Japanese men and women , which improved cognitive function reverting after cessation of treatment (274). Yamabushitake mushroom was also promoted expression of nerve growth factor in human astrocytoma cells via the JNK pathway (275), its extracts stimulated neurite outgrowth in NG108-15 cells (276) and prevented impairments of spatial short-term and visual recognition memory induced by Aβ(25-35) peptide in mice (272). Functional Independence Measure was improved in dementia patients by chemically-derivitised phospholipids from Yamabushitake due to protection against Aβ-specific toxicity (277). Thus, consumption of mushrooms and specific extracts has been linked to several possible modes of protection against dementia and possibly AD.

Button mushroom had shown blood glucose and cholesterol lowering ability in diabetic and hypercholesterolemic rats (278). *Lentinus edodes*, commonly called Shiitake mushroom is the second most popular and the third widely cultivated edible mushroom in the world (279). Several important compounds including bioactive polysaccharides (lentinan), dietary fiber, ergosterol, vitamin B1, B2 and C and minerals have been isolated from the fruiting body, mycelia, and culture medium of this mushroom. Many recent studies have shown its pharmacological attributes including antitumor, antimicrobial, liver function improving and cholesterol lowering activity (280–283). Active Hexose Correlated Compound (AHCC) is an α-glucan rich compound isolated from Shiitake (284) produced an anti-cancer effect in 269 human hepatocellular carcinoma patients (285), in 1 patient case study (286) and in 44 hepatocellular carcinoma patients (287). AHCC's immunomodulatory functions observed in mouse models (288,289) are also reproduced in a double-blind, placebo-controlled trial of 21 people

(281). AHCC administered by gavage reduced the bacterial load in the mice (290) and various other infectious agents (291). In addition to anti-microbial effects Shiitake can reduce platelet aggregation (292) and lowered cholesterol due to the presence of a compound known as eritadenine (2(R),3(R)-dihydroxy-4-(9-adenyl)-butyric acid) (293). Lentinan, a (1-3)-beta-β-glucan extracted from the mushroom L. edodes, is a potent immunostimulatory drug, and licensed in Japan for antitumor therapy. Credited to such property, the Korea Food & Drug Administration approved on January 2000 that the extracts of the mycelium of Shiitake mushrooms can protect and help recover the liver from substances such as alcohol. The immunomodulatory effects of lentinan range from enhanced host resistance to bacterial, fungal, viral or parasitic infections to antitumor effects (294–296). Shiitake mushrooms possess anti-bacterial properties (297–299) and anti-viral properties (300,301) including anti-HIV (Human Immunodeficiency Virus) (302–304) and anti-HSV-1 (Herpes Simplex Virus -1) (305). Shiitake mushrooms naturally contain anti-viral agents known as proteinase inhibitors (306). *In vivo*, orally administered AHCC increased resistance to pathogens such as influenza virus (307) and west nile encephalitis (283).

Bioactivity of *Hericium Erinaceum* was attenuated in the oven-dried product (308). Also variability in biological potency of extracts was related to the source and process history of the medicinal mushroom *Agaricus blazei* (309). Such phenomena of process instability of bioactive factors also coincided with our study in addition to the precedent for neuroprotective bioactivity of mushrooms.

L-ergothioneine was present at 0.2 to 2.6 mg/g dry weight across a range of cultivated mushrooms including White button, Shiitake and Oyster (310) as studied here. The mushroom metabolite, L-ergothioneine is a naturally occurring anti-oxidant in plants and animals, that cannot be synthesised in humans and levels in human tissues reflect dietary intake. L-ergothioneine together with Vitamins C and E, provided *in vivo* anti-oxidant protection, through metal chelation, scavenging of reactive oxygen species (310) and protection against lipid peroxidation (311),. This is also implicated in our study that L-ergothioneine had shown NO suppression by itself. It had shown no significant TNF-α suppression in the assays indicating that it might not play a role in the pro-inflammatory cytokine suppressing properties.

Despite the reports of exhibiting anti-oxidant activity in FRAP and DPPH assays (312), Wood Ear mushroom has not been previously associated with neuroprotection, which might be related to the process instability of bioactive factors. In addition, polysaccharide fractions present in an aqueous extract of Wood Ear exhibited anti-inflammatory activity in animal studies (313). The chemical composition of an aqueous extract from Wood Ear was total sugars, 42.5%; uronic acids, 19.6%; sulphate, 15.8%; nitrogen, 1.7% and ash, 20.3% (314). Hispidin, an analogue of resveratrol, found in the fruit and culture broth of the medicinal mushroom *Phellinus Linteus* has been reported to inhibit BACE1 (315) and might account for the activity observed in Wood Ear. In a study with LPS-induced RAW 264.7 macrophages, a new immunomodulatory protein was purified from the fruiting body of *Auricularia polytricha* enhanced the production of both NO and TNF-α (316). Another study with polysaccharides fractions from the same mushroom had also shown similar results of NO and TNF-α enhancement in RAW 264.7 macrophages (317). These results directly contradicted our observation where both NO and TNF-α were suppressed.

An extract of Oyster mushroom (*P. ostreatus*) has been demonstrated to protect against carbon tetrachloride-induced oxidative stress in rats i*n vivo* via anti-oxidant mechanism (Jayakumar, Sakthivel, Thomas, & Geraldine, 2008). Phytochemical analyses of *P. ostreatus* extracts revealed low to moderate levels of terpenoids, tannins, steroidal glycosides and carbohydrates and possessed antimicrobial and antioxidant potentials (318). Oyster mushroom concentrate (OMC) suppressed LPS-induced secretion of TNF-α, IL-6 and IL-12p40, in RAW264.7 macrophages and also suppressed PGE2 and NO by down-regulation of COX-2 and iNOS expression, respectively. OMC also inhibited LPS-dependent DNA-binding activity of AP-1 and NF-κB in RAW264.7 cells (319). Orally administered OMC markedly suppressed secretion of TNF-α and IL-6 in LPS challenged mice, *in vivo* (319). Anti-inflammatory activity of OMC was confirmed in concanavalin A (ConA)-stimulated mouse splenocytes by the inhibition of proliferation and secretion of interferon-γ (IFN-γ), IL-2, and IL-6 (319). Our study coincides with these observations.

Enokitake mushrooms (*Flammulina velutipes*) possess antioxidants like ergothioneine (320,321). Honey brown mushrooms (*Armillaria mellea)* also possess antioxidant and antimicrobial properties. Button mushroom (*Agaricus bisporus*) possesses high levels of ergosterol so that it could produce ergocalciferol by even brief exposure to UV light (322). Although they did not suppress TNF-α in our study, these three mushrooms were demonstrated to suppress NO in cells for the first time.

Anti-inflammatory activity of Shiitake mushroom extracts is not well studied in biological systems despite the promising results in chemical studies. A number of isolated mushroom constituents have been shown to modulate immunity. The water-soluble lignin in LEM (the extract of the solid culture medium of *Lentinus edodes* mycelia) has been known to have antiviral and immunopotentiating activities in vivo and in vitro. JLS-18, the water-soluble lignin rich fraction was prepared from LEM

using ultrafiltration and hydrophobic column chromatography, showed about 70 times higher antiviral activity than LEM *in vitro*. JLS-18 activated the cytotoxicity of NK cells of C3H mouse (male, 3 weeks old) and peritoneal macrophages of an ICR mouse (male, 7 weeks old), and activated T cells *in vitro*. JLS-18 also induced interleukin 6 (IL-6) secretion from human leukocytes infected with Sendai virus *in vitro*. These data showed that JLS-18, the water-soluble rich fraction of LEM, had antiviral and immunopotentiating activities (300).

In vitro studies shown that Shiitake extract co-stimulated with either lipopolysacharide or ovalbumin (OVA) induced TNF-α in the RAW 264.7 macrophages and OVA specific T cells, respectively. In bone marrow derived macrophages it slightly increased IL-10 by itself but inhibited IL10 when treated along with LPS. The same studies also indicated that stimulation with extracts alone had no effect, LPS stimulation alone induced IL-1β and extracts plus LPS increased the secretion of pro-inflammatory cytokine, IL-1β. The extracts mediated inhibition of IL-10 and enhancement of pro-inflammatory IFN-γ in Splenocytes from OT II mice. While *in vitro* data show that whole mushroom extracts regulate cytokine production by macrophage and T cell production boosting anti-tumor immunity and pro-inflammatory functions of edible mushrooms, it was harder to detect such phenomena *in vivo* (323). AHCC is a well-tolerated compound that possess antioxidant activity (324), and is metabolized via the CYP450 2D6 pathway (325).

Various publications describe the strong positive influence of glucans on the immune system comprising antibacterial, wound-healing and antitumour activities while antioxidant activity of β-glucan has been reported recently (326,327). Recent investigations have shown that Lentinan, a (1-3)-beta-β-glucan extracted from the mushroom L. edodes, possesses antioxidant activity (328).

The polysaccharides extracted from L. edodes were able to decrease the level of serum NO_2^-, serum total cholesterol (TC), triglyceride (TG), low density lipoprotein cholesterol (LDL-c) in animals fed high-fat-diet, as well as enhanced the level of serum high density lipoprotein cholesterol (HDL-c) (329). The same study also discovered that polysaccharides from L. edodes might not only improve oxidative injury induced by free radicals and increase antioxidant enzyme activity, but also decreased the plasma endothelin (ET) levels. Oral administration of polysaccharides from L. edodes shown to increase antioxidant enzyme activity and improve blood lipid levels in rats and inhibit the oxidative injury induced by accumulating free radicals caused by high-fat-diet to a certain extent (329).

Dose dependent antioxidant activity of methanol and water crude extracts from Shiitake mushroom was demonstrated with chemical assays investigating antioxidant capacity in three different assays, namely, the β-carotene and linoleic acid system, 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity, and inhibition of hemolysis of rat erythrocyte induced by peroxyl radicals (330). Stronger inhibition of lipid oxidation has occured at higher concentrations of the extracts in most cases. Moreover, the water extract of L. edodes was found to possess the strongest antioxidant activities among the four extracts. In general, a correlation between higher antioxidant activity and larger amount of total phenolics was found in the mushroom extracts. Though other antioxidants were probably present in these mushroom extracts, phenolic compounds could make a significant contribution to the antioxidant activity in these extracts (330). Another chemical study had shown that heat treated Shiitake mushroom may have increased health beneficial effects associated with the increase of antioxidant activities (331). The results from these chemical assays were in line with our biological assay showing NO suppression and these assays validated scavenging of free radicals generated during lipid peroxidation as one possible mechanism of the antioxidant activity of the mushroom extracts, e.g. by peroxy radicals, possibly through hydrogen-donating capacity.

It is possible that L-ergothioneine in its anti-oxidant capacity contributed to the anti-inflammatory effects of Shiitake and other mushrooms by suppression of NO levels (Table 3.2), as was observed for RAW 264.7 cells stimulated by the fruit of *Ganoderma lucidum* (332). Superior anti-oxidant activity was found in the aqueous extract which was associated with the phenolics [68]. In lung epithelial cells, L-ergothioneine exerted antioxidant and anti-inflammatory effect by inhibiting both hydrogen peroxide and TNF-α-mediated activation of the NF-κB inflammation cascade (333). However, anti-inflammatory activity of Shiitake tested in the DSS model of colitis in mice was inconclusive (323).

After screening 6 mushroom types, Shiitake and Oyster mushrooms were identified as the most potent anti-inflammatorymushroom species. In general, fresh mushrooms exhibited significantly higher bioactivity than processed mushrooms, highlighting the instability to processing of bioactive factors. Nevertheless, effects of processing can inform the development of functional food ingredients from mushrooms. Indeed, processing may be required to resolve positive and negative inhibitory factors. If bioactive properties can be verified in suitable animal models and ultimately in humans, then these mushroom products could be used as dietary (or pharmaceutical) tools against inflammatory diseases including AD.

CHAPTER 4

SCREENING OF CSIRO PLANT AND FOOD LIBRARY FOR SELECTING A LEAD WITH ANTI-INFLAMMATORY PROPERTIES

4.1 Introduction

Nutrition offers promising perspectives for the prevention of AD. Several cohort studies have recorded dietary behaviour and then documented cognitive decline and incidence of dementia through repeated neuropsychological testing over many years of follow-up. Evidences are increasing for a protective role of antioxidants, homocysteine-related vitamins (vitamin B12 and folate) and n-3 poly-unsaturated fatty acids (PUFA) against AD. Curcumin, a tumeric-derived antioxidant with anti-inflammatory capabilities, that is believed to be responsible for the low prevalence of AD in India (334). Natural antioxidants are found in plants, fruits, and vegetables and could be contributing to better health by balanced diet and several epidemiological studies have evidenced the lowered risk cognitive decline or dementia in association with higher consumption of vegetables. In addition to food, natural antioxidants are commonly available as complementary medicines, with examples being fruit-derived flavonoids, vitamins C and E, Ginkgo biloba, α-lipoic acid and β-carotene. The term 'antioxidant' covers a broad range of substances. The common action of these substances is the scavenging of extracellular and, depending on their membrane-permeability, intracellular free radicals including reactive oxygen (ROS) and reactive nitrogen species (RNS) (335). Understanding the molecular basis of the effects of food on cognition will help us to determine how best to manipulate diet in order to increase the resistance of neurons to insults and promote mental fitness.

Several steps in the AD inflammatory pathway involve the production of free radicals such as superoxide and nitric oxide (336,337). The production of these radicals may play a more dominant role in the progression of AD than previously conceived (338). ROS and RNS have several detrimental effects, including DNA and cell membrane damage, mitochondrial inhibition and the activation of redox-sensitive transcription factors such as NFκ-B, effects that all exacerbate the excessive inflammation in AD (339). Antioxidants could play an important role in preventing the age-related

disorders such as cancer, cardiovascular diseases, diabetes, inflammation, degenerative diseases, anaemia, and ischemia, as there is a growing evidence that reactive oxygen species (ROS) such as superoxide anion ($O_2^{\cdot -1}$, hydroxyl radicals, singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2) play an important role (340).

Currently there is an increase of interest in phytochemicals as potential sources of natural antioxidants with low cytotoxicity, which are candidates for the prevention of oxidative damage. Recent studies showed that a number of medicinal and dietary plant extracts containing polyphenols, flavonoids, and other compounds exhibited significant antioxidant activities (204). Several studies carried out on antioxidants showed their anti-inflammatory, anti-atherosclerotic, anti-mutagenic, anti-carcinogenic, anti-bacterial activities along with other potential health promoting properties (193). The neuroprotective properties of several antioxidants including vitamins E and C (341,342), α-lipoic acid (343) and Gingko biloba (344) might be due to the result of free radical scavenging. Results from our studies with Chinese medicinal plants (Chapter 2) and mushrooms (Chapter 3), suggesting that although radical scavenging may provide some neuroprotection, effective prevention of AD requires secondary mechanisms of antioxidants. Some antioxidants are known to possess side-effects, supporting the involvement of secondary factors in AD prevention. One of the effective antioxidants, α-Lipoic acid inhibits degradation of the inhibitor of NFκ-B (IκB) and also directly inhibits NFκ-B in a DNA-binding-dependant manner (345,346). Like α-Lipoic acid Curcumin also inhibited NFκ-B in DNA binding and IκB-dependant mechanisms, but has several other beneficial characteristics, such as enhancing glutathione production (347) and direct *in vitro* inhibition of Aβ aggregation and plaque formation (348). Curcumin is also an agonist of PPARs (349,350).

Varieties of healthcare remedies used in many parts of the world have a common beginning from wild plants. Aspirin, for instance, owes its origin to the willow tree. Hippocrates used powder extracted from willow bark to treat pain and reduce fever in the 5th century BC. Over 2000 years later, the chemical compound salicin was isolated from willow bark. The new drug, formally acetylsalicylic acid, Aspirin by Bayer AG™ was named after the old botanical name for meadowsweet, Spiraea ulmaria. Plants remain the source of new compounds which constitute the base for new pharmaceutical products and a major component of the burgeoning markets for herbal health care remedies and natural therapeutic products. A plant is said to be medicinal when "at least one part possesses therapeutic agent" (351). Several new therapeutic agents from plant materials have been derived like reserpine, deserpidine, rescinnamine, vinblastine and vincristine. Among the therapeutic agents currently used for the treatment of certain types of cancer, the most important drugs are plant based eg., vincristine and vinblastine which both are isolated from *Catharanthus roseus* (Winka rosea). From just 94 species of plants that are used globally as drugs, about 122 componds were identified of defined structure and demonstrated that 80% of these have had an ethnomedical use identical or related to the current use of the active elements of the plant (352). The first tranquiliser, reserpine, came from the Indian plant Rawolfia serpentine used in Ayurvedic medicine for mental disturbances. Plants being a very important source of drugs many plant species have been screened. To mention some, digoxin from foxglove used to treat heart failure, paclixatel from yew leaves is a promising anticancer agent and Bromelain from *Ananas comosus (L.) Merrill* is used therapeutically as an anti-inflammatory agent.

Drug development research conducted for the three to four decades has shown that plant materials are the potential sources of novel molecules (353). Often new drugs have originated from natural sources. Of about 250000 higher plant species in the world just less than 10% have been screened for biological activity (354) indicates the need to screen more for new drug development. The

aim of this study was to screen dietary processed plant foods (n = 238) provided by CSIRO, in order to evaluate anti-inflammatory activities and select potential lead compounds to proceed for bioactive identification. In this study, a generic food-compatible method for processing was applied to a library of plant and food samples (273). Processing, which involved heating, physical and chemical treatments to disperse solids including ultrasonication, was intended to select for process-stable or process-modified products and also investigate the stability of bioactive species to processing conditions.

4.2 Materials and Methods

Materials

Plant and food samples in the library included in the study are representative of a typical healthy Australian diet and were obtained from retail suppliers in Melbourne, Australia. EDTA, asorbic acid, lipopolysaccharide were obtained from Sigma-Aldrich (St Louis, Missouri USA); methanol from Merck KGaA (Darmstadt, Germany); ethanol from CSR Distilleries (Victoria, Australia), sodium phosphate from Univar (NSW, Australia), DMSO, sodium nitrate, hydrogen peroxide, 95% ethanol, Bovine serum albumin, Lipopolysaccharide (LPS) (*E.coli* serotype 0127:B8), N-(1-1-napthyl) ethylenediamine dihydrochloride, Penicillin G Sodium Benzyl, Resazurin Sodium 10%, Streptomycin, Sulfanilamide, Tetra methyl benzidine (TMB), Trypan blue 0.4% were purchased from Sigma-Aldrich (Castle Hill, Australia). Antibiotics, Dulbecco's modified Eagle's medium (DMEM), Foetal bovine serum (FBS) and Glutamine were purchased from GIBCO (Mulgrave, Australia). Interferon-γ (murine) and TNF-α – ELISA kits were purchased from Peprotech (NJ USA).

Methods

Sample Processing

Sample processing methods designated unprocessed, Stage 1 and Stage 2 processing were applied to plants at Food Science Australia, CSIRO as described in Chapter 3. Samplese were eluted with and without glucose, whereas the addition of a sugar in the process allows for the generation and selection of component susceptible to sugar mediated reactions, for example through the Maillard reaction, or by caramelization. Typically, glucose is added to the aqueous medium to a final concentration of 1% glucose.

Screening for anti-inflammatory properties using N11 microglia and RAW 264.7 macrophages

The samples were tested in two sets, one on N11 microglia and another on RAW 264.7 macrophages, both for the LPS and IFN-γ induced NO and TNF-α suppression by employing a method which was described in Chapter 2. Those which were active were then reprocessed and tested for concordant activity in RAW 264.7 macrophages. Usually the maximum dose of the extracts used was 2.5 mg/ml and a minimum of 6 doses made by serial dilution. Two independent experiments were conducted with each experiment having samples in duplicates and dose-dependent curves were obtained for each read-out. IC_{50} values were obtained by sigmoidal dose-response function in Graphpad Prism (v5.01 – v5.04). IC_{50} values from each experiment were averaged and SD between these averages was calculated. The results were expressed in mean ± SD.

4.3 Results

Lead selection process

The samples that had shown the LPS and IFN-γ induced NO and TNF-α downregulating activity on both N11 microglia and RAW 264.7 macrophages, were then reprocessed and tested for concordant activity on RAW 264.7 macrophages. The most potent sample in both the assays and without cytotoxicity was selected as the lead compound for further analysis and the whole selection process is as shown below as a flow chart (Figure 4.1).

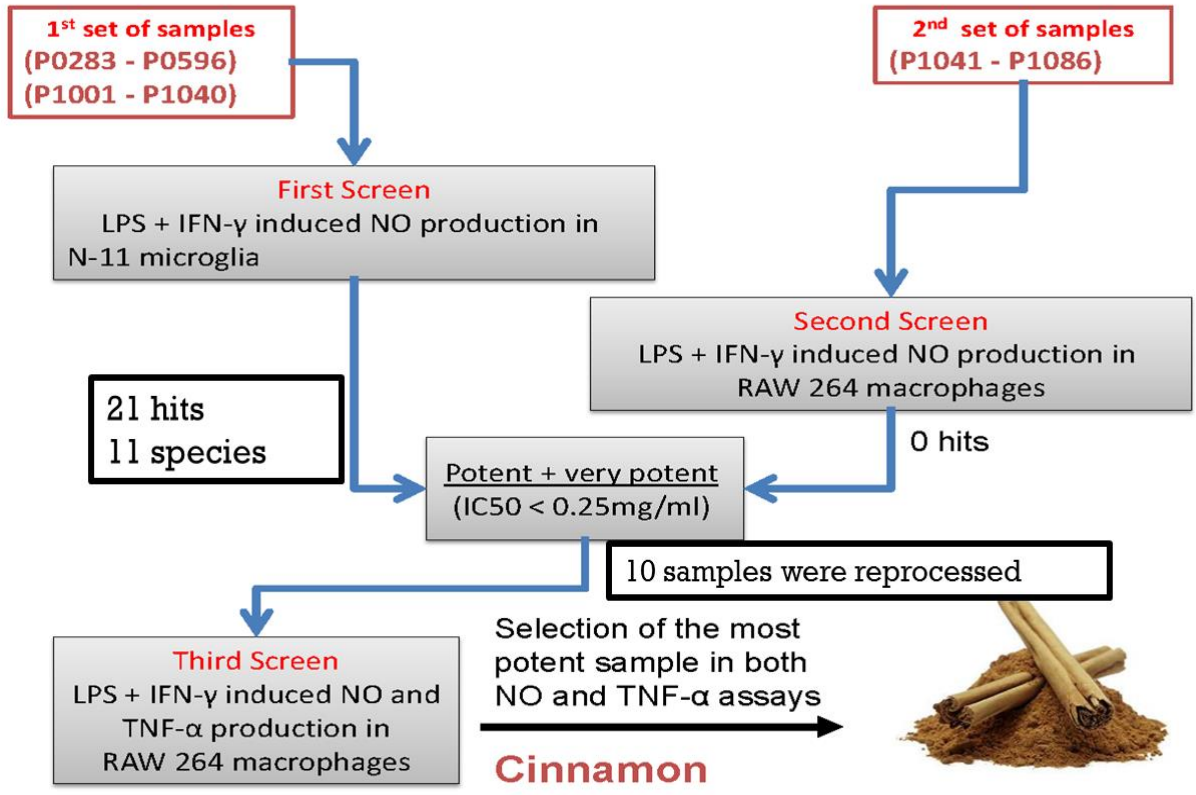


Figure 4.1 Lead selection process based on the anti-inflammatory properties exhibited on murine microglia and murine macrophages

Primary selection of active samples from the library using microglia

238 samples from the CSIRO library (Appendix 1) were screened for the LPS and IFN-γ induced NO down-regulating property in N11 microglia using a method explained in Chapter 2. The testing was done in two independent experiments with samples in duplicates. The active species was categorized as most potent and potent based on the IC_{50} values.

Table 4.1: Very potent (IC_{50} < 0.1mg/ml) anti-inflammatory samples tested in N11 microglia

Sample No	**Eluted in**	**Sample**	**IC_{50} for NO production inhibition (mg/ml) ± SD**	**Percentage Cell viability at IC_{50} (NO) ± SD**
P0347	mQ	onion	0.087 ± 0.03	99 ± 5.6
P0348	Glucose	onion	0.075 ± 0.01	101 ± 0
P0349	mQ	oregano	0.066 ± 0.02	96 ± 2.1
P0350	Glucose	oregano	0.083 ± 0.03	96 ± 2.8
P0351	mQ	oyster mushroom	0.47 ± 0.58	96 ± 5.6
P0352	Glucose	oyster mushroom	0.27 ± 0.30	98 ± 8.4
P0353	mQ	red sweet potato	0.067 ± 0.02	95 ± 7.1
P0354	Glucose	red sweet potato	0.054 ± 0	90 ± 0

Among the 238 samples, three plant species namely onion, oregano, and red sweet potato and one mushroom variety, Oyster were shown to be very potent (IC_{50} < 0.1mg/ml) and non-toxic (cell viability > 80% at IC_{50} for NO) in the primary screen (Table 4.1). These four species were shown to exhibit NO suppression both in water and glucose elutes.

Table 4.2: Potent (IC_{50}: 0.1 - 0.25 mg/ml) anti-inflammatory samples tested in N11 microglia

Sample No	Eluted in	Sample	IC_{50} for NO production inhibition (mg/ml) ± SD	Percentage Cell viability at IC_{50} (NO) ± SD
P0560	Glucose	Lime Zest	0.13 ± 0.04	93.00 ± 9.64
P0563	mQ	English Breakfast Tea leaves	0.24 ± 0.13	94.33 ± 2.89
P0564	Glucose	English Breakfast Tea leaves	0.30 ± 0.21	91.67 ± 2.08
P0571	mQ	Honey Brown mushroom	0.16 ± 0.13	106.50 ± 12.02
P0572	Glucose	Honey Brown mushroom	0.11 ± 0.04	104.00 ± 14.14
P0573	mQ	Button Mushroom	0.14 ± 0.01	105.00 ± 15.56
P0574	Glucose	Button Mushroom	0.35 ± 0.00	124.00 ± 0.00
P0583	mQ	cinnamon	0.21 ± 0.10	88.50 ± 19.62
P0584	Glucose	cinnamon	0.29 ± 0.34	89.17 ± 14.60
P0587	mQ	cloves	0.28 ± 0.30	83.00 ± 5.20
P0588	Glucose	cloves	0.15 ± 0.01	95.27 ± 9.19
P0595	mQ	White Zucchini	1.62 ± 1.31	115.33 ± 2.08
P0596	Glucose	White Zucchini	1.31 ± 1.08	113.67 ± 4.73

Another **7** species including 5 plants namely Lime zest, English breakfast tea leaves, cinnamon, cloves and white zucchini and two mushrooms namely honey brown and button, were shown to be potent (IC_{50}: 0.1 - 0.25 mg/ml) and non-toxic (cell viability > 80% at IC_{50} for NO) in the primary screen (Table 4.2). Except lime zest, all these samples inhibited NO irrespective of their elution method. Altogether, 21 samples were progressed into the next round of testing for NO and TNF-α suppression in murine macrophages.

Secondary screening of active samples from screen 1 using RAW macrophages

The potent and very potent samples from the primary screen, based on down-regulation of NO production, were re-tested for anti-inflammatory activity in a second cell line (RAW 264.7 macrophages) (Figure 4.1). Altogether, 10 samples were selected for reprocessing (Chapter 3) and used in the final screen.

Table 4.3: Reprocessed samples tested in RAW 264.7 macrophages

Samples No	Sample	IC_{50} for NO production inhibition (mg/ml) ± SD	Percentage Cell viability at IC_{50} (NO)	IC_{50} for TNF-α production inhibition (mg/ml) ± SD
RP0349FO	oregano	0.04 ± 0.01	121.0 ± 0.01	>2.5
RP0351FO	oyster mushroom	0.07 ± 0.01	115.0 ± 0.01	0.30 0.02
RP0353FO	red sweet potato	>2.5	NA	0.50 ± 0.01
RP0559FO	Lime Zest	0.47 ± 0.04	107.0 ± 5.73	0.83 ± 0.00
RP0571FO	Honey Brown mushroom	0.84 ± 0.01	123.0 ± 9.19	1.25 ± 0.04
RP0573FO	Button Mushroom	0.83 ± 0.00	102.0 ± 2.10	0.31 ± 0.01
RP0583FO	cinnamon	0.05 ± 0.00	86.0 ± 4.20	0.43 ± 0.00
RP0587FO	cloves	0.05 ± 0.01	79.5	>2.5
RP0595FO	White Zucchini	>2.5	NA	0.33 ± 0.00
RP1043FO	Madura Premium blend tea	0.05 ± 0.00	113.0 ± 0.01	>2.5

The individual IC_{50} value for the reprocessed samples and their dose-response curves are shown in Table 4.3 and Figure 4.2. Oregano, cloves and Madura premium blend tea were shown to possess NO suppressing property but with no TNF-α suppression. White zucchini and red sweet potato behaved exactly opposite to this and had shown TNF-α suppression but no NO suppression in RAW macrophages. Lime zest, Honey brown and button mushrooms and cinnamon had shown increased cell viability in the reprocessed samples (Figure 4.2).

Among the 10 samples shown in Table 4.5, cinnamon was chosen for bioactivity guided-fractionation (Chapter 5) as the most promising candidate since it not only showed both anti-NO (P0583: IC_{50} = 0.2087 mg/ml at 89% CV; P0584: IC_{50} = 0.2889 mg/ml at 89% CV) and anti-TNF-α activity at low concentrations and with low cell toxicity, but the activity was exhibited consistently (reprocessed sample IC_{50}: NO=0.050 mg/ml at 86.0% CV, TNF-α=0.433 mg/ml) throughout the processing stages (Chapter 3).

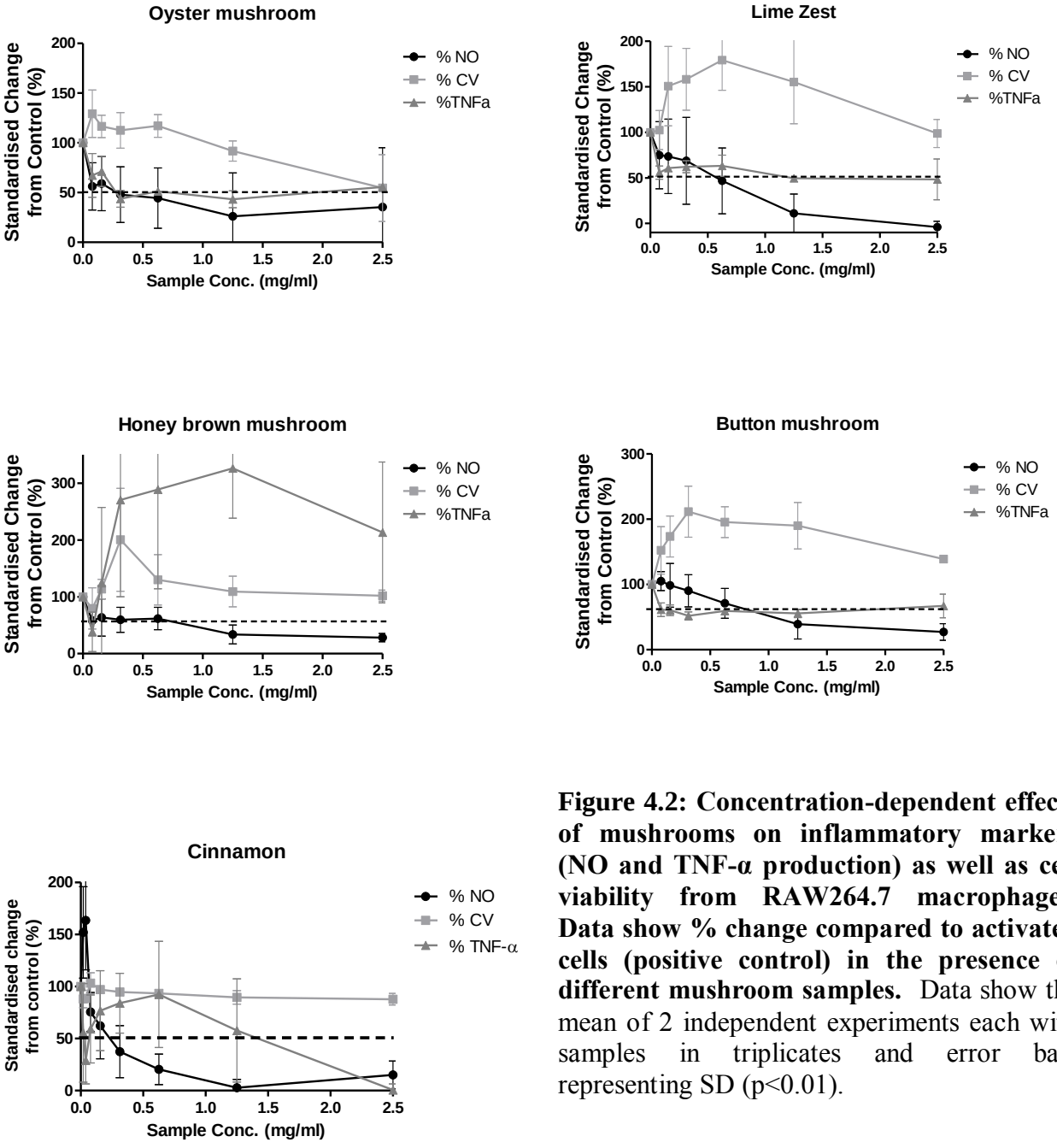


Figure 4.2: Concentration-dependent effects of mushrooms on inflammatory markers (NO and TNF-α production) as well as cell viability from RAW264.7 macrophages. Data show % change compared to activated cells (positive control) in the presence of different mushroom samples. Data show the mean of 2 independent experiments each with samples in triplicates and error bars representing SD ($p<0.01$).

4.4 Discussion

High throughput screening of plants from a large library of plant and food was made possible by employing *in vitro* bio-assays to test for the anti-inflammatory properties. Many plants were shown to be active against NO and TNF-α in murine microglia and murine macrophages. Commonly the selected plant and mushroom extracts decreased NO dose-dependently. Interestingly, with extracts such as Lime zest, honey brown and button mushroom we have observed increase in cell viability at initial doses (at a range of 1 to 1.25 mg/ml) followed by a gradual decrease. TNF-α was increased with some samples e.g., honey brown mushroom. While it is sensible to assume that the increase in cell viability would be leading to increased production of TNF-α, it is not always the case. With button mushroom cell viability is increased but TNF-α is decreased. It could be due to the improvement in cell viability due to lowered levels of TNF-α, which is an important cytokine that plays a role in apoptosis. One of the possible explanations could be enhanced proliferation of cells due to the extracts. However, the exact mechanism of such inconsistent behavior couldn't be predicted with these assays.

Considering the stability of activity during processing stages, activity on both the cell lines and suppression of both NO and TNF-α without cytotoxicity, cinnamon was selected as the lead candidate for further chemical analysis and bioactivity-guided fractionation. A variety of cinnamon species were extensively studied for the anti-diabetic, anti-microbial, anti-cancer and anti-arthritis properties. Despite the fact that cinnamon has been demonstrated to have anti-oxidant properties, there is not much evidence in support of anti-inflammatory properties. However, we suspect that the components of cinnamon are not only bioactive in relation to anti-oxidant properties, and hence we are investigating their anti-inflammatory properties. For achieving this, we needed to identify the active components of cinnamon by chemical analytical methods and to test them on anti-inflammatory *in vitro* bioassays.

CHAPTER 5

IDENTIFYING ACTIVE ANTI-INFLAMMATORY INGREDIENTS FROM *CINNAMOMUM ZEYLANICUM* (*CINNAMOMUM VERUM*)

5.1 Introduction

In traditional oriental therapies, cinnamon has a long history of medicinal use. Cinnamon has been used in Asia not only as a traditional food but also as a herbal medicine; hence it is likely that that cinnamon contains bioactive components. A variety of cinnamon species were extensively studied for their anti-diabetic and anti-microbial properties. There are studies of relevance to cinnamon's anti-cancer and anti-arthritis properties as well. Most of the work on cinnamon has focused on its antioxidant activity (355). For example, it was suggested that regular consumption of tea made from the bark of *C. zeylanicum* (100mg/300ml: daily) could be beneficial to oxidative stress related illness in humans (356).

However, research into the anti-inflammatory properties of cinnamon is limited. Studies on anti-inflammatory properties have been directed toward the species *Cinnamomum osmophloem* kaneh (357,358), belonging to the family Lauraceae, a tree that commonly grows naturally in the forests of Taiwan. This species is chosen generally for analysis because the chemical constituents of its oil are similar to those of Cinnamomum cassia bark oil (357), which is the common additive to foods and beverages termed 'cinnamon'. However, there is limited published research about a possible anti-inflammatory activity of the 'true' cinnamon of India, *Cinnamomum zeylanicum* (*C.zeylanicum*).

Therefore, the aim of this study was to examine *C.zeylanicum* for anti-inflammatory activity and to try to isolate and analyse of its active anti-inflammatory constituents.

5.2 Materials and Methods

Materials

Dried powder of the species *C.zeylanicum* was supplied by l, Bennett, CSIRO Food Science, Werribee. The sample P0583 was made from *C.zeylanicum* sticks and the samples P1105 was made from ground *C.zeylanicum* powder. DMSO, 95% ethanol, bovine serum albumin, lipopolysaccharide (LPS) (*E.coli* serotype 0127:B8), EDTA, N-(1-1-napthyl) ethylenediamine dihydrochloride, penicillin G sodium benzyl, resazurin sodium 10%, streptomycin, sulfanilamide, tetra methyl benzidine (TMB), trypan blue 0.4%, benzyl benzoate, phenacetin, furfural, imipramine hydrochloride, cinnamaldehyde, p-cymene, β-caryophyllene were purchased from Sigma-aldrich, Australia. Antibiotics, Dulbecco's modified Eagle's medium (DMEM), foetal bovine serum (FBS) and glutamine were purchased from GIBCO. IFN-γ (murine) and TNF-α – ELISA kits were purchased from Peprotech. Diatomaceous Earth was purchased from Dionex.

Methods

Preparation of the C.zeylanicum sample for HPLC purification

The cinnamon sample P1105 (prepared in a similar manner to the first tested cinnamon sample RP0583) was suspended in 1% ethanol (99% water), vortexed for 2 min and allowed to stand at room temperature overnight. Prior to HPLC analyses, insoluble precipitates were removed by filtration (using a 0.45 µm syringe filter) or by centrifugation (14000 rpm, 5 min).

HPLC fractionation of C.zeylanicum sample

The general principle of bioactivity guided purification assumes that the active constituents of *C.zeylanicum* are contained in defined fractions and the active constituents of these fractions could be

identified using mass spectroscopy and NMR. For optimization purpose, the initial HPLC fractionation of the selected lead, RP0583 from the CSIRO library, was performed using a 150 x 4.6 mm Phenomenex Luna C18 reversed phase column. Automatic injection was carried out at a flow rate of 1 ml/min and a linear gradient elution was performed with methanol ranging from 50 to 100% in an aqueous solution of 2 g/l formic acid at a flow rate of 1.0 ml/min for 30 min. Using a PDA detector, the spectrum between 200 and 400 nm was recorded during the chromatographic run.

The cinnamon sample P1105 (prepared in a similar manner to the first tested cinnamon sample RP0583) was suspended in 1% ethanol, vortexed for 2 min and allowed to stand at room temperature overnight. Then two different sample were prepared for HPLC analyses, one undergoing filtration (0.45 µm sringe filter) and another centrifugation (14000 rpm, 5 min) of the supernatant. Automatic fractionation of samples was carried out at the same conditions mentioned before using C18 column and collected at 2 min blocks. In turn 7 samples for filtered sample and another 7 for centrifuged sample were collected and concentrated to 1 ml by drying using nitrogen. These fractions were tested for bio-activity (down-regulation of LPS and IFN-γ induced NO and TNF-α production) on RAW 264.7 macrophages as mentioned before. Cytotoxicity of the fractions was also studied simultaneously.

Solvent extraction of C.zeylanicum - sequential extraction with multiple solvents

Extractions were performed on a Dionex Accelerated Solvent Extractor 350 at a temperature using 5 min static time and 2 cycles. Temperatures and heating duration were varied according to the solvent of extraction as suggested by the instructions of the Dionex Accelerated Solvent Extractor. DCM extract was heated at 100° C for 5 min. EA extract was heated at 100° C for 5 min. Ethanol extract was heated at 120° C for 6 min. Methanol extract was heated at 120° C for 6 min. Water extract was heated at 180° C for 9 min.

A 250 mg sample of *C.zeylanicum* powder was used in a 4:1 ratio with diatomaceous earth and was extracted sequentially in the following solvents of increasing polarity: DCM, EA, ethanol, methanol and water. The volume of the extracts was then reduced to 2 mL using a rotary evaporator. One-in-two dilutions were made of the concentrated extracts. A further one-in-two dilution was made in serial of the ethanol extract. These were evaporated with nitrogen gas and redissolved in 1 mL of either ethanol or water for chemical analysis using GC-MS techniques and biological assays.

Solvent extraction of C.zeylanicum - extraction with ethanol

A further 100 mg sample of *C.zeylanicum* powder was extracted in a 4:1 ratio with Diatomaceous earth (DE) in ethanol. The volume of the extract was reduced to 2 mL on a rotary evaporator. A one-in-two dilution was made of the concentrated extract. This was evaporated with nitrogen gas and redissolved in 1 mL of ethanol for analysis using GC-MS techniques and biological assays.

Solvent extraction of C.zeylanicum - extraction with water

A further 100 mg sample of *C.zeylanicum* powder was extracted in a 4:1 ratio with DE in water. The volume of the extract was reduced to 2 mL on a rotary evaporator. A one-in-two dilution was made of the concentrated extract. This was evaporated with nitrogen gas and redissolved in 1 mL of water for analysis using GC-MS techniques and biological assays.

Preparation of standards for GC-MS analysis

Standards were purchased according to the literature and standard solutions of β-caryophyllene (97.9 mM), cinnamaldehyde (153 mM), p-cymene (149 mM), trans-anethole (135 mM), trans cinnamic acid (3402 mM), α-amylcinnamaldehyde (99 mM), citral (131 mM), eugenol (135 mM), coumarin

(15.3 mM), benzyl benzoate (94.2 mM), furfural (208 mM), cinnamyl alcohol (50 mM), cinnamyl acetate (50 mM), estragole (100 mM) and o-methoxycinnamaldehyde (50 mM) were prepared in ethanol for GC-MS analysis.

GC-MS analysis

GC-MS analysis was performed on an Agilent (California, USA) 7890A gas chromatograph with 5975C inert XL EI/CI mass selective detector (MS) and CombiPral, a CTC-PAL autosampler. Gas chromatography separation was performed on J&W scientific (California, USA) HP-5MS (30 m × 0.25 mm ID, 0.25 μm). The injection volume of sample was 1 μL, using a split ratio of 10:1, at a temperature of 250°C. The injection syringe is pre-cleaned with ethanol once, then the sample five times before each injection. The syringe is then rinsed with ethanol five times after each injection. Separation was performed at a constant flow rate of 1 ml/min by programming the column pressure. The oven temperature was initially 80ºC for 1 min and then increased at a rate of 4°C min^{-1} until 200ºC was reached. The MS transfer line was set to a temperature of 250°C, the EI source to 230°C and the quadrupole at 150°C. Measurement was delayed for solvent for 3 min and the acquisition mode was set to scan 40 – 500 m/z. The components were identified based on the comparison of their GC retention times, interpretation of their mass spectra and confirmed by mass spectral library search using the National Institute of Standards and Technology (NIST) database (359). This work was supported and the spectral data interpreted by Dr. Samuela Lee from CompleMed, UWS.

Screening for anti-inflammatory activity using RAW 264.7 macrophages

The samples were tested on RAW 264.7 macrophages, for the LPS and IFN-γ induced NO and TNF-α downregulating activity by employing a method which was described in Chapter 2. Usually the

maximum dose of the extracts used was 2.5 mg/ml and a minimum of 6 concentrations made by serial dilution. Two independent experiments were conducted with each experiment having samples in duplicates and dose-dependent curves were obtained for each read-out. Data calculations were done using MS-Excel 2007 & MS-Excel 2010 softwares and IC_{50} values were obtained by sigmoidal dose-response function in Graphpad Prism (v5.01 – v5.04). IC_{50} values from each experiment were averaged and SD between these averages was calculated. Significant difference in the data is calculated by Bartlett's test for equal variances (p value <0.0001). The results were expressed in mean ± SD.

5.3 Results

Anti-inflammatory properties of *C.zeylanicum* fractions

In chapter 4 of this thesis, a library of food and plant samples were screened for their anti-inflammatory properties and cinnamon was selected as the lead compound (Table 4.5). Cinnamon was chosen for bioactivity guided-fractionation as the most promising candidate because it showed potent anti-inflammatory activity, since it inhibited both NO and TNF-α production in macrophages at low concentrations and also exhibited low toxicity. A newly produced cinnamon sample, freshly supplied by CSIRO (P1105) was again tested for its anti-inflammatory properties and found to be active against NO and TNF-α in macrophages cells, without affecting cell viability (Figure 5.1).

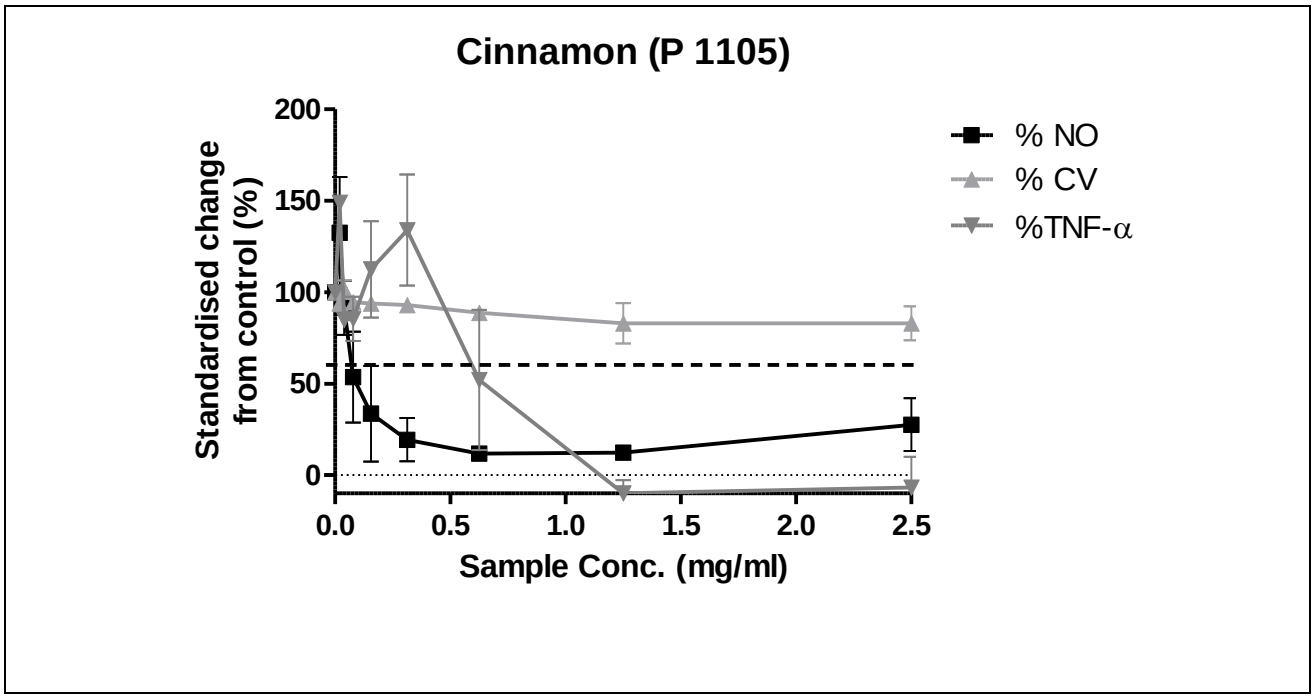
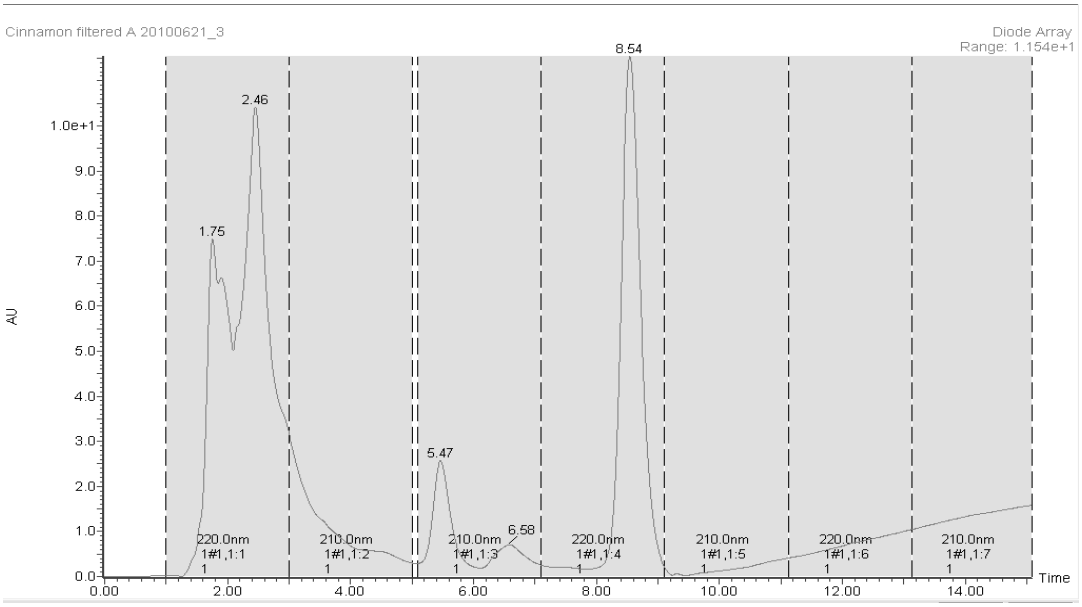
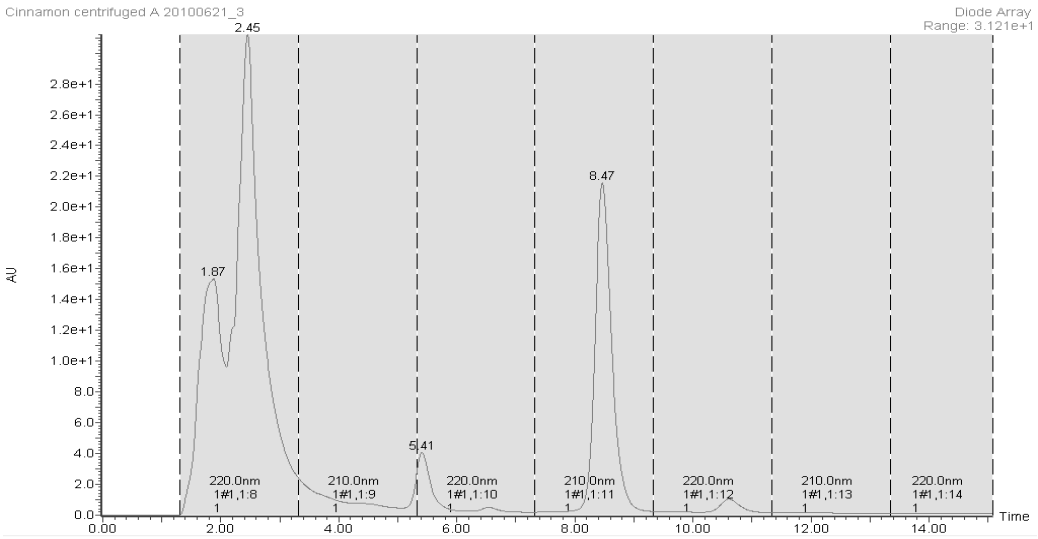


Figure 5.1 Concentration-dependent effects of cinnamon on inflammatory markers (NO and TNF-α production) as well as cell viability (CV) in RAW264.7 macrophages. Data show % change compared to activated cells (positive control) in the presence *of C. zeylanicum (P1105)* showing the mean of 2 independent experiments each with samples in triplicates and error bars representing SD ($p<0.0001$).

We assumed that the active constituent(s) of *C.zeylanicum* could be easily separated by HPLC fractionation and the active constituent(s) could be identified from the selected fraction(s) by using mass spectroscopy and NMR. Prior to analysis, two different sets were prepared for HPLC analyses, one by filtration and another by centrifugation of the supernatant. The two sets were prepared and tested to see if the particular method has advantage over the other. The results clearly implied that both methods do not affect much of the separation but filtration seems to provide a larger yield (evident by larger peaks) than the centrifugation method (Figure 5.2).



a Fractionation of the filtered cinnamon sample P 1105 by HPLC



b Fractionation of the centrifuged cinnamon sample P1105 by HPLC

Figure 5.2. HPLC fractionation: Fractionation of (a) filtered and (b) centrifuged samples at a flow rate of 1 ml/min and a linear gradient elution were performed for 30 min with methanol ranging from 50 to 100% in an aqueous solution of 2 g/ml formic acid. 7 fractions were collected in 2 min blocks.

In order to identify the most active fraction, the fractionated samples were tested for anti-inflammatory activity in RAW 264.7 macrophages using down-regulation of NO and TNF-α production as readouts. The data were collected from two independent experiments, performed in duplicates (Table 5.1).

Table 5.1 Summary of IC_{50} values for the sample fractions exhibiting significant attenuation of NO and TNF-α, without evidence of cell toxicity, in RAW264.7 macrophages

Fraction	**IC_{50} for NO production inhibition: filtered (mg/ml)**	**IC_{50} for NO production inhibition: centrifuged (mg/ml)**	**IC_{50} for TNF-α production inhibition: filtered (mg/ml)**	**IC_{50} for TNF-α production inhibition: centrifuged(mg/ml)**
P1105-Fr1	0.15	0.08	0.12	0.08
P1105-Fr2	0.08	0.08	0.06	0.08
P1105-Fr3	0.15	0.08	0.11	0.13
P1105-Fr4	0.12	0.11	0.11	0.17
P1105-Fr5	0.08	0.08	0.06	0.08
P1105-Fr6	0.08	0.08	0.09	0.08
P1105-Fr7	0.08	0.08	0.09	0.08

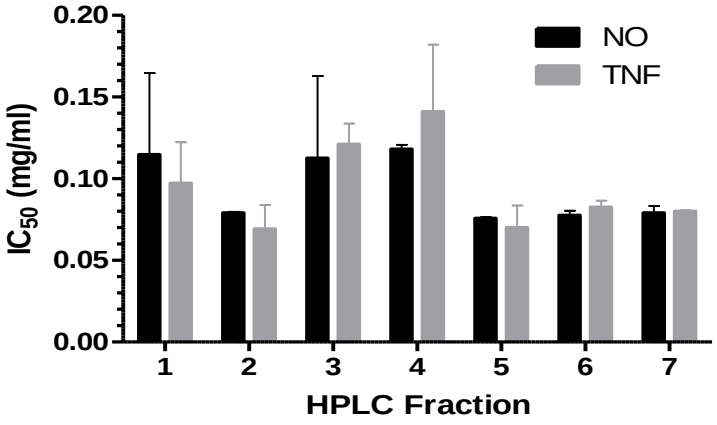


Figure 5.3. Anti-inflammatory activity of cinnamon fractions: Data show the IC_{50} values for NO and TNF-α production in activated RAW 264.7 macrophages in the presence of the fractions of *C. zeylanicum* showing the mean of 2 independent experiments each with samples in triplicates and error bars representing SD.

Surprisingley, all fractions showed anti-inflammatory activity but among the cinnamon fractions the most active compounds are found to be in Fractions 2, 5, 6 & 7 (Figure 5.3). It could be possible that the activity is contained in so many different fractions, because many different due anti-inflammatory compounds with different polarity might be present in *C. zeylanicum*. Therefore, this approach was abandoned. A different approach involving the extraction of cinnamon with solvents of different polarity, followed by identification of the all compounds CG-MS, and their individual testing by anti-inflammatory bioassyas was followed instead.

Determination of anti-inflammatory properties of *C.zeylanicum* sequential and direct extracts

C. zeylanicum was extracted in a sequential extraction procedure using solvents in an increasing polarity gradient. This served the purpose of fractionating the cinnamon sample to extract its individual components. These components were then identified using GC-MS. We then compared this sequential extraction procedure against a direct extraction procedure using a single polar solvent, either ethanol or

water, for the anti-inflammatory activity and cell-viability as measured by NO and TNF-α inhibition and an Alamar blue assay.

Identification of constituents of C.zeylanicum bark extractsby GC-MS

After accelerated solvent extraction (ASE) of *C. zeylanicum* bark, a total of 9 compounds were identified in the bark extracts, as summarised in Table 5.2.

Table 5.2: Compounds identified in extracts of *C. zeylanicum*

Solvent	***Constituent identified***	***Retention time (min)***	***Concentration (µM)***
Sequential Extracts			
Dichloromethane (DCM)	Trans-cinnamaldehyde	12.78	120.09
	Cinnamyl alcohol	13.97	1.72
	β-Caryophyllene	16.99	0.78
	Benzyl benzoate	26.83	40.84
	p-cymene	8.63	65.22
	Eugenol	15.29	0.82
Ethylacetate (EA)	Trans-cinnamaldehyde	12.72	0.07
	Eugenol	15.30	0.04
	β-Caryophyllene	17.03	0.52
	Benzyl benzoate	26.84	4.73
Ethanol	None		
Methanol	Eugenol	15.30	0.02
	Benzyl benzoate	26.83	1.90
Water	None		
Direct extracts			
Ethanol	Trans-cinnamaldehyde	12.75	42.24
	Cinnamyl alcohol	14.01	0.80
	Eugeno l	15.30	0.36
	β-Caryophyllene	16.99	1.94
	o-methoxycinnamaldehyde	20.44	36.25
	α-amyl cinnamaldehyde	22.49	0.01
	Benzyl benzoate	26.84	15.4
Water	Furfural	4.33	109.01
	o-methoxycinnamaldehyde	20.54	2.68

6 compounds namely, cinnamaldehyde, cinnamyl alcohol, eugenol, β-caryophyllene, p-cymene and benzyl benzoate were identified in the sequential extracts. 3 compounds namely α-amyl cinnamaldehyde, o-methoxy cinnamaldehyde and furfural were identified exclusively in direct extract. Cinnamaldehyde and β-caryophyllene were identified in DCM, EA and direct ethanol extracts. Benzyl benzoate and eugenol, both were identified in DCM, EA, methanol and direct ethanol extracts. Cinnamyl alcohol was identified in DCM and direct ethanol extract. In the direct water extract furfural and o-methoxy cinnamaldehyde were identified. α-amyl cinnamaldehyde was exclusively identified in direct ethanol extract. No constituents were identified in water and ethanol extracts from the sequential extraction procedure.

The identification of furfural is arguable as there is no previous evidence of its presence in natural plants. Considering the total yield of compounds identified, the sequential extraction procedure has been shown to be more effective than direct extraction, as it exhibits a significantly higher retention rate. Sequential extracts compared for 236.75 µM of constituents against 101.6 µM in direct extracts, excluding furfural. This is likely to be due to the gradient of polarity created by sequential extraction being able to interact with a wider variety of constituents with differing electronic properties. However the exclusive presence of few compounds such as α-amyl cinnamaldehyde, o-methoxy cinnamaldehyde and also furfural justifies the fact that direct extraction cannot be ruled out completely.

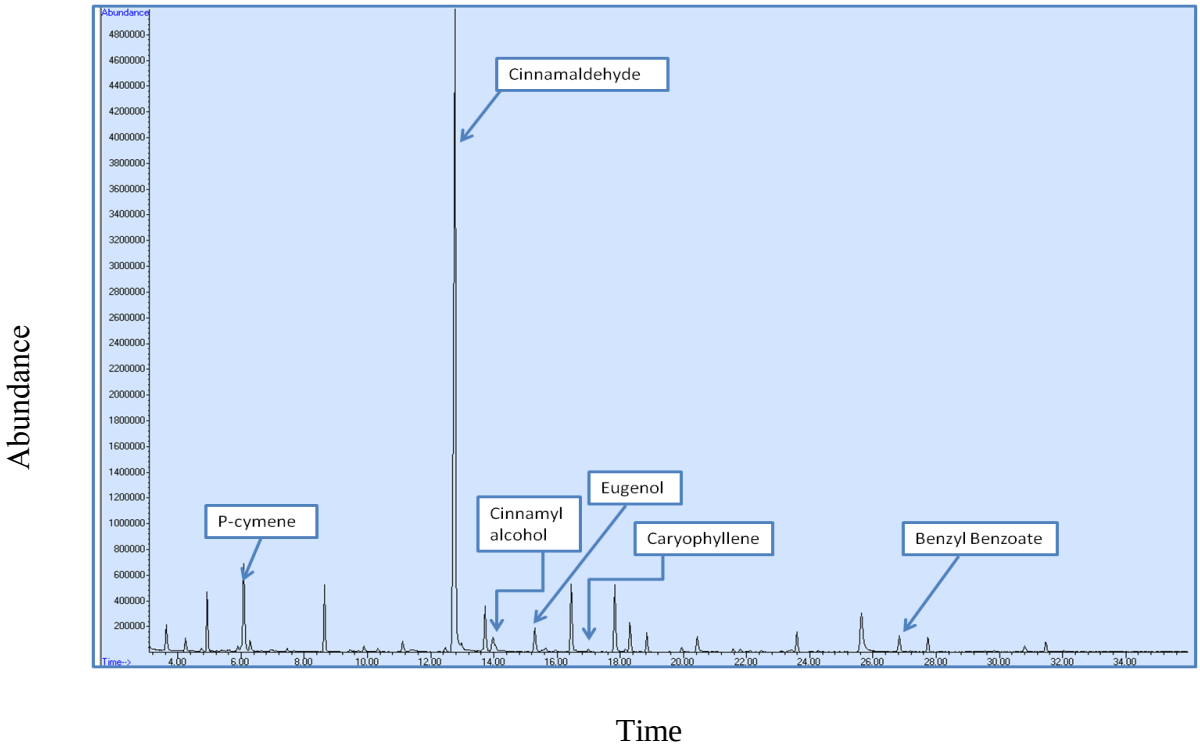


Figure 5.4. GC-MS chromatogram of the DCM extract of *C.zeylanicum*

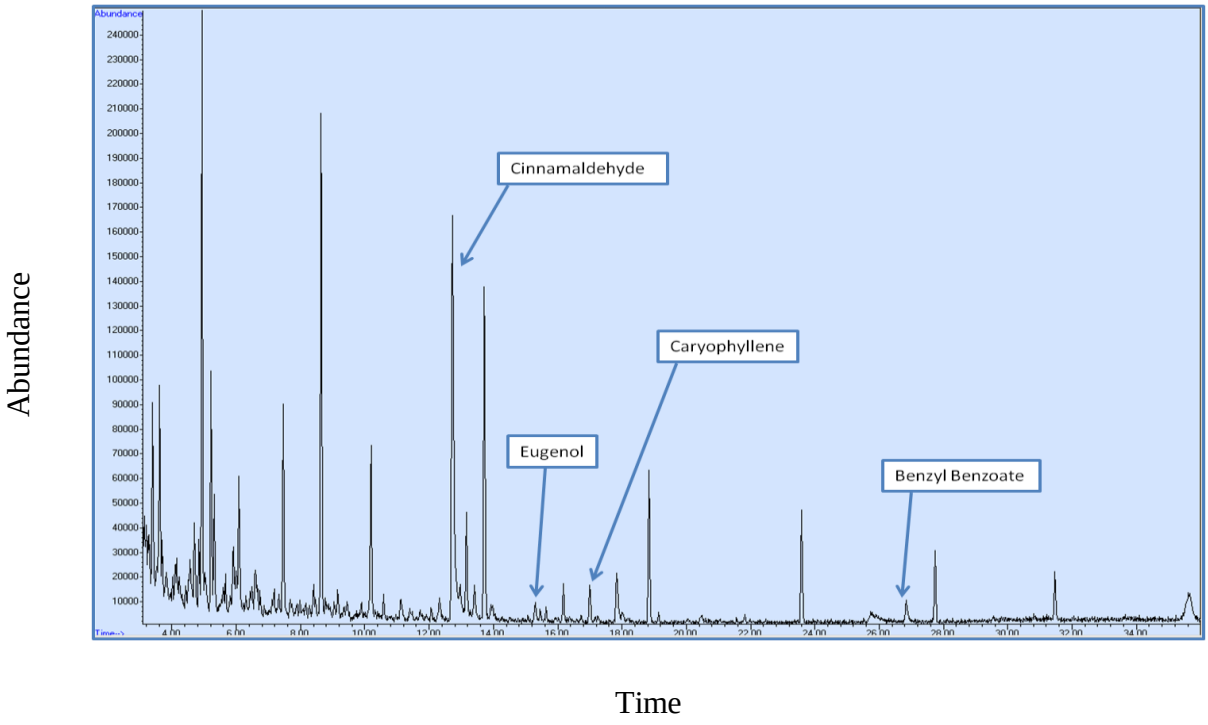


Figure 5.5. GC-MS chromatogram of the EA extract (sequential) of *C.zeylanicum*

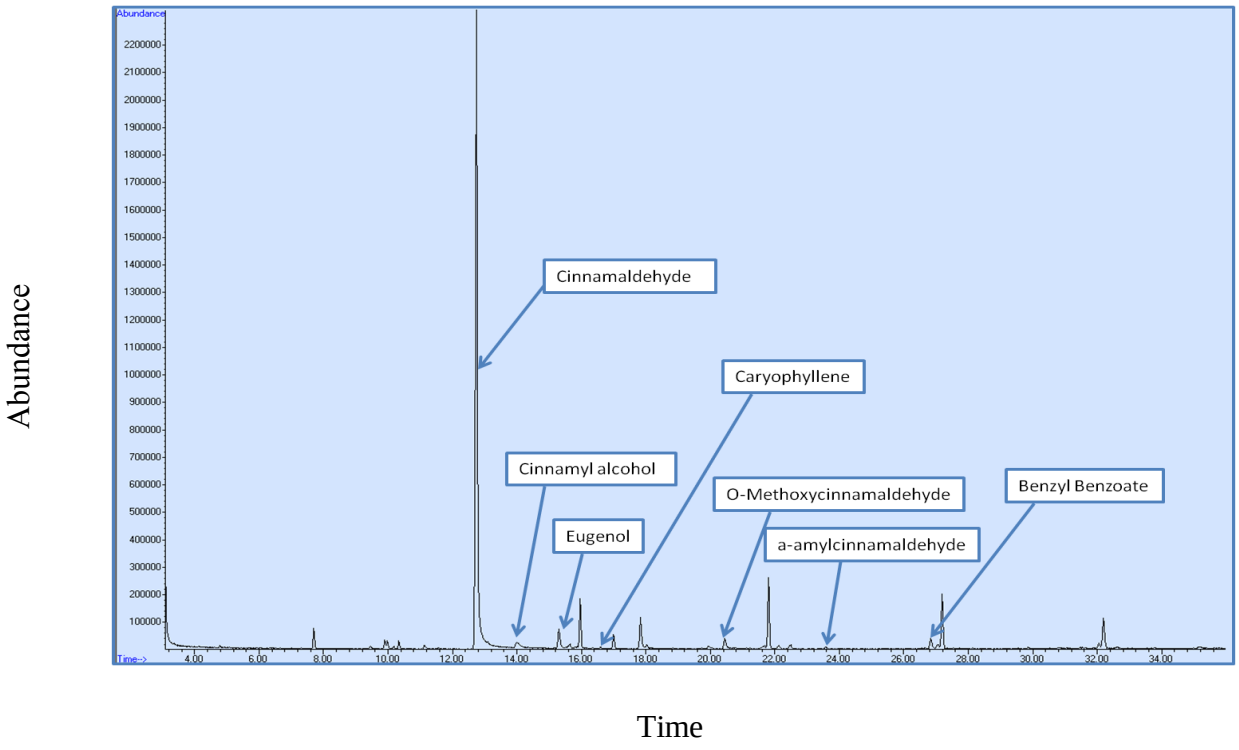


Figure 5.6. GC-MS chromatogram of *C.zeylanicum* extracted **directly** with ethanol

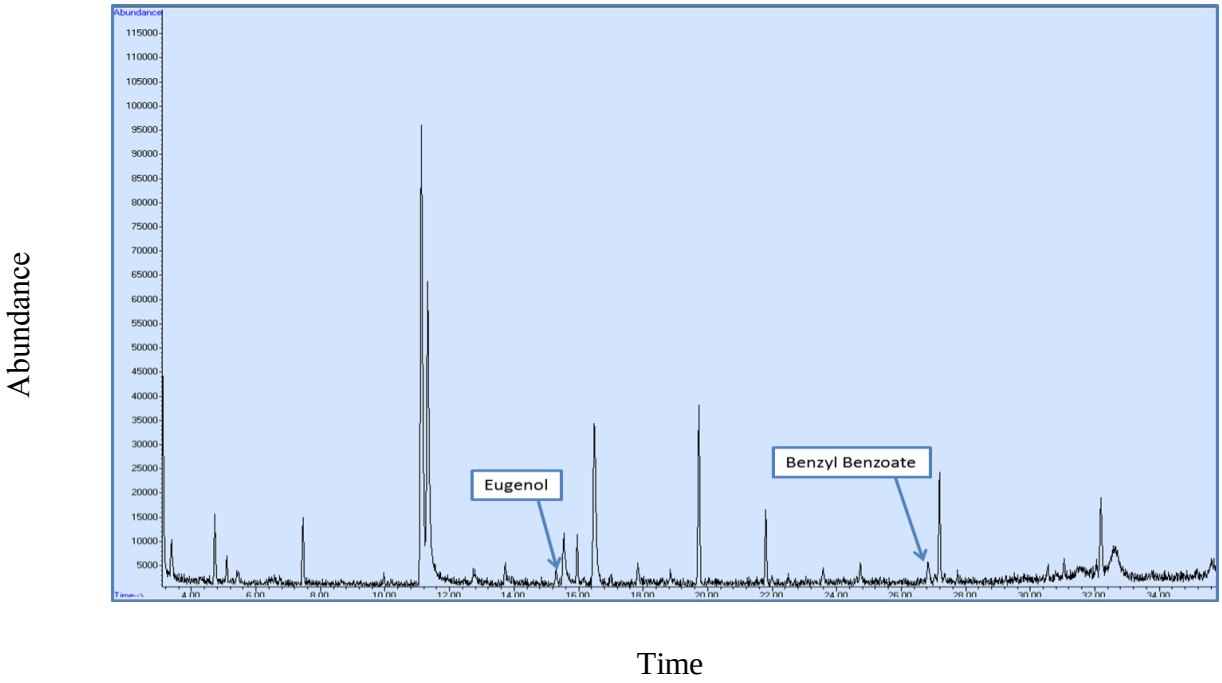


Figure 5.7. GC-MS chromatogram of the methanol extract (sequential) of *C.zeylanicum*

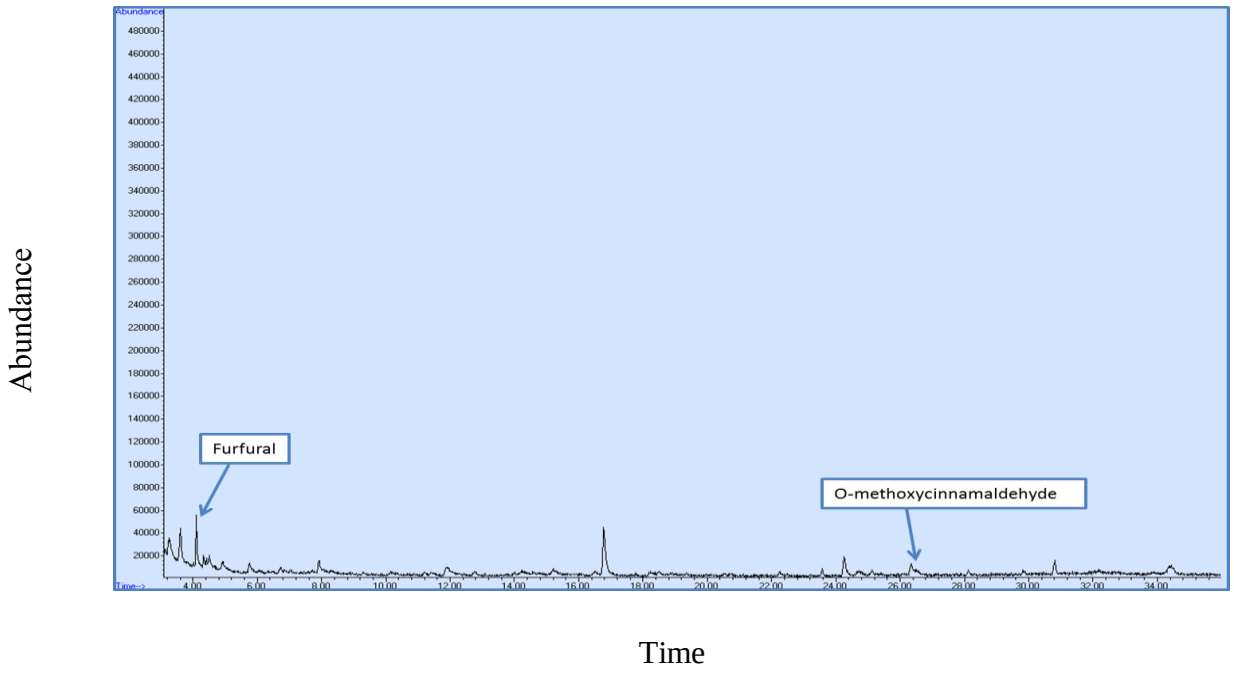


Figure 5.8. GC-MS chromatogram for *C.zeylanicum* extracted **directly** with water

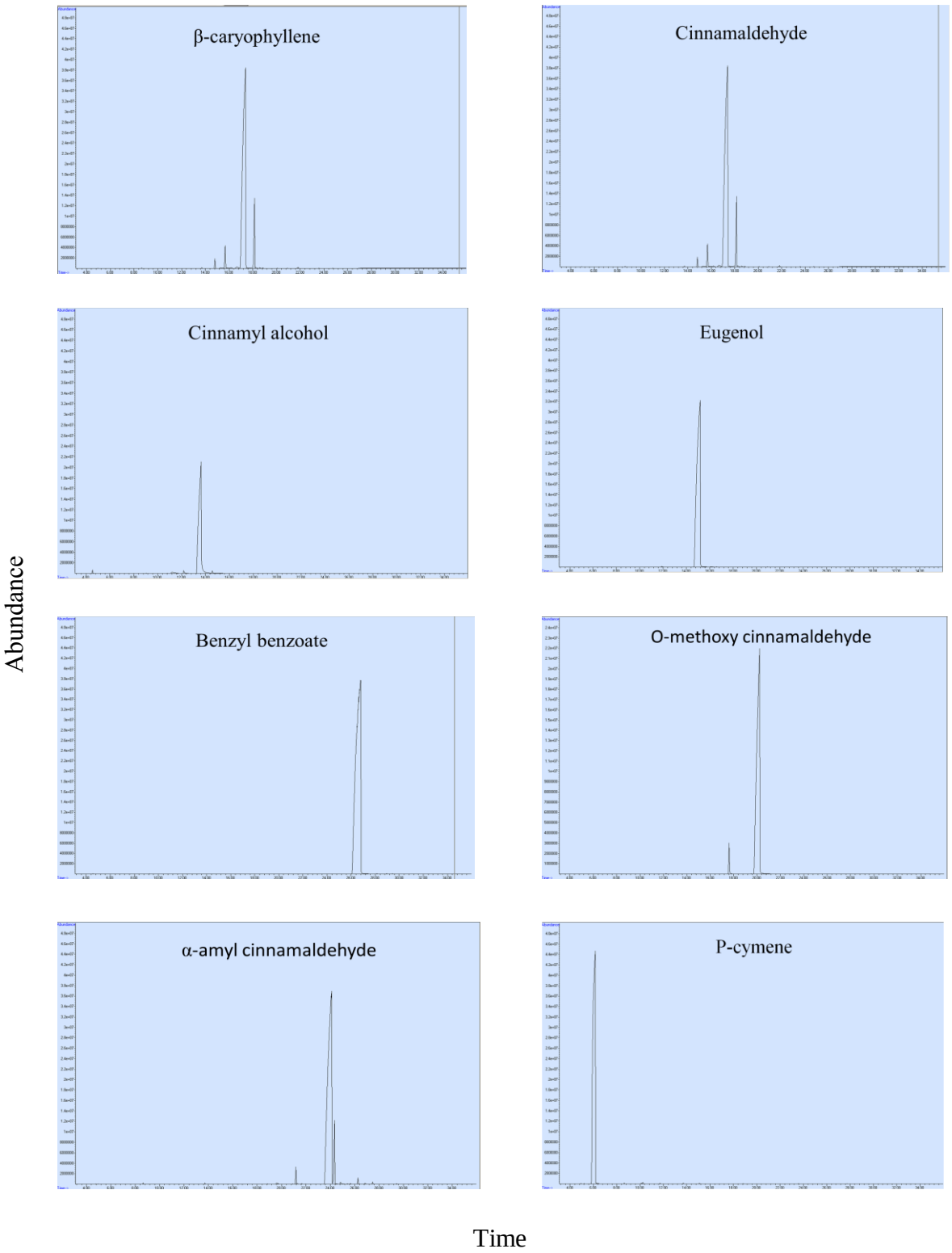


Figure 5.9. GC-MS chromatograms of standard compounds

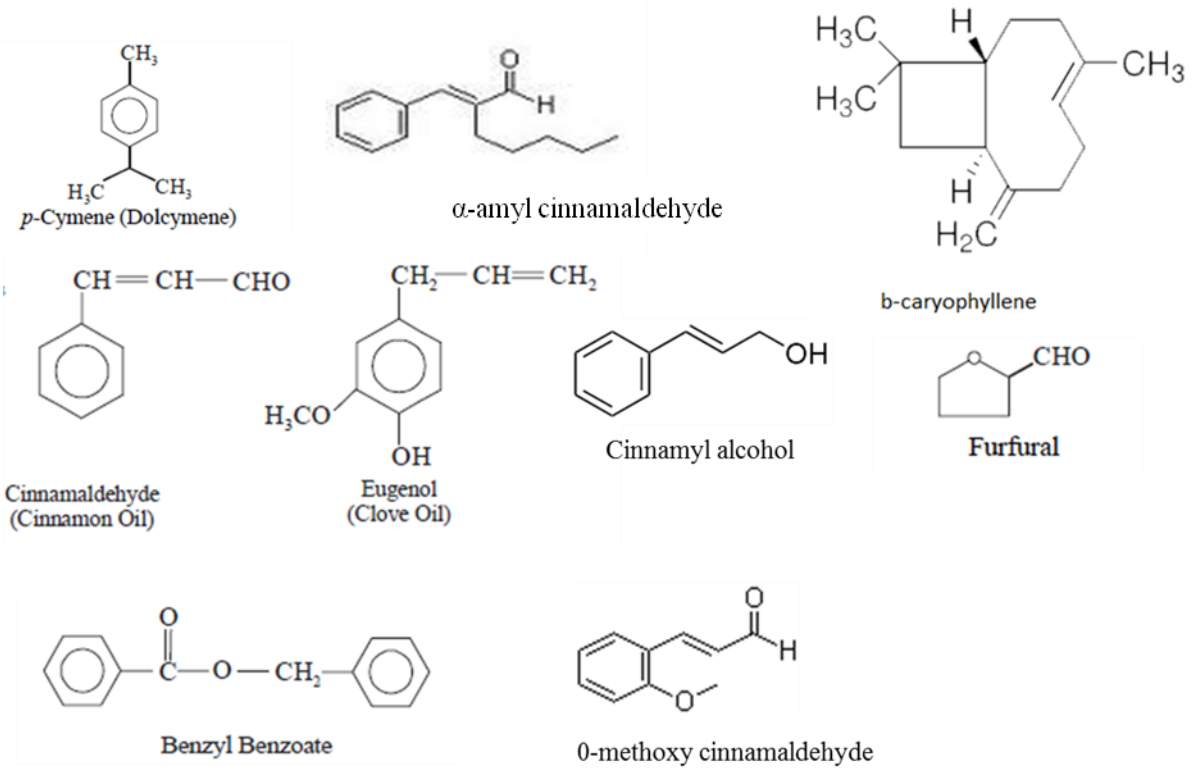


Figure 5.10: Chemical structures of active compounds identified from *C.zeylanicum* extracts.

Effects of extracts and their individual constituents on NO production in LPS-stimulated RAW 264.7 cells

Anti-inflammatory effects of the *C. zeylanicum* extracts was tested using inhibition of LPS induced NO production. It was found that the sequential extracts in DCM, EA, ethanol, methanol, water and also direct ethanol and water extracts all suppressed NO production (Table 5.3). In addition, these extracts produced no considerable cytotoxicity (Table 5.3).

Table 5.3: Anti-inflammatory activity and cell-viability of sequential and direct extracts of *C. zeylanicum in RAW 264.7 macrophages* showing the mean of 2 independent experiments each with samples in triplicates and SD.

Extract	**IC_{50} for NO production inhibition (mg/ml) ± SD**	**Percentage Cell viability at IC_{50} (NO) ± SD**	**IC_{50} for TNF-α production inhibition (mg/ml) ± SD**	**Percentage Cell viability at IC_{50} (TNF- α) ± SD**
Sequential				
DCM	0.06 ± 0	92.4 ± 5.52	0.17 ± 0.04	61.2 ± 5.52
EA	0.02 ± 0	84.7 ± 3.96	0.07 ± 0.01	53.2 ± 9.90
Ethanol	0.05 ± 0.01	113 ± 9.90	0.03 ± 0.04	113.5 ± 16.26
Methanol	0.11 ± 0.01	123.5 ± 4.95	0.24 ± 0.02	95.2 ± 3.39
Water	0.16 ± 0.01	122 ± 15.56	0.82 ± 0.03	87 ± 17.54
Direct				
Ethanol	0.03 ± 0.01	98.65 ± 6.15	0.09 ± 0.01	78.4 ± 4.10
Water	0.07 ± 0	108 ± 11.31	0.49 ± 0.01	91.1 ± 5.52

These results are comparable to previous results reported by Lee et al. (360), where the extracts of *C. cassia* had significant inhibitory effects of NO production, with an IC_{50} value between 1 and 5 μg/ml. Additionally, Tung et al (357) reported an IC_{50} value of 11.2 μg/ml of the essential oil from *c. osmophloeum* twigs. Hence the extracts of *C. zeylanicum* are comparably lower in anti-inflammatory activity in relation to *C. cassia* and *C. osmophloeum*, however some of our extracts are still quite potent, such as the EA extract which showed an IC_{50} value of 20 μg/ml. Because this extract also showed a good potential to promote cell viability, constituents which contribute could possibly be considered as a possible treatment for inflammatory diseases.

The direct extracts in ethanol and water direct extracts were also found to have significant capacity to inhibit the production of NO (Table 5.3). As in the sequential extracts, similarly, the ethanol extract has a higher level of NO inhibition than the water extract. While the direct water extract had

similar non-cytotoxic effects on the cells when compared with the sequential water extract, the direct ethanol extract was higher in cytotoxicity than its sequential analogue (Table 5.3). Hence it is possible to once again validate the use of the sequential extraction procedure, as it produced less cytotoxic fractions. This may be due to non-polar toxic constituents being first removed by the use of the non-polar solvents, before they can affect the cells in ethanol solution, i.e. the sequential ethanol extract has less cytotoxic components because they were removed prior to extraction in Ethanol in one of the non-polar solvents such as DCM or EA.

In examining the correlation of the chemical constituents of the extracts to the complete extracts, some of the constituents of the *C. zeylanicum* extracts were tested in the same manner as the extracts. The constituents are listed in Table 5.4 and all of them showed NO down-regulation.. β-caryophyllene, p-cymene, 2-methoxy cinnamaldehyde, citral, estragole and α-amyl cinnamaldehyde were highly active with IC_{50} values below 0.1 mg/ml. In addition, neither of these constituents showed significant levels of cytotoxicity. However, Trans-anethole, trans-cinnamic acid, coumarin, benzyl alcohol showed significant cytotoxicity (data not presented).

Effects of extracts and their constituents on TNF-α production in LPS-stimulated RAW 264.7 cells

Large amounts of TNF-α have been demonstrated to be released in response to LPS (361). Hence therefore, another method of examining the inhibitory effects of the *C. zeylanicum* is by measuring the inhibition of TNF-α production. It was observed that the sequential extracts in DCM, EA, ethanol, methanol and water all inhibit the production of the TNF-α (Table 5.3). Therefore the extract with the highest anti-inflammatory properties in reagrds to TNF-a production was the ethanolic extract. These results are comparable to previous results reported by Chao *et al.* (358), who demonstrated that the essential oil from the leaves of *C. osmophloeum* had significant inhibitory effects

of TNF-α production, with a reduction of 16 and 17 ng/ml from a positive control with a 30 and 60 µg/ml addition of essential oil. Hence the extracts of *C. zeylanicum* are similar in TNF-α inhibition in relation to the essential oil of *c. osmophloeum*, for example, the IC_{50} value of the ethanol extract was 34 µg/ml. Direct extracts in ethanol and water also can inhibit the production of TNF-α. The IC_{50} values of the direct extracts are also summarised in Table 5.3. As in the sequential extracts, similarly, the ethanol extract demonstrated a higher level of TNF-α inhibition than the water extract.

In addition, neither of these constituents showed significant levels of cytotoxicity at doses inhibiting NO (Percentage cell viability <80%), but Citral, p-cymene and α-amyl cinnamaldehyde have shown cell death at IC_{50} doses of TNF-α inhibition.

Table 5.4: Anti-inflammatory activity of cinnamon constituents tested in LPS activated RAW 264.7 macrophages

Standard (mM)	**IC_{50} for NO production inhibition ± SD**	**Percentage Cell viability at IC_{50} (NO) ± SD**	**IC_{50} for TNF-α production inhibition ± SD**	**Percentage Cell viability at IC_{50} (TNF- α) ± SD**
β-caryophyllene	0.04 ± 0.01	105 ± 1.42	0.03 ± 0.01	103 ± 1.41
p-cymene	0.05 ± 0.02	106.5 ± 17.68	0.2 ± 0.11	75.8 ± 25.74
Cinnamaldehyde	0.07 ± 0.02	104.8 ± 6.51	0.16 ± 0.02	87.75 ± 5.06
Cinnamalacetate	0.18 ± 0.03	91.15 ± 1.59	>2.5mM	NA
Cinnamylalcohol	0.14 ± 0.01	95.6 ± 2.12	>2.5mM	NA
2-methoxy cinnamaldehyde	0.05 ± 0.02	109.7 ± 7.28	0.27 ± 0.01	96.3 ± 8.98
Eugenol	0.23 ± 0.07	93.35 ± 3.71	>2.5mM	NA
Citral	0.02 ± 0	92.6 ± 0	0.06 ± 0	77.6 ± 0
α-amyl cinnamaldehyde	0.02 ± 0	90.4 ± 0	0.05 ± 0.01	76.15 ± 3.71
Benzyl benzoate	0.2 ± 0.01	95.25 ± 0.81	0.31 ± 0.02	87.75 ± 2.93
Furfural	0.26 ± 0.01	96 ± 1.84	0.61 ± 0.02	83.65 ± 0.53
Estragole	0.02 ± 0.01	101.5 ± 1.59	0.03 ± 0.01	104 ± 1.41

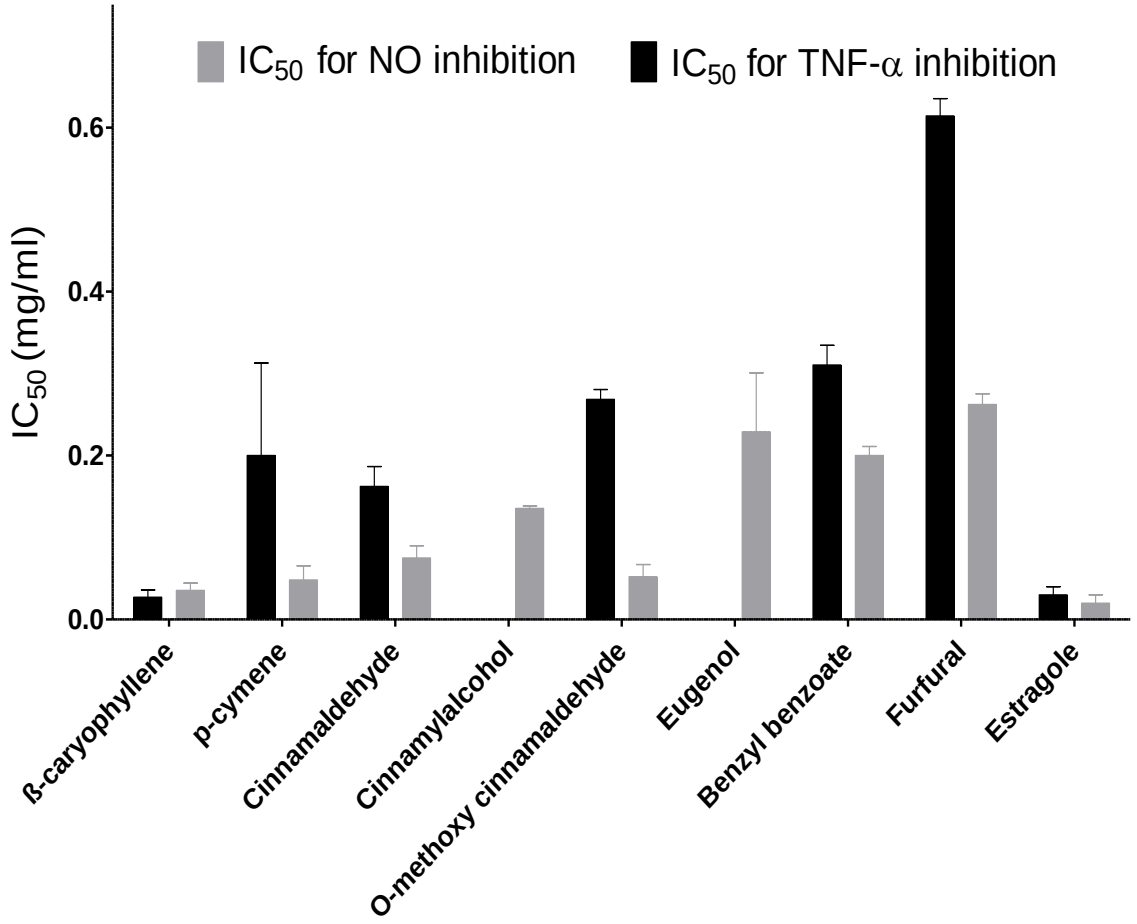


Figure 5.11: Comparison of anti-inflammatory activity of cinnamon constituents in RAW 264.7 macrophages showing the mean of 2 independent experiments each with samples in triplicates and error bars representing SD.

5.4 Discussion

This analysis demonstrated that extraction and analysis procedure is more effective than fractionation and subsequent mass spectroscopic analysis, in case of cinnamon. Extracts taken from the bark of *C. zeylanicum* have high anti-inflammatory activities. There was also a good indication that the chemical constituents of the extracts including β-caryophyllene, Cinnamaldehyde, 2-methoxy cinnamaldehyde, benzyl benzoate and furfural also have strong anti-inflammatory activities, both through the suppression of NO production and through the down-regulation of TNF-α, as demonstrated

in LPS-stimulated macrophages. These compounds may be promising as leads in the development of anti-inflammatory treatments in diseases of pathological inflammation.

Cinnamaldehyde, cinnamic acid and cinnamyl alcohol, major constituents of *Cinnamomum cassia*, have been shown to possess antioxidant, anti-inflammatory, anticancer and other activities. One constituent which has shown significant bioactivity is cinnamaldehyde, which has been previously extracted from the leaf essential oil of the *C. osmophloem* plant. It has been shown to induce apoptosis, i.e. that it has potential in anti-cancer activity, both through mitochondrial permeability transition in human promyelocytic leukemia HL-60 cells (362), and through activation of Bcl-2 proteins in human hepatoma cells (363). It has activated the Nrf2-dependent antioxidant response in human epithelial colon cells (364). It has also been shown to be a potential anti-bacterial agent (365). Cinnamaldehyle suppressed the proliferation of T cells from murine splenocytes after they had been activated by the growth inducers concanavalin A and LPS(366) and suppressed DNA binding activity and transcriptional activity of NF-κB in LPS stimulated RAW 264.7 macrophages(367). Through this inhibition of NF-κB, cinnamaldehyde is able to inhibit the inducible nitric oxide synthase (iNOS) and subsequently nitric oxide (NO) production(360). Moreover, cinnamaldehyde inhibited the IL-1β induced cyclooxygenase-2 activity, which subsequently led to the inhibition of PGE2 in rat cerebral microvascular endothelial cells (368). These latter three findings then suggest strongly that cinnamaldehyde has great potential as an immuno-modulatory agent. Hence the mechanism of anti-inflammatory activity of the cinnalmaldehyde in the essential oil extracted from the leaves of C. osmophloeum is able to inactivate the cytokines involved in proliferating the immune response, rather than inhibiting the causative agent or blocking receptor binding sites of cell-surface markers. This validates our study examining the anti-inflammatory activities of constituents in the essential oil extracted from the bark of *C. zeylanicum* which deserves further investigation as a possible treatment

for inflammation associated diseases, due to the high of level of anti-inflammatory activity exhibited by this study.

Trans-cinnamaldehyde and 2-methoxycinnamaldehyde were both identified previously as the NF-κB inhibitors and both inhibited LPS-induced DNA binding activity of NF-κB in addition to NF-κB transcriptional activity (367). This report is coinciding with our data that had shown NO and TNF-α suppression in LPS and IFN-γ induced RAW macrophages.

Plant essential oils are typically composed of volatile aromatic terpenes and phenylpropanoids. β-caryophyllene, a sesquiterpene, has been reported as a dietary cannabinoid which exerted anti-inflammatory effects by activating CB_2 receptor through selective binding to the CP55,940 binding site (i.e., THC binding site) (369). In our study we have found that it could also affect NF-κB pathway due to its NO and TNF-α down-regulation.

Eugenol, an o-methoxyphenol and a naturally occurring food flavouring agent, is also shown to be present in cinnamon that shows significant NO production inhibition. Previously it has been reported for antioxidant, anti-inflammatory and DNA-protective properties in thioacetamide-induced liver injury in rats (370). Our study is in line with an *in vivo* study anti-inflammatory effects of eugenol suppressing cyclooxygenase-2 expression, while eugenol dimers prevent nuclear factor-κB (NF-κB) activation and inflammatory cytokine expression in lipopolysaccharide-stimulated macrophages (371).

One very interesting result of our study is the outcome of benzyl benzoate, a component of the essential oil, exhibiting good NO and TNF-α down regulating properties with no cytotoxicity. It is not investigated for such anti-inflammatory potential, before. One study on rescuing muscular damage in

rats by diclofenac used nano-tubes made of benzyl benzoate and has shown the anti-inflammatory benefits in comparison to the diclofenac solution (372). In that study the difference in activity was attributed to the formulation using nano-tubes but our study shows that it could be due to anti-inflammatory effects provided directly by benzyl benzoate.

This study has identified the presence of furfural in trace amounts in cinnamon extracts, might be due to contamination of the sample. Nevertheless, furfural had shown the anti-inflammatory properties without cytotoxicity. Trans-anethole, trans-cinnamic acid, coumarin, estragole, citral, cinnamyl acetate and benzyl alcohol were not identified in this analysis. But estragole had shown strong NO and TNF-α suppression without cytotoxicity, inspite the suspected to carcinogenic and genotoxic properties documented by Committee on Herbal Medicinal Products (373).

In addition these findings indicate a refined method for extraction of cinnamon products, by sequential extraction in solvents of increasing polarity, rather than by direct extraction in a single solvent of high polarity. This has been indicated by increased cytotoxicity of direct extracts in comparison with the sequential extracts of the same solvent, as demonstrated by an Alamar Blue cell viability assay. Hence, in future studies, to improve cell survival, a similar sequential extraction procedure should be employed.

CHAPTER 6

DISCUSSION

Oxidative stress in AD

The brain is especially sensitive to oxidative damage due to its high content of unsaturated and oxidation-susceptible fatty acids, high use of oxygen, and low levels of antioxidants. Free radical–induced oxidative damage may play a role in the pathogenesis of AD (169). ROS production could activate NF-κB, the nuclear transcription factor, by triggering a change in the cell that results in phosphorylation of the subunit of an inhibitory protein, I-κB (203). Downstream products of NF-κB activation include NO and inflammatory cytokines such as, IL-1, IL-6 and TNF-α. Antioxidants are playing an important role in preventing these diseases by counteracting these oxidants that modulate NF-κB activation. Due to their low cytotoxicity, phytochemicals are potential sources of natural antioxidants, which are candidates for the prevention of oxidative damage. A number of medicinal and dietary plants extracts containing polyphenols, flavonoids, and other compounds exhibited significant antioxidant activities (204). To mention a few, anti-inflammatory activities was shown by alkaloids from *Forsythia suspensa* in rats (205), the aerial part of *Helichrysum italicum* extracts in various *in vivo* and *in vitro* experimental models (206) and aqueous extract of *Scrophularia frutescens L,* in carrageenan-induced oedema test on the rat paw (207).

Microglial cells were used in the preliminary screening and macrophages were used in further stages of screening. This procedure had provided us with a dual advantage that those potent plants identified from both cell lines could act against brain-specific inflammation and other forms of inflammation. Macrophages were reported to contribute amyloid angiopathy by shuttling Aβ from neurons to vessels (374). Autoimmune animal models shown the involvement of these two related cell types in brain pathology relevant to multiple sclerosis and experimental allergic encephalomyelitis (375). It is also evidenced that COX-2-positive macrophages infiltrate into AD brain damage the blood-brain barrier (376). Massive tissue destruction due to high macrophage number in neural tissues is also

reported (377). We have tested a set of Chinese medicinal plants and a library of plant and mushrooms for anti-inflammatory properties. We have tested the toxicity exerted by these samples on the cells by measuring the cell viability, simultaneously.

Anti-inflammatory properties of Chinese medicinal plants

Among the screened 58 Chinese medicinal plant extracts (Chapter 2), we have identified that all plant extracts, except S.nigrum were shown to suppress NO while the extracts of *S. flavescens Ait.*, *S. coicis* L., *P. vulgaris* L., *M. fortunei (Lindl.)*Fedde, *U. rhyncophylla* Miq, *E. ulmoides* Oliv., *L. lucidum* Ait., *A. officinarum, A. quinata (Houtt.)*Decne., *A. arguta, S. suberectus* Dunn., *S. barbata* Don.were shown to inhibit both NO and TNF-α production with very low toxicity. This study coincided with previously evidences of anti-inflammatory activity of *P. vulgaris* L. (212), *U. rhyncophylla* Miq (233) and *A. quinata (Houtt.)* Decne (249). We have demonstrated the anti-inflammatory properties of the anti-microbial *S. flavescens* Ait. (260), *M. fortunei (Lindl.)*Fedde (256), *E. ulmoides* Oliv. (246) and *S. barbata* Don (259); the anti-tumorigenic *E. ulmoides* Oliv. (246), *S. barbata* Don (259) and *A. arguta* (244); the antioxidants *P. vulgaris L.* (212) and *U. rhyncophylla* Miq (233) and the anti-diabetic *S. coicis* L. (213). These extracts were found be the most potent and could be promising candidates against inflammatory diseases. In addition, the findings from our correlation studies show that phenolics and flavonoids from these medicinal herbs might not be essential for the anti-inflammatory activities. As shown in the antioxidant assays, namely DPPH-scavenging activity and yeast oxidative inhibition, NO inhibition correlated significantly to antioxidant levels of the plant, but TNF-α inhibition did not correlated significantly. This could imply that antioxidants might not be directly influencing TNF-α release.

This study has established the anti-inflammatory properties of the whole herbal extracts rather than the individual constituents, therefore further systematic investigations are needed to characterise the bioactive compounds of these plant extracts. If bioactive properties can be verified in suiTable animal models and ultimately in humans, these plant extracts might represent useful dietary (or pharmaceutical) tools for use against inflammatory diseases including AD.

Anti-inflammatory properties of mushrooms

In this study, a generic food-compatible method for processing was applied to a library of plant and food samples. Processing, which involved heating, physical and chemical treatments to disperse solids including ultrasonication, was intended to select for process-stable or process-modified products and also investigate the stability of bioactive species to processing conditions.

6 mushroom types were 'processed' in successive stages (Chapter 3) and were tested for their anti-inflammatory properties. In general, fresh mushrooms exhibited significantly higher anti-inflammatory activity than processed mushrooms, highlighting the instability to processing of bioactive constituents. Among them, Shiitake and Oyster were identified as the potent anti-inflammatory candidates as they suppressed both NO and TNF-α in RAW macrophages without killing the cells.

Oyster mushrooms protected against carbon tetrachloride-induced oxidative stress in rats i*n vivo* via anti-oxidant mechanism (Jayakumar, Sakthivel, Thomas, & Geraldine, 2008); suppressed LPS-induced secretion of TNF-α, IL-6 and IL-12p40, in RAW 264.7 macrophages, suppressed PGE2 and NO by down-regulation of the expression of COX-2 and iNOS respectively, inhibited LPS-dependent DNA-binding activity of AP-1 and NF-κB in RAW 264.7 cells (319); markedly suppressed secretion of

TNF-α and IL-6 in LPS challenged mice, *in vivo* (319) and inhibited proliferation and secretion of IFN-γ, IL-2, and IL-6 in concanavalin A-stimulated mouse splenocytes by the inhibition of (319). Our study coincides with the observations of anti-inflammatory activity of oyster mushrooms.

Antioxidant properties were reported for polysaccharides from shiitake mushrooms that increased antioxidant enzyme activity and improve blood lipid levels in rats and inhibit the oxidative injury induced by accumulating free radicals caused by high-fat-diet to a certain extent (329). Dose dependent antioxidant activity of methanol and water crude extracts from Shiitake mushroom was demonstrated with chemical assays investigating antioxidant capacity in three different assays, namely, the β-carotene and linoleic acid system, 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity, and inhibition of hemolysis of rat erythrocyte induced by peroxyl radicals (330). In general, in mushroom extracts higher antioxidant activity is correlated to larger amount of total phenolics and inspite the probable presence of other antioxidants, phenolic compounds could make a significant contribution to the antioxidant activity in these extracts (330). Not only the results from these chemical assays were in line with our biological assay showing NO suppression but also these assays postulated scavenging of free radicals generated during lipid peroxidation as one possible mechanism of the antioxidant activity of the mushroom extracts.

Antioxidant properties of Enokitake (320,321), Honey brown and Button mushrooms (322) were known previously but this is the first study that demonstrated their ability to suppress NO in cells.

Anti-inflammatory effects of Shiitake and other mushrooms could be due to the presence of antioxidant, L-ergothioneine as its inhibition of NO (Table 3.2), atleast partially. L-ergothioneine exerted antioxidant and anti-inflammatory effect by inhibiting both hydrogen peroxide and TNF-α-

mediated activation of the NF-κB inflammation cascade in lung epithelial cells (378). These properties need to be verified in suitable animal models and ultimately in humans and if successful, then these mushroom products could represent useful dietary (or pharmaceutical) tools for use against inflammatory diseases including AD.

High throughput screening of plant and food library and identification of the most potent candidate

High throughput screening of plants from the library of plant and food provided by CSIRO had shown many plants to be active against NO and TNF-α in murine microglia and murine macrophages. Considering the stability of activity during processing stages, activity on both the cell lines and suppression of both NO and TNF-α without cytotoxicity, Cinnamon stood out as the most potent candidate. Hence Cinnamon has been selected as the lead candidate for further chemical analysis. Though a variety of cinnamon species were previously studied (chapter 3) for the anti-oxidant, anti-diabetic, anti-microbial, anti-cancer and anti-arthritis properties, there was not much evidence in support of anti-inflammatory properties especially relevant to the species *C.zeylanicum*.

Anti-inflammatory properties of *C.zeylanicum*

We expected to identify the active constituents of cinnamon by using HPLC fractionation followed by mass spectroscopy. But anti-inflammatory assays had shown that the activity was spread out through the fractions which might be due to the variety of ingredients present in *C.zeylanicum*, hence we have opted for extraction followed by mass spectroscopy analysis.

With solvent extraction and subsequent GC-MS analysis we were able to identify 9 constituents of *C.zeylanicum.* β-caryophyllene, p-cymene, cinnamaldehyde and its derivatives 2-methoxy cinnamaldehyde and α-amyl cinnamaldehyde, citral, benzyl benzoate and furfural have shown both NO and TNF-α inhibitory activity while cinnamalacetate, cinnamyl alcohol and eugenol have shown NO inhibitory activity but not TNF-α inhibition. Neither of these constituents showed significant levels of cytotoxicity at doses inhibiting NO (Percentage cell viability <80%), but citral, p-cymene and α-amyl cinnamaldehyde have shown cell death at IC_{50} doses of TNF-α inhibition. Hence β-caryophyllene, cinnamaldehyde, 2-methoxy cinnamaldehyde, benzyl benzoate and furfural may be appropriate choices as leads compound in the development of anti-inflammatory treatments in diseases of pathological inflammation.

In summary, the use of natural extracts and compounds in inflammatory conditions including AD is promising, with added benefits of low or no toxicity, low cost, ease of access for treatment. These compounds could provide neuroprotection via separate anti-inflammatory and antioxidant pathways hence might allow a safe, multi-targeted therapeutic neuroprotective approach to the prevention of AD.

7. Further research

Permeability of the blood-brain barrier (BBB) of the potential drug candidates remains a big obstacle in AD. From our study we were able to identify a compound, o-methoxy cinnamaldehyde which suppressed the inflammatory markers NO and TNF-α without cytotoxicity. Also we were able to show the anti-inflammatory properties exerted by water extracts of *C.zeylanicum*. This could indicate the presence of potential but yet unidentified compounds in the extract that needs to be identified. Also the extracts of Chinese medicinal plants and mushrooms were shown to possess anti-inflammatory properties that need to be further analysed for their chemical constitution. While the further chemical research towards the chemical composition of these mentioned extracts suggested, it is also recommended to formulate the active but less polar compounds such as cinnamaldehyde, eugenol so that they could be shuttled into the brain.

The anti-inflammatory compounds identified in this study require confirmation of their efficacy in vivo. Despite the positive outcomes in murine models of AD, they could be analysed on a higher model representing the disease conditions more perfectly such as neuron-microglia co-culture. The activity could be confirmed in models such as the mouse with brain overexpressing TNF-α. A direct comparison between the neuroprotective potential observed *in vitro* with outcomes in mice, including both behavioral and physical measures such as Aβ plaque and NFTs, microglial proliferation, levels of NO and pro-inflammatory markers in addition to TNF-α, neurite retraction and mitochondrial respiration of neurons would further distinguish effective compounds for human trials.

The intervention trial in human would be planned after confirming the activity of the identified compounds in animal models. This could be done with a single compound or combination of more than one compound. In addition to the drug efficiency, the adverse effects need to be documented.

8. Conclusion

1. This study adds to the fact that diet and medicinal plants contain potential anti-inflammatory properties that could be helpful in inflammatory conditions including AD.
2. This study emphasises the possibility of high throughput screening and selection of potent compound from a library by using cell-based anti-inflammatory assays.
3. Our selected plant, *C.zeylanicum* had shown strong anti-inflammatory properties and the identified constituents such as β-caryophyllene, cinnamaldehyde, o-methoxy cinnamaldehyde, benzyl benzoate and furfural exerted such properties without cytotoxicity.
4. We have concluded that in case of identification of active anti-inflammatory compounds of *C.zeylanicum*, extraction procedure followed by chemical analysis is more effective than fractionation and subsequent analysis procedure.

Appendices

Appendix 1: CSIRO plant and food fractions library

Code	Eluted in	Sample
P0283	mQ	Almonds
P0284	Glucose	Almonds
P0285	mQ	asparagus
P0286	Glucose	asparagus
P0287	mQ	avocado
P0288	Glucose	avocado
P0289	mQ	basil green
P0290	Glucose	basil green
P0291	mQ	basil purple
P0292	Glucose	basil purple
P0293	mQ	basil thai
P0294	Glucose	basil thai
P0295	mQ	Dark Columbia coffee beans
P0296	Glucose	Dark Columbia coffee beans
P0297	mQ	Blueberries
P0298	Glucose	Blueberries
P0299	mQ	bok choi
P0300	Glucose	bok choi
P0301	mQ	broccoli
P0302	Glucose	broccoli
P0303	mQ	brussel sprouts
P0304	Glucose	brussel sprouts
P0305	mQ	carrot
P0306	Glucose	carrot
P0307	mQ	Chinese broccoli
P0308	Glucose	Chinese broccoli
P0309	mQ	Chinese cabbage
P0310	Glucose	Chinese cabbage
P0311	mQ	chives
P0312	Glucose	chives
P0313	mQ	choi sum
P0314	Glucose	choi sum
P0315	mQ	choko flesh
P0316	Glucose	choko flesh

P0317	mQ	Mocha supreme coffee beans
P0318	Glucose	Mocha supreme coffee beans
P0319	mQ	continental parsley
P0320	Glucose	continental parsley
P0321	mQ	coriander leaf
P0322	Glucose	coriander leaf
P0323	mQ	corn
P0324	Glucose	corn
P0325	mQ	cucumber
P0326	Glucose	cucumber
P0327	mQ	fennel
P0328	Glucose	fennel
P0329	mQ	maitake mushroom
P0330	Glucose	maitake mushroom
P0331	mQ	garlic
P0332	Glucose	garlic
P0333	mQ	ginger
P0334	Glucose	ginger
P0335	mQ	green beans
P0336	Glucose	green beans
P0337	mQ	green peas
P0338	Glucose	green peas
P0339	mQ	Jew's ear
P0340	Glucose	Jew's ear
P0341	mQ	kiwi gold
P0342	Glucose	kiwi gold
P0343	mQ	kiwi green
P0344	Glucose	kiwi green
P0345	mQ	leek
P0346	Glucose	leek
P0347	mQ	onion
P0348	Glucose	onion
P0349	mQ	oregano
P0350	Glucose	oregano
P0351	mQ	oyster mushroom
P0352	Glucose	oyster mushroom
P0353	mQ	red sweet potato
P0354	Glucose	red sweet potato
P0355	mQ	pecans
P0356	Glucose	pecans
P0357	mQ	pine nuts

P0358	Glucose	pine nuts
P0359	mQ	pumpkin
P0360	Glucose	pumpkin
P0361	mQ	quince
P0362	Glucose	quince
P0363	mQ	radiccio
P0364	Glucose	radiccio
P0365	mQ	raspberries
P0366	Glucose	raspberries
P0367	mQ	red cabbage
P0368	Glucose	red cabbage
P0369	mQ	red coral lettuce
P0370	Glucose	red coral lettuce
P0371	mQ	rhubarb
P0372	Glucose	rhubarb
P0373	mQ	rosemary
P0374	Glucose	rosemary
P0375	mQ	sage
P0376	Glucose	sage
P0377	mQ	savoy cabbage
P0378	Glucose	savoy cabbage
P0379	mQ	Seaweed, spiralina
P0380	Glucose	Seaweed, spiralina
P0381	mQ	shallot
P0382	Glucose	shallot
P0383	mQ	spinach
P0384	Glucose	spinach
P0385	mQ	spring onion
P0386	Glucose	spring onion
P0387	mQ	squash
P0388	Glucose	squash
P0389	mQ	strawberries
P0390	Glucose	strawberries
P0391	mQ	sweet potato
P0392	Glucose	sweet potato
P0393	mQ	tarragon
P0394	Glucose	tarragon
P0395	mQ	thyme
P0396	Glucose	thyme
P0397	mQ	tomato
P0398	Glucose	tomato

P0399	mQ	Walnuts
P0400	Glucose	Walnuts
P0401	mQ	watercress
P0402	Glucose	watercress
P0403	mQ	yellow mustard seed
P0404	Glucose	yellow mustard seed
P0405	mQ	silver beet
P0406	Glucose	silver beet
P0407	mQ	rainbow silverbeet
P0408	Glucose	rainbow silverbeet
P0409	mQ	coconut
P0410	Glucose	coconut
P0411	mQ	turmeric
P0412	Glucose	turmeric
P0413	mQ	ruby grapefruit
P0414	Glucose	ruby grapefruit
P0415	mQ	lime
P0416	Glucose	lime
P0417	mQ	mandarine (imperial)
P0418	Glucose	mandarine (imperial)
P0419	mQ	brown linseed
P0420	Glucose	brown linseed
P0421	mQ	red onion
P0422	Glucose	red onion
P0423	mQ	beetroot
P0424	Glucose	beetroot
P0425	mQ	peanuts
P0426	Glucose	peanuts
P0427	mQ	macadamia
P0428	Glucose	macadamia
P0429	mQ	brazil nut
P0430	Glucose	brazil nut
P0431	mQ	cashews
P0432	Glucose	cashews
P0433	mQ	cocoa beans
P0434	Glucose	cocoa beans
P0435	mQ	red delicious apples
P0436	Glucose	red delicious apples
P0437	mQ	Lemon flesh
P0438	Glucose	Lemon flesh
P0552		Vegemite

P0553		Vegemite
P0555	mQ	Lemongrass
P0556	Glucose	Lemongrass
P0557	mQ	Black Carrot
P0558	Glucose	Black Carrot
P0559	mQ	Lime Zest
P0560	Glucose	Lime Zest
P0561	mQ	white potato
P0562	Glucose	white potato
P0563	mQ	English Breakfast Tea leaves
P0564	Glucose	English Breakfast Tea leaves
P0565	mQ	Japanese Sencha tea leaves
P0566	Glucose	Japanese Sencha tea leaves
P0567	mQ	Navel Oranges
P0568	Glucose	Navel Oranges
P0569	mQ	Valencia Oranges
P0570	Glucose	Valencia Oranges
P0571	mQ	Honey Brown mushroom
P0572	Glucose	Honey Brown mushroom
P0573	mQ	Button Mushroom
P0574	Glucose	Button Mushroom
P0575	mQ	Enoki mushroom
P0576	Glucose	Enoki mushroom
P0577	mQ	Shiitake mushroom
P0578	Glucose	Shiitake mushroom
P0579	mQ	orange zest
P0580	Glucose	orange zest
P0581	mQ	lemon zest
P0582	Glucose	lemon zest
P0583	mQ	cinnamon
P0584	Glucose	cinnamon
P0585	mQ	cardamom
P0586	Glucose	cardamom
P0587	mQ	cloves
P0588	Glucose	cloves
P0589	mQ	bay leaves
P0590	Glucose	bay leaves
P0591	mQ	Brewer's yeast
P0592	Glucose	Brewer's yeast
P0593	mQ	Corriander Seeds
P0594	Glucose	Corriander Seeds

P0595	mQ	White Zucchini
P0596	Glucose	White Zucchini
P0597	mQ	Choko Skin
P0598	Glucose	Choko Skin
P0701	-	Grains
P0702	-	Grains
P0703	-	Grains
P0704	-	Grains
P0705	-	Grains
P0706	-	Grains
P0707	-	Grains
P0708	-	Grains
P0709	-	Grains
P0710	-	Grains
P1001	mQ	yellow citrus flesh
P1002	Glucose	yellow citrus flesh
P1003	mQ	yellow citrus skin
P1004	Glucose	yellow citrus skin
P1005	mQ	red citrus flesh
P1006	Glucose	red citrus flesh
P1007	mQ	red citrus skin
P1008	Glucose	red citrus skin
P1009	mQ	green citrus
P1010	Glucose	green citrus
P1015	mQ	M48-42
P1016	Glucose	M48-42
P1017	mQ	Chambourcin
P1018	Glucose	Chambourcin
P1021	mQ	Cabernet Sauvignon
P1022	Glucose	Cabernet Sauvignon
P1023	mQ	MI28-41
P1024	Glucose	MI28-41
P1025	mQ	Sunmuscat (control grape)
P1026	Glucose	Sunmuscat (control grape)
P1027	mQ	Concord
P1028	Glucose	Concord
P1037	mQ	CR101-13
P1038	Glucose	CR101-13
P1039	mQ	CR101-10
P1040	Glucose	CR101-10

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